Depletion of Endogenous Nitric Oxide Enhances Cisplatin-induced Apoptosis in a p53-dependent Manner in Melanoma Cell Lines*

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The expression of inducible nitric-oxide synthase in melanoma tumor cells was recently shown to correlate strongly with poor patient survival after combination biochemotherapy (p < 0.001). Furthermore, evidence suggests that nitric oxide, a reaction product of nitric-oxide synthase, exhibits ant apoptotic activity in melanoma cells. We therefore hypothesized that nitric oxide antagonizes chemotherapy-induced apoptosis. Whether nitric oxide is capable of regulating cell growth and apoptotic responses to cisplatin treatment in melanoma was evaluated. We demonstrate herein that depletion of endogenously produced nitric oxide can inhibit melanoma proliferation and promote apoptosis. Moreover, our data indicate that the depletion of nitric oxide leads to changes in cell cycle regulation and enhances cisplatin-induced apoptosis in melanoma cells. Strikingly, we observed that the depletion of nitric oxide inhibits cisplatin-induced wild type p53 accumulation and p21Waf1/Cip1/Sdi1 expression in melanoma cells. When cisplatin-induced p53 binding to the p21Waf1/Cip1/Sdi1 promoter was examined, it was found that nitric oxide depletion significantly reduced the presence of p53-DNA complexes after cisplatin treatment. Furthermore, dominant negative inhibition of p53 activity enhanced cisplatin-induced apoptosis. Together, these data strongly suggest that endogenously produced nitric oxide is required for cisplatin-induced p53 activation and p21Waf1/Cip1/Sdi1 expression, which can regulate melanoma sensitivity to cisplatin.

Melanoma is the deadliest form of skin cancer and exhibits extremely aggressive growth characteristics. The prognosis for patients with disseminated disease is invariably very poor. Melanoma is highly resistant to chemotherapy and radiation therapy, and therapies for melanoma have generally been ineffective at best and rarely result in sustained responses. Resistance of cancer to therapy is attributable in part to mechanisms such as altered drug uptake or transport, intracellular detoxification, altered DNA repair, and the dysregulation of the apoptotic pathway (1). The tumor suppressor p53 is a sensor of diverse cellular stresses including DNA damage, oxidative stress, and hypoxia, and helps to direct cell cycle arrest and apoptosis at least in part through transcriptional activation of target genes like p21Waf1/Cip1/Sdi1 (p21), GADD45a, 14-3-3, and bax (2). Not surprisingly, p53 is the most commonly mutated gene in a broad spectrum of cancers, and its inactivation is frequently associated with tumor progression, resistance to therapy, and poor prognosis. However, the p53 gene is rarely mutated in melanoma (3), and increased expression is associated with tumor progression (4). Results from several studies suggest that the p53 pathway is dysregulated in melanoma cells, which in turn may lead to inactivation or alteration of p53 function (5). For instance, many human melanoma cell lines containing wild type p53 showed abnormalities in the p53 pathway in response to γ-irradiation (6). In recent years, studies have suggested that the retention of wild type p53 may actually confer resistance to therapy in a number of tumor systems (7). However, the role of p53 function in melanoma remains unclear.

Activated p53 can bind to p53-responsive elements in the promoter of the potent cell-cycle regulator p21 and induce its expression (8). When sufficiently expressed, p21 induces G1 arrest by direct binding of cyclin-dependent kinases (9). Expression of p21 has been implicated as a survival mechanism against therapy (10). For instance, inhibition of p21 by antisense technology sensitized glioblastoma cells to chemotherapeutic agents (11) and to radiation therapy (12). p21 also appears to protect human colon carcinoma cells and prostate cancer cells against apoptosis (13, 14). Additionally, there is evidence that p21 may protect against p53-mediated apoptosis in human melanoma cells (15). The precise involvement of p21 in the resistance of melanoma to therapy remains largely undefined.

Nitric oxide (NO) has recently been implicated in the regulation of p53. Specifically, treating cells with NO donors resulted in conformational and functional changes and possible tyrosine nitration of p53 protein (16, 17). In addition, it was shown that NO can regulate the ability of p53 to stimulate radiation-induced cell cycle arrest in the MCF-7 breast cancer cell line (18). NO is generated as a reaction product of the NO synthase (NOS) family of enzymes that catalyze the conversion of L-arginine into NO and L-citrulline. Three isoforms of NOS have been identified: constitutive NOS (cNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). The expression of iNOS is induced by inflammatory cytokines and mediates tissue injury during inflammation, while cNOS and nNOS are constitutively expressed in the brain and nervous system respectively. Recent studies have suggested a role for iNOS in tumor growth and metastasis, as well as in resistance to therapy (19). However, the role of iNOS in melanoma remains unclear.

In melanoma tumor cells, NO has been shown to inhibit apoptosis induced by various stimuli, including chemotherapy, radiation, and cytokines (20). In particular, NO has been shown to inhibit apoptosis induced by cisplatin, a commonly used chemotherapeutic agent in the treatment of melanoma (21). The mechanism by which NO inhibits cisplatin-induced apoptosis is not fully understood, but it has been proposed that NO may interfere with the activation of p53 downstream targets such as p21Waf1/Cip1/Sdi1, which play a critical role in mediating the antiproliferative effects of p53 (22).

The role of NO synthase in apoptosis is complex and may depend on the cell type and the stimulus. While NO has been shown to have pro-apoptotic effects in some contexts, it can also have anti-apoptotic effects in other contexts. For example, in some cell lines, treatment with NO donors has been shown to enhance apoptosis induced by tumor necrosis factor-α (TNF-α) (23).

In this study, we aimed to investigate the role of NO in regulating the sensitivity of melanoma cells to cisplatin-induced apoptosis. We used a melanoma cell line, M14, which expresses wild type p53 and is sensitive to cisplatin. We treated these cells with a nitric oxide synthase inhibitor, L-NMMA, and a nitric oxide donor, SIN-1, and measured cell viability and apoptosis using flow cytometry and TUNEL assay.

Our results showed that depletion of nitric oxide significantly enhanced cisplatin-induced apoptosis in M14 cells, as evidenced by increased Annexin V-positive cells and TUNEL-positive nuclei. Moreover, we observed a decrease in the expression of the anti-apoptotic protein, bcl-2, in cells treated with SIN-1, which suggests that NO may inhibit apoptosis in melanoma cells by promoting the expression of pro-apoptotic proteins.

In conclusion, our data suggest that nitric oxide depletion can enhance cisplatin-induced apoptosis in melanoma cells. This finding has important implications for the development of novel therapeutic strategies that target NO synthase in melanoma and other cancer types. Further studies are needed to elucidate the mechanisms by which NO regulates the sensitivity of melanoma cells to chemotherapy and radiation therapy.
enzymatic conversion of l-arginine to l-citrulline by three iso-
types of NO synthase (NOS): endothelial (eNOS), neuronal
(nNOS), and inducible (iNOS). NOSs are expressed in various
tissue types. eNOS and nNOS are typically considered to be
to be constitutively expressed, whereas iNOS, as its name implies, is
inducible (19). NO is involved in neurotransmission, vasodila-
tion, inflammation, and immunity (20) and is also believed to
play roles in multiple stages of various cancers (21). It exhibits
both proapoptotic and antiapoptotic characteristics, depending
on concentration and cell type (22, 23). Recently, NO has been
shown to covalently modify proteins that can lead to regulation
of activity, including caspasas (24, 25), retinoblastoma protein
(26), c-Src (27), and p53 (17).

Our laboratory observed that the expression of iNOS and the
presence of nitrotirosine in tumor cells of melanoma patients
affected strongly with poor patient outcome (p < 0.001 and
0.02, respectively) (28), suggesting an important role for NO in
melanoma resistance to therapy. Presently, samples from
0.02, respectively) (28), suggesting an important role for NO in
expression with melanoma progression (29). Recently, Salvucci et al. (30) pro-
vided evidence that endogenous NO may serve as a survival
factor in human melanoma cells.

Using human melanoma cell lines and cisplatin as a model
system, we tested the hypothesis that NO regulates p53 sig-
aling and is required in melanoma resistance to chemotherapy.
We present evidence that the depletion of endogenous NO in
melanoma cell lines leads to increased sensitivity to cisplatin as a
consequence of the inhibition of cisplatin-induced p53
activation which acts as a protective factor against apoptosis
in melanoma cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-
onyl-3-oxide (c-PTIO) (31), 3-morpholinosydnonimine (SIN-1), (Z)-1-2-
(2-aminoethyl)-N-(2-ammonioethy)amine-1-ium-1,2-diolate
(DETA), aminoguanidine (AMG), and NOS-monomethyl-
t-arghine monocarboxate (l-NMMA) were obtained from Alexis Corp. (San
Diego, CA). N-[3-(Ammoniomethyl)benzy]acetamide dihydrochloride (1400W)
was purchased from A. G. Scientific (San Diego, CA). Cisplatin was
purchased from Bristol-Myers Squibb Co. [3H]Thymidine was obtained from
PerkinElmer Life Sciences. [γ-32P]ATP was purchased from ICN
Pharmaceuticals, Inc. (Costa Mesa, CA). Antibodies for p53 and actin
were purchased from Oncogene Research Products (San Diego, CA).
The antibody for p53 was purchased from Upstate (Lake Placid, NY). The
poly(ADP-ribose) polymerase (PARP) antibody was purchased from BD
Pharmingen. The antibody for p21 was obtained from Upstate (Lake Placid, NY). The
antibodies for p53 and actin
were purchased from Oncogene Research Products (San Diego, CA).

For the cleaved p3 assay, cells were washed once with Tris-
buffered saline and incubated in propidium iodide (PI)/RNase I buffer (Phoenix Flow
Systems, San Diego, CA) for an additional 30 min in the dark at 37
°C. The cells were analyzed by flow cytometric analysis on a FACScan fluor
flow cytometer (BD Biosciences). A sample population of 10,000 cells
was used for analysis with CellQuest software.

**Immunoblot for p21, p53, Cleaved Caspase 3, PARP, and Actin**—
Cells were washed with PBS, trypsinized, and washed once with ice-
cold PBS before lysis. Cells were lysed in a buffer containing
25 mM Tris-HCl (pH 7.5), 140 mM NaCl, 10 mM NaF, 5 mM EDTA, 1% Nonidet
P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 20%
glycerol with the aforementioned supplements to remove the detergent.

**Electrophoretic Mobility Shift Assay—** The double-stranded oligonu-
cleotide probe corresponding to the p53-binding site of the
tumor promoter

**Oxymyoglobin Preparation—** Oxymyoglobin was prepared from
Equine heart myoglobin as previously described (32). Briefly, the oxy-
lized myoglobin was resuspended in water, reduced with a molar
excess of sodium dithionite (sodium hydrosulfite, Sigma), and passed
through a Sephadex G-25 column (PD-10, Amersham Biosciences). Ox-
myglobin is recovered as a single bright red band from the column flow.

**[3H]Thymidine Uptake Assay—** The [3H]thymidine uptake assay was
performed essentially as previously described (33). Briefly, cells were
harvested by trypsinization, washed once in PBS, and pelleted by
centrifugation at 1,000 × g for 5 min. Cells were fixed in 4% formaldehyde,
PBS for 10 min at ambient temperature, pelleted, and resuspended in
ambient temperature for storage at −80°C. On the day of staining, cells were
rehydrated in Tris-buffered saline for 10 min at ambient temperature and
permeabilized by protein K treatment for 5 min. Cells were then incubated for 15 min in the manufacturer-supplied equilibration buffer,
after which the buffer was removed and the cells were incubated in TdT
labeling reaction mixture in the dark for 1.5 h at 37
°C. After the mixture
was removed, cells were washed once with Tris-buffered saline
and incubated in propidium iodide (PI)/RNase I buffer (Phoenix Flow
Systems, San Diego, CA) for an additional 30 min in the dark at 37
°C. The cells were analyzed by flow cytometric analysis on a FACScan fluor
flow cytometer (BD Biosciences). A sample population of 10,000 cells
was used for analysis with CellQuest software.

**Terminal Deoxynucleotidyl Transferase-medi-ated dUTPFITC Nick-
end Labeling (TUNEL) Assay—** The TUNEL assay (Fluorescence DNA
Fragmentation Detection Assay, Oncogene Research Products) was
performed according to the manufacturer’s protocol. Briefly, cells were
harvested by trypsinization, washed once in PBS, and pelleted by
centrifugation at 1,000 × g for 5 min. Cells were fixed in 4% formaldehyde,
PBS for 10 min at ambient temperature, pelleted, and resuspended in
ambient temperature for storage at −80°C. On the day of staining, cells were
rehydrated in Tris-buffered saline for 10 min at ambient temperature and
permeabilized by protein K treatment for 5 min. Cells were then incubated for 15 min in the manufacturer-supplied equilibration buffer,
after which the buffer was removed and the cells were incubated in TdT
labeling reaction mixture in the dark for 1.5 h at 37
°C. After the mixture
was removed, cells were washed once with Tris-buffered saline
and incubated in propidium iodide (PI)/RNase I buffer (Phoenix Flow
Systems, San Diego, CA) for an additional 30 min in the dark at 37
°C. The cells were analyzed by flow cytometric analysis on a FACScan fluor
flow cytometer (BD Biosciences). A sample population of 10,000 cells
was used for analysis with CellQuest software.
were treated in triplicate for 24 h with c-PTIO (NO scavenger). [3H]Thymidine was added simultaneously with the NO modulators. Cells were harvested as described under “Experimental Procedures,” and [3H]thymidine incorporation was measured with a scintillation counter. B, cells were treated for 24 h with DETA. C, cells were treated with c-PTIO, SIN-1, or both for 24 h. D, c-PTIO and/or DETA were added to melanoma cells and incubated at 37 °C for 1 h. Cisplatin and [3H]thymidine were added to the culture medium, and the cells were incubated for 24 h at 37 °C before harvesting. Data are expressed as the mean ± S.D. The data shown are representative of at least two separate experiments.

was synthesized by Sigma-Genosys (The Woodlands, TX) with the sequence 5′-GAGACCTGCCACACGTGG-3′ (8). The complementary oligonucleotides were annealed to form the probe, which was then labeled with γ-32P]ATP using T4 polynucleotide kinase and resolved on a NAP-5 Sephadex G-25 column (Amersham Biosciences) to remove the unincorporated label. The DNA binding reaction was performed with 6 μg of nuclear extracts in a solution containing 20 mM Tris-HCl (pH 7.5), 2 mM NaCl, 5 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 100 ng of poly(dIdC), and 1 ng of labeled probe in 40 μl of total volume for 30 min at ambient temperature, after which 0.5 μg of monoclonal anti-p53 antibody pAb421 was added and incubation was continued for another 30 min at ambient temperature. DNA-protein complexes were resolved on a non-denaturing 5% polyacrylamide gel and detected by autoradiography using Bio-Max MR film (Eastman Kodak Co.).

Inhibition of p53 Activity by Transfection of a Dominant Negative Expression Vector—Transfection of A375 melanoma cells was performed according to the manufacturer’s protocol. Briefly, cells were plated one day prior to transfection in DMEM supplemented with 10% fetal bovine serum, HEPES, and glutamine, without antibiotics to give an approximate cell density of 95% confluence at the time of transfection. LipofectAMINE 2000 (1.0 μg/ml) and plasmids (0.4 μg/ml) were added individually to Opti-MEM I (Invitrogen) for 5 min, combined, and again incubated for 20 min. Prior to the addition of the liposome-DNA complexes, wells are rinsed with Opti-MEM I and refilled with fresh supplemented DMEM without antibiotics. The transfection was carried out for 24 h, after which the cells are treated with appropriate reagents for another 24 h before harvesting. Transfection was confirmed by Western blotting against hemagglutinin.

RESULTS

The Effect of NO on Melanoma Cell Proliferation—Because NO has been implicated in increased melanoma progression and metastatic potential, we evaluated whether endogenously produced NO enhanced the proliferation of iNOS-expressing A375 melanoma cells. The NO-specific scavenger c-PTIO and the NO donor DETA were used to modulate NO levels in the cells overnight, after which [3H]thymidine uptake was measured. NO depletion resulted in a dose-dependent decrease in proliferation (Fig. 1A). The addition of 50 μM DETA lead to an increase in [3H]thymidine uptake (Fig. 1B). Treatment of cells with more than 150 μM DETA caused a dose-dependent decrease in [3H]thymidine uptake, suggesting that low levels of NO enhances cell growth, whereas higher NO concentrations lead to inhibition of growth. Similar results were observed in A375.S2 melanoma cells (data not shown).

In addition to iNOS, the presence of nitrotyrosine also correlated with poor outcome in melanoma patients (28). To assess whether increased protein tyrosine nitration enhances melanoma proliferation, cells were treated with SIN-1, which donates NO and superoxide at a 1:1 ratio, and [3H]thymidine incorporation was measured (Fig. 1C). NO and superoxide form peroxynitrite, which is responsible for protein tyrosine nitration, at a near diffusion controlled rate. SIN-1 treatment of up to 300 μM had little affect on cell growth. SIN-1 was also able to reverse the inhibition of proliferation by NO depletion.

To assess whether NO depletion regulates growth inhibition after cisplatin treatment, we incubated cells with c-PTIO prior to the addition of cisplatin (Fig. 1D). [3H]Thymidine uptake decreased in a dose-dependent manner after cisplatin treatment. When cells were preincubated with both cisplatin and c-PTIO, proliferation decreased 2-fold over that which was induced by cisplatin alone, suggesting an additive or synergistic effect between cisplatin treatment and NO depletion. The
FIG. 2. Endogenous NO depletion enhances cisplatin-induced apoptosis. TUNEL assay and flow cytometric analysis. A, A375 melanoma cells were pretreated for 1 h with c-PTIO prior to 24 h of incubation with 0–8 μg/ml of cisplatin at 37 °C. Cells were fixed, and TUNEL staining and flow cytometric analysis were performed as described under “Experimental Procedures.” Ten thousand cells were counted for each treatment. B, graphic representation of TUNEL staining shown in A. The quenched scavenger control did not differ from its corresponding control. C, immunoblot against cleaved caspase 3 and PARP. D, cells were incubated with oxymyoglobin for 1 h prior to 24 h cisplatin treatment. Cells were harvested and analyzed as in A.
FIG. 3. **Cell cycle regulation by NO.** A375 melanoma cells are treated with c-PTIO and/or DETA and cisplatin for 24 h, fixed, stained, and analyzed by flow cytometry. **A**, TUNEL/PI staining with TUNEL positivity plotted along the ordinate and PI staining along the abscissa. Approximate locations of cells in each cell cycle phase are indicated by arrows. The data shown are representative of at least three separate experiments. **B**, synchronized A375 cells are treated for the specified amount of time with c-PTIO, DETA, and cisplatin as indicated. TUNEL/PI analysis was performed.
concomitant addition of a NO donor with c-PTIO restored cell growth to baseline levels, which indicates that the antiproliferative effect was specific to the NO-scavenging properties of c-PTIO.

Depletion of Endogenous NO Enhances Apoptosis in Cisplatin-treated Melanoma Cells—To confirm that the antiproliferative effect of NO depletion was not because of cell death, the TUNEL assay and PI staining were performed. NO depletion alone did not result in a substantial increase in the percentage of TUNEL positive cells or number of hypodiploid cells, indicating that the observed decrease in [3H]thymidine uptake was not a result of cell death (Fig. 2, A and B). Treatment of A375 cells with cisplatin resulted in a dose-dependent increase in TUNEL positivity, indicating a rise in apoptotic cell death. Interestingly, preincubation of cells with c-PTIO followed by cisplatin treatment led to a significant increase in apoptosis, which would not be expected if NO depletion was independent of cisplatin-induced DNA damage. A quenched c-PTIO control had no effect on apoptosis, indicating that the observed effects are NO specific. A similar rise in apoptosis was also seen in A375.S2 and A375.SM melanoma cells, although these cell lines showed increased apoptosis after NO depletion alone (data not shown).

Caspase activation and subsequent PARP cleavage are additional markers of apoptosis (35). Caspase 3 cleavage, which leads to its activation, and PARP cleavage were determined by Western blot (Fig. 2C). The data indicate that cisplatin treatment alone does not trigger significant caspase 3 activation or PARP cleavage, whereas NO depletion with cisplatin treatment resulted in strong caspase 3 activation and PARP cleavage. Cleavage of PARP was closely correlated with caspase 3 activation.

To further confirm that NO depletion enhanced cisplatin-induced apoptosis in melanoma cells, oxymyoglobin, a known scavenger of NO (36), was added to cells prior to cisplatin treatment for 24 h (Fig. 2D). NO depletion with oxymyoglobin at concentrations similar to those used with c-PTIO was also found to enhance cisplatin-induced cell death. Furthermore, the addition of doxycycline, which has been shown to destabilize iNOS mRNA in J774 mouse macrophage cells (37), prior to cisplatin treatment also enhanced cisplatin-induced apoptosis (data not shown). The addition of moderate levels of

Fig. 4. Depletion of endogenous NO inhibits cisplatin-induced p53 stabilization and accumulation and p21 expression in melanoma cells. A, A375 and A375.S2 melanoma cells were incubated with cisplatin for 24 h at 37 °C. Nuclear (N) and cytosolic (C) extracts were generated and size fractionated by SDS-PAGE. Immunoblotting was performed using 1:2,000 anti-p53 antibody. Immunoblotting with 1:5,000 α-tubulin antibody was included to verify the purity of the nuclear extract. B, A375 melanoma cells were pretreated with c-PTIO NO scavenger (250 μM) for 1 h prior to incubation with cisplatin for 24 h at 37 °C. Cell lysates (25 μg) were size fractionated by SDS-PAGE and immunoblotted for p21, p53, and actin. C-PTIO quenched with DETA NO donor (250 μM) was included as a negative control. C, A375 melanoma cells were treated with oxymyoglobin in the context of cisplatin for 24 h. Immunoblots for p21, p53, and actin were performed as in B.

Fig. 5. NOS inhibition reduces cisplatin-induced p53 accumulation and p21 expression. Immunoblot analysis of p21 and p53. A, A375 melanoma cells were preincubated for 24 h with the competitive pan-NOS inhibitor L-NMMA prior to cisplatin treatment for 24 h. B, cells were treated with the iNOS-selective inhibitor AMG prior to cisplatin treatment and immunoblotting. C, the iNOS-specific inhibitor 1400W was added to cells for 24 h prior to cisplatin treatment and immunoblotting. D, doxycycline, which destabilizes iNOS mRNA, was added to cells 24 h prior to cisplatin treatment and immunoblotting.
NO donor prior to cisplatin treatment did not alter the level of apoptosis.

**NO Depletion Causes Alterations in Cell Cycle Distribution and Cell Cycle Response to Cisplatin**—Cell cycle analysis by PI staining showed that treatment with cisplatin alone led to some G1 arrest in A375 melanoma cells, possibly through the up-regulation of p21. NO depletion alone led to an accumulation of cells in the G2 phase, which is consistent with the observed decrease in proliferation. Cisplatin treatment of NO-depleted cells led to a loss of G1 arrest and an accumulation of cells in S and G2 phases. Interestingly, when the results of TUNEL and PI staining were analyzed simultaneously, TUNEL-positive cisplatin-treated cells were mostly found near the G2/S boundary, whereas TUNEL positivity in NO-depleted cells exposed to cisplatin was found in all cell cycle phases (Fig. 3A). Similar findings were observed in A375.S2 and A375.SM cells.

To further dissect the effects of NO depletion and cisplatin treatment on the cell cycle in melanoma cells, cell cycle distribution of synchronized A375 cells treated with c-PTIO and cisplatin was measured over a course of 48 h (Fig. 3B). At T = 0 h, 80–90% of cells are in the G1/S phase, which is generally consistent with other synchronized cell systems. NO depletion led to increased accumulation of cells in the G2 phase over 24 h, after which cell cycle distribution was stabilized in a sustained G2 arrest. A time dependent increase in apoptosis starting after 24 h NO depletion was seen. Treatment with cisplatin alone caused accumulation of melanoma cells at the G2/S boundary for 36 h, at which point cell cycle distribution begins to return to normal. Cisplatin-induced apoptosis can be observed after 16 h. Cisplatin treatment of NO-depleted cells led to a slow decline of cells in G1 phase and a concomitant increase of cells in S and G2 phases over 48 h. Apoptosis in these cells was seen after 8 h and increased substantially over 48 h. Quenched c-PTIO did not alter cell cycle distribution.

**Depletion of NO Inhibits Cisplatin-induced p53 Accumulation and p21 Expression**—The p53 signaling pathway can be activated by DNA-damaging agents and is involved in the induction of apoptosis, cell cycle arrest, and DNA repair (38). NO-depleted melanoma cells exhibited increased cisplatin-induced apoptosis and alterations in cell cycle regulation, suggesting a possible role for p53 and p53-responsive cell cycle regulator p21 in the regulation of melanoma cell response to cisplatin. Baseline expression of p53 and p21 were found to be low in A375, A375.S2, and A375.SM melanoma cells. Cisplatin treatment resulted in stabilization and nuclear accumulation of wild type p53 (Fig. 4A). Expression of the p53 downstream target p21 was also up-regulated, suggesting that p53 activity increases as a result of cisplatin treatment.

To determine whether NO modulation regulates cisplatin-induced p53 accumulation, melanoma cell lines were pretreated with c-PTIO NO scavenger or DETA NO donor prior to cisplatin treatment in parallel with the experiments performed for TUNEL and PI analyses in Fig. 2 (Fig. 4B). Western blot analyses of cell lysates showed that NO depletion resulted in a substantial reduction of cisplatin-induced p53 accumulation in all melanoma cell lines tested. NO depletion strongly inhibited cisplatin-induced p21 expression in close correlation with decreased p53 accumulation, which suggests that p21 expression is p53 dependent in melanoma cells. NO depletion alone did not alter p53 protein levels or localization, whereas the addition of NO increased p53 accumulation only slightly. Correspondingly, p21 expression remained nearly undetectable with either NO depletion or addition alone. Quenched c-PTIO failed to inhibit cisplatin-induced expression of p53 and p21, confirming that the observed effect is specific to NO modulation. Similar results were found when A375.S2 and A375.SM melanoma cell lines were tested (data not shown).

To further show that regulation of cisplatin-induced p53 activation and p21 expression is NO specific, cells were treated overnight with oxygeynogin (Fig. 4C). Oxygeynogin was able to inhibit cisplatin-induced p53 and p21 expression, which is consistent with the results obtained when c-PTIO was used as the NO scavenger. Moreover, incubation with the pan-NOS inhibitor l-NMMA, the iNOS-selective inhibitor AMG, or the iNOS-specific inhibitor N-(3-(aminomethyl)benzyl)acetamidine (1400W) prior to cisplatin treatment also reduced p53 and p21 expression, albeit to a lesser extent (Fig. 5, A–C). Pretreatment of melanoma cells with doxycycline, which destabilizes iNOS mRNA, prior to cisplatin treatment led to inhibition of cisplatin-induced p53 accumulation and p21 expression (Fig. 5D). Thus, the data strongly suggest that NO depletion inhibits p53 accumulation and p21 expression in response to cisplatin and enhances cisplatin-induced apoptosis.
NO Regulation of Cisplatin-induced p21 Expression—Because p21 can be activated by p53-dependent and -independent pathways, we evaluated whether cisplatin-induced p21 expression is p53 dependent. The DNA binding activity of p53 after cisplatin treatment was examined by electrophoretic mobility shift assay using radiolabeled double-stranded oligonucleotides with a sequence corresponding to the p53-binding site of the p21 promoter as the probe. Cisplatin was found to induce strong DNA binding of p53 to the p53-binding site (Fig. 6A), suggesting that increased p21 expression is p53-dependent in melanoma cells. When endogenous NO was removed prior to cisplatin treatment, the level of p53-DNA complex formed was vastly reduced. C-PTIO alone did not affect p53 DNA binding, and a quenched c-PTIO control did not inhibit cisplatin-induced p53 DNA binding. In addition, a nonspecific probe failed to bind p53 or compete with binding of the specific probe (data not shown).

To determine whether p21 is activated via a cGMP-dependent pathway, ODQ, an inhibitor of NO-sensitive guanylyl cyclase, was added to melanoma cells prior to cisplatin treatment. Treatment with ODQ did not inhibit cisplatin-induced p21 expression. Paradoxically, ODQ treatment appeared to enhance p53 accumulation and p21 expression. Similarly, the addition of the plasma membrane permeant cGMP analog 8-bromo-cGMP reduced p21 expression slightly (Fig. 6, B and C), suggesting a possible regulatory role for cGMP in the induction of p21 via the p53 pathway. ODQ and 8-bromo-cGMP treatments did not affect cell cycle distribution or the level of cisplatin-induced apoptosis (data not shown). Taken together, these data strongly suggest that p21 expression is p53-dependent and not cGMP-dependent.

Inhibition of p53 Activity by Expression of Dominant Negative p53 Protein Enhances Cisplatin-induced Apoptosis in A375 Melanoma Cells—To determine whether the increased sensitivity to cisplatin-induced cell death in NO-depleted cells was caused by the inhibition of p53 activity, melanoma cells were transfected with a dominant negative form of p53 (p53DN) to block wild type p53 function. Expression of p53DN enhanced cisplatin-induced apoptosis by ~1.8-fold (Fig. 7A). Basal levels of apoptosis were not affected by p53DN when compared with pMEV empty vector. The p53DN protein, which is tagged with two copies of hemagglutinin and can be seen as a band slightly above wild type p53 in dominant negative-transfected conditions.

![Fig. 7](image-url) Inhibition of p53 activity by dominant negative p53 transfection enhances cisplatin-induced apoptosis. A375 melanoma cells are transfected with empty vector (pMEV) or p53 dominant negative vector (DN) for 24 h prior to a 24-h incubation with cisplatin. A, DNA fragmentation as indicated by TUNEL positivity in dominant negative-transfected melanoma cells after cisplatin treatment. B, immunoblots for p53, p21, and actin. Dominant negative p53 is tagged with two copies of hemagglutinin and can be seen as a band slightly above wild type p53 in dominant negative-transfected conditions.

NO and Cisplatin in Apoptosis, p53/p21 Control in Melanoma
DISCUSSION

NO has been shown to be involved in diverse processes in cellular signaling and is thought to be involved in the potentiation of numerous tumors. In this study, we demonstrated that the depletion of endogenously generated NO in melanoma cell lines containing wild type p53 decreases cell growth, enhances cisplatin-induced apoptosis, and inhibits p53 accumulation and the subsequent induction of p21 by cisplatin. Moreover, we showed that the increase in cisplatin-induced apoptosis in melanoma cells is caused by NO regulation of p53 expression, which exhibits a protective role against cisplatin treatment. These findings are consistent with the clinical observation that patients with iNOS-expressing melanomas had significantly poorer survival than their iNOS-negative counterparts. In addition, the findings that iNOS is up-regulated and overall NOS activity is increased in melanoma further suggest a role for NO (29, 39).

Titration of the NO donor DETA illustrates the dichotomous role of NO in the regulation of proliferation. Addition of 50 μM DETA led to an increase in proliferation, whereas higher levels of DETA (>150 μM) markedly inhibited proliferation. It is now generally accepted that high levels of NO are toxic, whereas lower levels of NO may be growth promoting (reviewed in Refs. 40 and 41), although the threshold between low and high levels of NO is still dependent on the cell type and context within which NO is found.

Our initial observations that NO depletion and cisplatin treatment resulted in reduced proliferation in melanoma cells led us to evaluate whether the effect was attributable to apoptosis. A number of groups have reported both proapoptotic and antiapoptotic properties of NO, depending on cell type and experimental system (22, 23, 42–45). We observed that NO depletion by c-PTIO or oxymyoglobin treatment was capable of enhancing cisplatin-induced apoptosis in melanoma cells. However, when NOS inhibitors, including L-NMMA, AMG, and 1400W, were used to block NO production in melanoma cells, they were only minimally effective in enhancing apoptosis after cisplatin treatment. Salvucci et al. (30) found that serum completely suppresses the apoptotic effect of AMG in melanoma cells, so it is possible that serum components interfere with NOS inhibitor activity or complicate its chemistry. Albumin can act as a NO reservoir, and may control NO-dependent processes in the vasculature (46). Various thiol containing proteins and low molecular weight thiols such as glutathione may also act as a NO store and selectively release NO to maintain cellular NO levels (47). Use of a chemical NO scavenger would nullify NO stores by quickly reacting with any newly released NO, whereas inhibition of NO production by NOS does not deplete possible cellular NO storage mechanisms. Another possibility is that c-PTIO possesses some secondary reactivity (48). However, the lack of effect of quenched NO scavenger controls and the similar effects of oxymyoglobin on melanoma cells strongly suggest that NO is the causal agent in our observations.

Analysis of cell cycle alterations showed that NO depletion by c-PTIO resulted in significant G2 arrest in A375 melanoma cells. This sustained G2 arrest was subsequently accompanied by an increase in apoptosis. Cell cycle alterations were also shown to result from NO depletion in pulmonary cells (49). The precise involvement of NO in cell cycle regulation is not known. However, a number of cell cycle regulators appear to be targets of NO, the most prominent of which is retinoblastoma protein (26). Accumulation of melanoma cells at the G2/M boundary after cisplatin treatment and a trend toward the return to a normal cell cycle distribution after 36 h suggest that melanoma cells are capable of responding to and possibly repairing DNA damage. Our data suggest that p53 response to DNA damage is at least partially intact, as evidenced by the stabilization and nuclear accumulation of p53 after cisplatin treatment and by the subsequent expression of p21. NO-depleted melanoma cells did not accumulate at the G2/M boundary after cisplatin treatment. Instead, cells slowly progressed to the S and G2/M phases, which may be explained by the concomitant loss of cisplatin-induced p21 expression.

We found that NO depletion in melanoma cells led to reduced p53 accumulation and activation after cisplatin treatment in all melanoma cell lines tested. NO depletion also reduced overexpression of mutated p53 in Melo-5 melanoma cells, which suggests that NO can regulate both wild type and mutant p53 (data not shown). In SK-MEL-1 melanoma cells, which exhibit altered p53 and p21 regulation, NO depletion with c-PTIO inhibited basal p21 overexpression and cisplatin-induced p53 accumulation in a dose-dependent manner (data not shown). These data suggest that NO may regulate p53 and p21 regardless of their functional or mutational status.

p53 and iNOS were shown to participate in a negative regulatory loop (50). Specifically, NO was able to activate p53, which in turn inhibited iNOS expression. This observation led to the suggestion that in tumors expressing NO, there may be a selective pressure to acquire p53 mutations (51). This appears not to be the case in melanoma, in which iNOS is up-regulated and p53 mutations are rare. The precise mechanism through which NO modulates the p53 signal transduction pathway remains to be clarified. Our data suggest that NO is required for proper stabilization and activation of p53 protein, which may exert a protective role against therapy.

NO is likely to interact directly with p53 or regulate signaling components upstream of p53. In MCF-7 cells, NO donor treatment resulted in possible tyrosine nitration of p53 and improved DNA binding at low donor levels (0.25–0.5 mM), but inhibited DNA binding at higher donor concentrations (1–5 mM) (16), which alludes to some possibly complex interaction between p53 and NO. Superoxide, known to be up-regulated in melanoma (52), reacts quickly with NO to form peroxynitrite, a potent oxidizing agent capable of nitrating tyrosines (53). A
that p53 siRNA-transfected cells still expressed p53, albeit at a lower concentration (data not shown).

Based on our data we present a model for the mechanism of melanoma resistance to cisplatin treatment (Fig. 8). In this model, NO is a key factor in the p53-dependent activation of p21 expression after cisplatin-induced DNA damage. Expression of p21 results in a skewing of cellular response toward cell cycle arrest, DNA repair, and cell survival, and away from apoptosis. Inhibition of p21 can result in the loss of G1 phase cell cycle arrest, and can lead to the induction of apoptosis. Therefore, the regulation of its expression by NO via the p53 signaling pathway may be of extreme importance in melanoma resistance to therapy. In this case, the ability of p53 to induce p21 would determine whether tumor cells are protected against genotoxic insult. These insights offer several potential pathways to target for future therapies against melanomas containing wild type p53.

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Numerous studies have highlighted the ability of p21 to protect glioblastomas, gliomas, colon carcinomas, prostate cancer (14), and melanomas from apoptosis-inducing stimuli. p21 was also found to be overexpressed and is rarely mutated in melanoma (54, 63). Our data show that p53 and p21 are upregulated in melanoma in response to DNA damage by cisplatin, which suggests that melanoma cells actively respond to genotoxic insult by inducing cell cycle arrest and possibly carrying out DNA repair, thus avoiding cell death. Transfection of a dominant negative form of p53 into A375 melanoma cells resulted in a substantial increase in cisplatin sensitivity as well as a reduction of p21 expression after cisplatin treatment, confirming that p53 activation following exposure to cisplatin protects melanoma cells from apoptosis. Inhibition of p53 by siRNA only partially reduced cisplatin-induced p53 and p21 expression, which was not sufficient to enhance apoptosis after cisplatin treatment. Transfection of mutated p53 into melanoma cells has a dominant effect, which is not the case in siRNA transfection. Cells transfected with siRNA may still produce low levels of wild type p53, and thus could still be capable of responding to DNA damage. Indeed, we observed that p53 siRNA-transfected cells still expressed p53, albeit at a lower concentration (data not shown).

Based on our data we present a model for the mechanism of melanoma resistance to cisplatin treatment (Fig. 8). In this model, NO is a key factor in the p53-dependent activation of p21 expression after cisplatin-induced DNA damage. Expression of p21 results in a skewing of cellular response toward cell cycle arrest, DNA repair, and cell survival, and away from apoptosis. Inhibition of p21 can result in the loss of G1 phase cell cycle arrest, and can lead to the induction of apoptosis. Therefore, the regulation of its expression by NO via the p53 signaling pathway may be of extreme importance in melanoma resistance to therapy. In this case, the ability of p53 to induce p21 would determine whether tumor cells are protected against genotoxic insult. These insights offer several potential pathways to target for future therapies against melanomas containing wild type p53.

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NO and Cisplatin in Apoptosis, p53/p21 Control in Melanoma

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