Motexafin Gadolinium Enhances p53-Mdm2 Interactions, Reducing p53 and Downstream Targets in Lymphoma Cell Lines

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Abstract. Background: Loss of p53 renders cells more susceptible to acute oxidant stress induced by oxidant-generating agents such as motexafin gadolinium (MGd). We hypothesized that reactive oxygen species (ROS)-generating MGd results in low-level p53 expression, making cells more susceptible to oxidant stress. Materials and Methods: Lymphoma cells were incubated with different concentrations of MGd with or without zinc (Zn) and ascorbate, and ROS, apoptosis, proteins, and oxidant genes were measured. Results: MGd, with ascorbate and Zn, induced apoptosis in lymphoma cells. This was accompanied by reduction of p53 protein but not message, and by reduction of p53 downstream targets p21, glutathione peroxidase 1 (GPx1), and p53 up-regulated modulator of apoptosis (PUMA). p53 protein reduction was reversed by MG132, and nutlin-3. Conclusion: Our data are consistent with a pathway of cell death that is independent of p53-mediated induction of PUMA; the cellular response to reduce p53 represents a cell survival adjustment to ROS-mediated stress.

Numerous studies have indicated a role for p53-dependent or -independent apoptotic pathways in a variety of tumor systems (1, 2). p53 is a tumor suppressor gene and is frequently inactivated or mutated in tumors and in transformed cells (3-6). It is considered a guardian of the genome, maintaining genomic stability (5-7). In normal cells, p53 has a rapid turnover, while it is induced by stress-related signals in abnormal cells (8).

The survival of lymphoma cells treated with radiotherapy or chemotherapy depends in part on the regulated expression of pro-survival vs. pro-death genes. Paradoxically, low constitutive activation of p53 promotes the expression of antioxidant genes, such that loss of basal p53 function leads to an increase in intracellular oxidant stress (9). This suggests that low levels of p53 activity act to limit oxidative stress and to inhibit cell proliferation when mild cellular damage occurs.

We used the novel redox-active expanded porphyrin, motexafin gadolinium (MGd), to investigate the effect on p53 expression, reactive oxygen species (ROS), and apoptosis in lymphoma cells. MGd is a small molecule anticancer drug with a novel mechanism of action (10). Inside the cell, MGd disrupts redox-dependent pathways and induces apoptosis. It accepts electrons, in the presence of oxygen, from reducing metabolites and forms ROS by redox cycling (10). In murine models, MGd enhances tumor response to radiation and chemotherapeutic agents. It has demonstrated synergy with ionizing radiation, several chemotherapy agents, and rituximab in both in vitro and in vivo tumor models (11). We used Zn and ascorbate in our study because ascorbate at physiological concentrations enhances MGd redox cycling and apoptosis (12) and was necessary for MGd-induced apoptosis in myeloma cell lines (13). Alteration in heavy metal ion homeostasis may be important in cancer (14), and we have found that MGd disrupts Zn metabolism in human cancer cell lines, leading to increased apoptosis, presumably due to increase in intracellular free Zn (13). It is not surprising, therefore, that we saw more apoptosis in our cell lines with the addition of physiological concentrations of either Zn or ascorbate, and the combination of MGd, Zn and ascorbate resulted in the most extensive apoptosis and also resulted in the largest reduction of p53. In the present study, we investigated the regulation of p53 protein and message by MGd, which generates ROS and induces apoptosis in lymphoma cells.
Materials and Methods

Cells and reagents. Ramos (Burkitt’s lymphoma) (American Type Culture Collection (ATCC), Manassas, VA, USA) and HF-1 (folicular lymphoma) (Pharmaceuticals, Sunnyvale, CA, USA) cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum and in the presence of penicillin, streptomycin and glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO₂. MGd and mannitol solution were kind gifts from Pharmociecs, Inc.; ascorbic acid and zinc acetate from Sigma (St. Louis, MO, USA); 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Molecular Probes, Inc. (Carlsbad, CA, U-S.A), propidium iodide (PI) was from Biosource (Camarillo, CA, USA); FITC-annexin-V Apoptosis Kit from Invitrogen. The following antibodies were used: p53 (Santa Cruz, CA, USA), PUMA, (Cell Signaling, Danvers, MA, USA), and GAPDH (Chemicon, Temecula, CA, USA).

ROS production. ROS production in live Ramos cells was measured by flow cytometry (13, 15). Cells were seeded at a density of 1.0×10⁶ cells/well in 24-well plates and treated with either individual or combined treatments of MGd (100 μM), Zn (100 μM), and ascorbate (100 μM) for 4 and 12 h. Cells were then stained with 5 μM H₂DCFDA and 2 μg PI per well and incubated for 30 min at 37°C in a humidified CO₂ incubator. Cells were washed with phosphate-buffered saline (PBS) and ROS measured by flow cytometry with a Beckman Coulter EPICS XL-MCL Cytometer. All experiments were performed in triplicate.

Measurement of apoptosis. Cellular apoptosis was measured by flow cytometry (13, 15). In brief, cells (1.0×10⁶ cells/well) were treated with either individual or combined treatments of MGd (100 μM), Zn (100 μM), and ascorbate (100 μM) at 37°C in a 5% CO₂ atmosphere for 24 h, washed with ice cold PBS and re-suspended in binding buffer containing 2.5 μl FITC-annexin V and 5.0 μl PI for 15 min at 37°C in a CO₂ incubator. Subsequently, flow cytometric measurements were made on a Beckman Coulter EPICS XL-MCL Cytometer. All experiments were performed in triplicate.

Western blot analysis. Treated cells were washed with PBS, centrifuged, and cell pellets treated with RIPA buffer containing protease inhibitors. Cell lysates were centrifuged and supernatants stored at -80°C. An aliquot of each supernatant was used for protein determination (16). Fifty μg protein of each sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane and probed with the antibodies specified above. Immune complexes were visualized by enhanced chemiluminescence.

RNA isolation and PCR. Granta cells were treated with 100 μM each MGd, ascorbate and Zn for 5 h. Total RNA was isolated by the Qiagen method and dissolved in RNase-free water. One μg RNA was used for reverse transcription using Invitrogen’s one-step superscript Taq DNA polymerase system. PCR amplification was performed at 94°C for 30 s (denaturation), 58°C (annealing) and 72°C (extension) for 40 cycles. Anti-sense and sense primers for p53 and GAPDH (17) (IDT Technologies, Coralville, IA, USA) were used and DNA was identified by 1% agarose gel electrophoresis. Anti-sense and sense primers for sestrin1-T2 variant (SESN1 (T2), sestrin2 (SESN2), glutathione peroxidase 1 (GPX1), cyclin-dependent kinase inhibitor 1A (CDKN1A), and peptidyldprolyl isomerase A (PPIA) (cyclophilin A) (9) (IDT Technologies) were used and DNA identified by 1% agarose gel electrophoresis.

Statistical analysis. Data from ROS measurements in live cells were analyzed and expressed as mean fluorescence, and data from apoptosis assays were expressed as the percentage of apoptotic cells (annexin V-positive and PI-positive) over total cells. Statistical significance was performed by one-way ANOVA and Newman-Keuls multiple comparison test (GraphPad Software, Inc., San Diego, CA, USA) to assess the effects of drugs on apoptosis and ROS production. All experiments were performed in triplicate.

Results

MGd increases intracellular ROS production and apoptosis in Ramos lymphoma cells. MGd, Zn and ascorbate combination significantly increased ROS production when compared to controls (Figure 1A). MGd, Zn, or ascorbate alone did not alter ROS production. N-Acetylcyesteine (NAC; 10 mM) significantly inhibited the combined effects of MGd, Zn, and ascorbate. These data demonstrate that MGd, with physiological concentrations of ascorbate and Zn, increases the production of intracellular ROS in Ramos cells. We next determined the effects of MGd on apoptosis. Following 24 h of incubation, MGd combined with Zn and Asc induced significant apoptosis in Ramos cells compared to apoptosis seen with individual treatments of MGd, Zn, and ascorbate (Figure 1B).

MGd reduces p53 protein expression in lymphoma cell lines. Because of the dual role of p53 in ROS-mediated pathways, we next determined p53 protein expression after MGd (0.01-100 μM), with Zn (100 μM), and ascorbate (100 μM). In HF-1 cells (Figure 2A), MGd elicited a dose-dependent decrease in p53 in HF-1 cells. Similarly, in Ramos cells, at 100 μM, MGd combined with Zn and ascorbate dramatically reduced p53 expression at 5 h (Figure 2B), but not at 0.5, 1 or 2 h (data not shown). To determine ROS effects on p53, ROS generation was blocked with NAC (10 mM) and cells treated with MGd (100 μM), Zn (100 μM) and ascorbate (100 μM) for 5 h. In the presence of NAC, p53 levels were restored with MGd/Zn/Asc treatment as shown in Figure 2B.

MGd does not reduce p53 message in Ramos cells. To determine if the observations on p53 protein were related to alteration of p53 message, p53 was examined by RT-PCR after RT-PCR after MGd, Zn and ascorbate. mRNA levels detected by RT-PCR suggested that there was no loss of p53 message under these conditions in the Ramos cells (Figure 2C).

p53 restoration after MG132 or Nutlin-3 in Ramos lymphoma cells. The ubiquitin-proteasome pathway has been implicated in several forms of malignancy as it plays a major role in the degradation of proteins involved in cell
cycle, proliferation, and apoptosis. Since proteasome antagonists inhibit the ubiquitin-proteasome pathway blocking the selective degradation of intracellular proteins, we examined p53 degradation pathways in Ramos cells after MGd. In the absence of MG132, p53 levels significantly decreased with MGd/Zn/Asc as shown in Figure 3A. However, MG132 reversed the effects of MGd/Zn/Asc by restoring p53 expression in Ramos cells (Figure 3A). In addition, the level of the p53 downstream target PUMA was enhanced following inhibition of proteasome function with MG132 (Figure 3A). Since Mdm2, a p53-specific ubiquitin ligase, which is a negative regulator of p53, mediates the ubiquitin-dependent proteolysis of p53 (18), we also examined the role of Mdm2 in p53 degradation by MGd. We found that p53 protein expression was almost completely restored when cells were co-incubated with Nutlin-3 and MGd/Zn/Asc in both Ramos as well as in HF-1 cells (Figure 3B).

Changes in pro- and antioxidant gene expression. We next determined if transcriptional targets of p53 involved in oxidant pathways might be altered after MGd-induced reduction of p53. We studied GPX1, p53-regulated SESN1 and SESN2, and p21 (CDKN1A). The T2 transcript of the SESN1 gene was absent from these cells. Co-treatment of MGd/Zn/Asc notably reduced GPX1, SESN2, and CDKN1A transcripts but these transcripts were restored with the Mdm2 inhibitor Nutlin-3 (Figure 4).

Discussion

In the current study, we examined the effects of the redox inducer, MGd, on ROS and p53 pathways in Ramos and HF-1 lymphoma cells. MGd is a small molecule anticancer drug with a novel mechanism of action, which selectively accumulates in cancer cells due to their increased metabolic rates (10, 19). Inside the cell, MGd disrupts redox-dependent pathways and induces apoptosis. It accepts electrons in the presence of oxygen from reducing metabolites and forms ROS by redox cycling (10). In vitro studies with various cancer cell lines have shown an increase in ROS and intracellular free Zn in cells treated with MGd (13). We have shown that MGd, in a dose- and time-dependent manner, with the addition of Zn and ascorbate at physiological concentrations, down-regulates p53 protein without affecting p53 message, suggesting that the mechanism involves post-translational modification of p53. That this might be mediated by ROS is suggested by the data presented in Figure 1 showing significant production of ROS in Ramos cells after MGd/Zn/Asc and partial reversal by NAC.

These observations are not the result of altered p53 message, as there was no effect on p53 message after MGd exposure. Furthermore, interference with p53 degradation pathways by MG132 and Nutlin-3 reliably restored p53 protein expression. The decreased p53 protein resulted in a decrease in PUMA expression and also resulted in down-regulation of genes engaged in oxidant and antioxidant pathways (GPX1, SESN2, and CDKN1A) (20, 21). Importantly, these transcripts were
restored by inhibition of Mdm2, a protein involved in a p53 degradation pathway, by Nutlin-3. Reduction of GPX1 as a result of decrease of p53 is consistent with the observation that p53 induces GPX1 promoter (22), and restoration by Mdm2 inhibition is consistent with a functional role for p53 in regulation of genes involved in redox regulation.

The observation that downstream targets PUMA, p21 and GPX1 were reduced along with p53 is important in relation to the cell death pathways in cancer cells. p53, a transcriptional activator, increases the expression of pro-apoptotic BCL-2 family members to mediate apoptosis. However, we found that MGd with Zn and ascorbate increased apoptosis, and that this was associated with ROS production and reduction of p53 and its downstream targets. We can speculate that the cell death that we observed in the lymphoma cell lines was a consequence of extensive production of ROS, that the usual p53-mediated pathways were not engaged, and that the cell, directly attacked by ROS, attempted to avoid death by reducing p53 and turning off downstream pro-apoptotic signals. Consistent with this observation are recent data that show that ROS are not involved in apoptosis mediated by the proteasome inhibitor MG132 in colon cancer cells, but that p53 downstream targets PUMA and BAX are (23). This supports the idea that in our experiments, apoptosis was mediated through ROS generation and could be separated from p53 and its downstream targets. We can also speculate that the cell has adapted further by attempting to turn to other survival tactics, such as activating autophagy pathways, and a search for markers of autophagy would be informative (24). There are data that suggest that ROS and nuclear factor-kappaB (NFkB) transcription factors may regulate autophagy and that this may be a mechanism involved in cell death after exposure to stress (25).

We found that the reduction of p53 protein by MGd was not due to a reduction in message but rather post-translational modification of p53 protein, perhaps as a consequence of increased p53 degradation (26). While we did not find that Mdm2 was increased after MGd, we did find that p53 and its downstream intermediates PUMA, p21 as well as GPX1 could be restored after addition of either the proteasome inhibitor MG132 or the E3 ubiquitin ligase Mdm2 inhibitor Nutlin-3.

Figure 2. MGd reduces p53 protein expression, but not message in lymphoma cells. A: This experiment illustrates the effects of a 5-h treatment with a range of concentrations of MGd (0.01-100 μM), with Zn (100 μM), and ascorbate (100 μM) on p53 in HF-1 cells. B: In Ramos cells, MGd (100 μM), in the presence of Asc (100 μM), and Zn (100 μM) reduced p53 protein. Co-incubation of cells with N-acetylcysteine (NAC; 10 mM) with MGd (100 μM), Zn (100 μM) and Asc (100 μM) for 5 h restored p53 levels. C: RT-PCR of p53 RNA showing no alteration in p53 message in Ramos lymphoma cells. Ramos cells were treated for 5 h with the following: Zn/ascorbate (100 μM each); MGd (100 μM); MGd/Zn/ascorbate; NAC (10 mM); and NAC/MGd/Zn/ascorbate.
In response to stress signals, the normal interaction between p53 and Mdm2 is altered, and p53 protein undergoes post-translational modification. That this is reversed by the Mdm2 inhibitor Nutlin-3 suggests that Mdm-2 is involved in the ROS-mediated stress induced by MGd.

The role of Zn and ascorbate in MGd-mediated cell death has been studied. Ascorbate at physiological concentrations enhances MGd redox cycling and apoptosis, and was necessary for MGd-induced apoptosis in myeloma cell lines (13). Alteration in heavy metal ion homeostasis may be important in cancer (14), and we have found that MGd disrupts Zn metabolism in human cancer cell lines leading to increased apoptosis, presumably due to increase in intracellular free Zn (13). It is not surprising, therefore, that we saw more apoptosis in our cell lines with the addition of physiological concentrations of either Zn or ascorbate, and that the combination of MGd, Zn and ascorbate resulted in the most extensive apoptosis and also resulted in the largest reduction of p53. We have not measured intracellular free Zn in these studies, but correlation of apoptosis and ROS production with intracellular levels of free Zn would be informative (28).

(27). Figure 3. Reduction of p53 protein and PUMA is mediated by proteosomes and Mdm2. Cells were treated with 100 μM each of MGd, Zn, and ascorbate alone and in combination for 5 h at 37°C in 5% CO2 humidified atmosphere. Cells were incubated in the presence or absence of the given doses of MG132 (A) or Nutlin-3 (B). Following incubations, cells were washed, lysed, and whole cell lysates were electrophoresed and Western blots performed as described in the Materials and Methods.

Figure 4. p53-regulated oxidant genes in Ramos lymphoma cells. Ramos cells were treated for 5 h with the following: Zn, ascorbate (100 μM each); MGd (100 μM); MGd/Zn/ascorbate; Nutlin (15 μM); and Nutlin/MGd/Zn/ascorbate. Following incubation, total RNA (sestrin1-T2 variant (SESN1 (T2), sestrin2 (SESN2), glutathione peroxidase 1 (GPX1), cyclin-dependent kinase inhibitor 1A (CDKN1A), and peptidylprolyl isomerase A (PP1A) (cyclophilin A)) was isolated and amplified as described in Materials and Methods.
In summary, we have demonstrated that the redox cycler MGD, with ascorbate and/or Zn, induces apoptosis in lymphoma cell lines, that this is related to reduction of p53 protein but not message, and that this is accompanied by reduction of p53 downstream targets p21, GPX1, and PUMA. These effects are reversed by the proteasome inhibitor, MG132, and the Mdm-2 small molecule inhibitor, Nutlin-3, suggesting that the loss of p53 is related to post-translational modification of p53. We suggest that these observations are consistent with a pathway of cell death that is not dependent on p53-mediated induction of pro-apoptotic protein PUMA and that the cellular response to reduce p53 represents a cell survival adjustment to ROS-mediated stress.

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Author Contributions

A.T.K.S. designed and performed experiments, analyzed data, prepared figures, and wrote the manuscript; L.I.G. designed experiments, analyzed data and wrote the paper; A.M.E. contributed to data analysis and review; and S.P. performed part of the experiments.

References

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