Wild-Type p53 Suppresses Transcription of the Human O6-Methylguanine-DNA Methyltransferase Gene

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Abstract

High-level expression of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) correlates with cellular resistance to the chloroethylnitrosourea (CENU) class of alkylating agents. Consequently, tumors expressing low levels of MGMT are sensitive to CENU chemotherapy, and any mechanism that can be used to reduce MGMT levels could sensitize resistant tumors. We have demonstrated that transient transfection of wild-type, but not mutant, p53 protein into a p53-null cell line, Saos-2, suppresses MGMT promoter activity in a reporter gene system. In addition, following a 24-h transduction of IMR90 fibroblasts with a wild-type p53-adenoviral vector, endogenous MGMT protein is down-regulated and is no longer detectable 5 days following infection. Because p53 is inducible by ionizing radiation, we propose that existing cancer therapy regimens that combine radiotherapy with CENU chemotherapy may be improved by altering scheduling and allowing enough time between the two therapies for the relatively stable MGMT protein to degrade.

Introduction

MGMT\(^\text{1}\) (EC 2.1.1.63) is a DNA repair protein that is responsible for the removal of O6-alkylguanine adducts from DNA (1). It is expressed constitutively in normal cells and tissues (1); however, the level of expression in tumors does not correlate with that of the tissue of origin, and a proportion of tumors (20%) is totally deficient in MGMT protein (2). The level of MGMT expression correlates with cellular resistance to alkylating agents (1), a class of drugs that generate O6-alkylguanine lesions, and consequently, human tumor xenographs deficient in MGMT are sensitive to such agents (3). An understanding of MGMT gene regulation may enable mechanisms to be devised by which protein levels of resistant tumors may be manipulated. We have previously cloned and characterized the MGMT promoter (4), and we and others have suggested that regulation is controlled at the level of transcription (1, 5).

p53 is a tumor suppressor gene commonly mutated in human tumors (6). It encodes a phosphoprotein that has the capacity to act as a transcription factor by binding to a specific site contained within promoter sequences of a number of genes e.g., p21 (7), BAX (8), and MDM2 (9). Although the wild-type protein up-regulates the expression of genes containing a p53-binding site, it has also been shown to down-regulate expression of a variety of genes without this site, e.g., c-fos, β-actin, and hsc70 (10). In addition, mutant p53 protein does not interact with the p53-binding site; yet, various p53 mutants have been shown to up-regulate the expression of certain genes (10). Owing to its transcriptional activity, we decided to investigate the effect of both wild-type and mutant p53 expression on MGMT gene transcription and elucidated that MGMT was in the class of genes lacking a specific p53-binding site but having expression that was down-regulated by the wild-type protein. The implications of this observation for using p53 to down-regulate MGMT clinically in drug-resistant tumors are discussed.

Materials and Methods

Cell Lines. The Saos-2 osteosarcoma cell line (a gift from G. Zambetti, St. Jude Children’s Research Hospital) was grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS (HyClone Laboratories, Inc., Logan, UT). IMR90 normal human lung fibroblasts and the human embryonic kidney cell line 293 were obtained from the American Type Culture Collection (Rockville, MD) and grown in MEM (Life Technologies) containing nonessential amino acids (Life Technologies) and DME, respectively. All cell lines were maintained in a humidified atmosphere of 95% air at 37°C. IMR90 cells required 5% CO\(_2\), whereas Saos-2 and 293 cells required 10% CO\(_2\) for growth.

Plasmids. pKTBCAT is a CAT reporter gene plasmid containing a 1.2-kb BamHi-Sal I MGMT promoter fragment described previously as construct 1 by Harris et al. (5). pCOSXlCAT (a gift from Dr. A. J. Levine, Princeton University, Princeton, NJ) is an MDM2 promoter-CAT construct described previously (9), which is referred to as MDM2CAT in this article. The negative controls pOCA1 and p1643CAT are the backbone plasmids for pKTBCAT and pCOSXlCAT, respectively. The p53 expression plasmids are: pC53-CIN (wild-type p53); pC53-Cx3, (mutant p53, V143A); pC53-Cx22AN (mutant p53, R175H); pC53-248 (mutant p53, R248W); pC53-4.2N (mutant p53, R273H); and pC53-Cx7AN (mutant p53, D281G). The negative control plasmid containing no p53 is PCMVneo-Bam.

Transient Transfections and CAT Assay. Plasmids were transfected into Saos-2 cells by electroporation using a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA) at 960 microfarads and 200 V, as described previously (11). The CAT reporter gene plasmids (5 μg) and the p53 expression plasmids (10 μg) were cotransfected into 1 × 10\(^7\) cells. Transfections were repeated at least three times per experimental variable, and for each one, the total amount of plasmid remained constant. At 48 h, cells were harvested, and whole cell extracts were assayed for CAT activity by a two-phase liquid scintillation counting assay using \(^{3}H\)acetylcoenzyme A (Amersham, Arlington Heights, IL) as a substrate (12). CAT activity was calculated as cpm \(^{3}H\)acetylchloroformic acid/bmg protein.

Adenoviral Vectors. The recombinant, wild-type p53 adenovirus was obtained from Genetic Therapy, Inc. (Gaithersburg, MD) and expresses p53 under the control of the Rous sarcoma virus promoter. The virus was amplified in 293 cells, which contain adenoviral E1A and E1B genes, allowing viral replication, and purified on two cesium chloride gradients, followed by dialysis (13). The concentration of the virus was determined by titering on 293 cells using a standard procedure (13). Experimental cells were transduced at a multiplicity of infection necessary to infect 100% of the cells. This amount was determined by using an adenoviral vector expressing β-galactosidase (Genetic Therapy, Inc.). Cells were plated into four-well-chamber slides (Nunc, Inc., Naperville, IL) at a density of 40,000 cells/well, and following overnight attachment, they were transduced for 24 h with a range of viral concentrations.

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3The abbreviations used are: MGMT, O6-methylguanine-DNA methyltransferase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; VC, vector control; HRP, horseradish peroxidase; TBP, TATA box-binding protein; PCNA, proliferating cell nuclear antigen.
At this time, X-Gal, a substrate for the β-galactosidase enzyme, was added to elucidate cellular enzyme content (14). The minimum number of virus particles required to infect 100% of the cells was determined by visualizing the blue cells by light microscopy.

**Antibodies.** p53-DO1-HRP, a HRP-conjugated, mouse monoclonal antibody, which can detect both wild-type and mutant human p53 proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), was used to detect the wild-type p53 expressed from the adenoviral vector. The mouse antihuman p21 monoclonal antibody was obtained from Pharmingen (San Diego, CA), and the mouse antihuman β-tubulin antibody was obtained from Sigma Chemical Co. (St. Louis MO). The antihuman MGMT antibody MT 3.1 was generated in this laboratory (15). The antimonouse secondary HRP-conjugated antibody necessary for detection of the p21, tubulin, and MGMT proteins was obtained from Amersham.

**Western Analysis.** Cell extracts (50 µg) were electrophoresed on a 12.5% SDS-polyacrylamide gel using the Bio-Rad Mini Protein II system, and proteins were electroblotted to polyvinyllidene fluoride membranes (Immobilon; Millipore, Bedford, MA) using the Bio-Rad Mini electroblotter. Membranes were blocked in BLOTTO (5% nonfat dried milk in TBS-T: 20 mM Tris, 137 mM NaCl (pH 7.6), and 0.1% Tween-20) for 1 h at room temperature, followed by incubation with the primary antibody diluted in BLOTTO for a further hour. After three washes in TBS-T (two for 5 min and one for 15 min), the secondary antibody diluted in BLOTTO was added for 1 h. The final washes in TBS-T consisted of two for 15 minutes and one for 30 min, prior to the addition of enhanced chemiluminescence (Amersham) detection reagents, as described by the manufacturer. Proteins were visualized by exposure to X-ray film (Kodak X-OMat AR; Eastman Kodak Co., Rochester, NY) for time intervals ranging from a few seconds to 1 h, depending on the protein abundance.

**Results**

**Wild-Type p53 Expression Inhibits MGMT Promoter Function.** Saos-2 cells were transiently transfected with 5 µg of either the MGMT promoter-CAT reporter gene construct (pKTBCAT) or pOCAT1 (negative control), together with 10 µg of either a p53 expression plasmid or pCMVneo-Bam (negative control). Saos-2 cells were used for these experiments, because they are null for p53 and, therefore, contain no endogenous p53 proteins, wild-type or mutant, which could influence the transfection results. The CAT activity produced in cells transfected with the two control plasmids (background) was subtracted from experimental values. Fig. 1A shows the relative MGMT promoter activity detected following cotransfection with either a wild-type p53 expression plasmid or one of five plasmids expressing different p53 mutants. The first column represents MGMT promoter activity generated following cotransfection with pCMVneo-Bam containing no p53 gene; all of the other results have been calculated relative to this value. In the presence of wild-type p53, the MGMT promoter produced only 4% of the relative promoter activity generated without p53 expression, whereas the five different p53 mutants had little effect, if any, on MGMT promoter function.

To demonstrate that the wild-type p53 protein was functioning normally, a similar experiment was conducted to examine the effect of the wild-type p53 promoter on a promoter known to be inducible by wild-type p53, i.e., MDM2. The MDM2 promoter generated a low level of CAT activity compared with the VC without the presence of wild-type p53 (Fig. 1B). However, when MDM2CAT was cotransfected with the wild-type p53 expression plasmid, CAT activity increased 9-fold, demonstrating the presence of a functional protein (Fig. 1B).

**Location of the p53-negative Regulatory Element to a 271-bp Region of the MGMT Promoter.** The effect of wild-type p53 expression on the transcriptional activity of MGMT promoter deletion fragments was investigated by performing transient transfections into Saos-2 cells, as described above. As before, the total plasmid concentration remained constant using pCMVneo-Bam in place of the p53 expression plasmid in the samples without p53 expression. pKTBCAT (MGMT promoter) activity, in the absence of p53 expression, decreased as deletions were made from the 5' end of the fragment (Fig. 2). Consistent with data that have previously been published following transfection into NIH3T3 cells (4). Once pKTBCAT was cotransfected with the wild-type p53 expression plasmid, p53-CIN. the results have been calculated relative to the value generated by cotransfection of the CAT-VC plasmid. p1643CAT with pCMVneo-Bam (VC for the p53 expression plasmid). Columns, mean promoter activity from at least three independent transfections; bars, SD. B, histogram of relative promoter activity generated by the MDM2 promoter-CAT construct when cotransfected with the wild-type p53 expression plasmid, p53-CIN. The results were calculated relative to the value generated by cotransfection of the CAT-VC plasmid p1643CAT with pCMVneo-Bam (VC for the p53 expression plasmid). Columns, mean promoter activity from at least three independent transfections; bars, SD. This full-length promoter construct was the only one of the promoter fragments tested able to generate any detectable CAT activity when in the presence of p53. The smallest fragment to generate any promoter activity without p53 in Saos-2 cells was the 271-bp Smal–SstI fragment, and this also lost function when cotransfected with wild-type p53. Therefore, the p53-negative regulatory element responsible for inhibition of MGMT transcription must be contained within this 271-bp promoter region.

**Endogenous MGMT Protein Can Be Down-Regulated by Overexpression of Wild-Type p53.** To demonstrate that overexpression of wild-type p53 could effect endogenous MGMT protein, as well as its promoter function, IMR90 cells were transduced with a wild-type p53 adenovirus. The virus was removed after a 24-h infection period, and cells were harvested at 24-h intervals up to 120 h. Whole cell extracts were prepared and subjected to Western analysis as described in "Materials and Methods." Saos-2 cells could not be used for this

Fig. 1. A, histogram of relative MGMT promoter CAT activity generated from the 1.2-kb BamHI–SstI maximal promoter fragment. The MGMT promoter-CAT construct was cotransfected with either the VC plasmid (pCMVneo-Bam; −/p53), or various p53 expression plasmids containing wild-type or mutant genes. The different mutant p53 genes used for transfection are identified by their mutation (m) sites. The values for the CAT-VC plasmid (pOCAT1) cotransfected with pCMVneo-Bam have been subtracted. Columns, mean promoter activity from at least three independent transfections; bars, SD. B, histogram of relative promoter activity generated by the MDM2 promoter-CAT construct when cotransfected with the wild-type p53 expression plasmid, p53-CIN. The results have been calculated relative to the value generated by cotransfection of the CAT-VC plasmid p1643CAT with pCMVneo-Bam (VC for the p53 expression plasmid). Columns, mean promoter activity from at least three independent transfections; bars, SD. Wi, wild-type.
experiment, because they are deficient in MGMT expression. Normal IMR90 cells were chosen, because they express 0.64 pmol/mg MGMT (16), contain wild-type p53, and do not require a high concentration of virus to achieve 100% cell transduction; a multiplicity of infection of only 1 is required for all of these cells to be infected.

Fig. 3 shows Western blots of these extracts probed with p53, p21, β-tubulin, and MGMT antibodies. After exposure to the virus, p53 protein, which is present at low levels in IMR90 cells (data not shown), was detected 48 h after the start of p53 viral transduction. However, p53 levels were increasing prior to this time, as determined by the induction of p21 visible at 24 h. MGMT protein levels began to decrease at about 48 h and had completely disappeared by 120 h, indicating that wild-type p53 was capable of down-regulating MGMT protein expression as well as the MGMT promoter. To demonstrate that the effect of adenoviral infection was selective, a β-tubulin antibody was used as a loading control. In contrast to MGMT, β-tubulin levels did not decrease (Fig. 3).

Discussion

Wild-type p53 transcriptionally activates genes containing a p53 recognition sequence in their promoter regions (10). However, this report demonstrates that overexpression of wild-type p53 can down-regulate the MGMT promoter, which contains no p53-binding site (Fig. 1A). Wild-type p53 protein has previously been shown to inhibit transcription of a number of genes, e.g., c-fos, β-actin, and hsc70 (10), all containing TATA box sequences in their promoters. Because promoters of genes such as H-ras and epidermal growth factor have no TATA box and are unaffected by p53, it has been suggested that a TATA box is a prerequisite for p53 transcriptional suppression (10). However, genes such as β-actin, which is present at low levels in IMR90 cells (data not shown), was detected 48 h after the start of p53 viral transduction. However, p53 levels were increasing prior to this time, as determined by the induction of p21 visible at 24 h. MGMT protein levels began to decrease at about 48 h and had completely disappeared by 120 h, indicating that wild-type p53 was capable of down-regulating MGMT protein expression as well as the MGMT promoter.

To demonstrate that the wild-type p53 protein was functioning correctly, its effect on MDM2 gene transcription was investigated. The MDM2 promoter contains p53-binding sites and, therefore, can be transactivated by wild-type p53 (9). Fig. 1B demonstrates that wild-type p53 protein transiently expressed from the pC53-CIN plasmid transactivated the MDM2 promoter, as expected.

Mutant p53 has no known DNA-binding specificity, does not bind TBP, and does not activate promoters containing p53-binding sites (10). However, several mutant genes, e.g., p53 143A, 175H, 249W, 273H, and 281G, have been shown to transactivate the multidrug resistance promoter (10), and p53 mutants 175H, 249W, 273H, and 281G are able to transactivate the PCNA promoter (18). We chose to investigate the effects on MGMT promoter function of five p53 mutants: 143A, 175H, 249W, 273H, and 281G, all of which have been previously shown to have transactivating potential with other promoters, as described above. None of these mutants affected MGMT promoter function when overexpressed in Saos-2 cells (Fig. 1A), demonstrating that MGMT transcription is not sensitive to mutant p53.

Based on our observations that wild-type p53 suppresses MGMT transcription, one would expect cell lines containing wild-type genes to have lower MGMT levels than those with mutant p53. In general, this seems to be true; normal lung fibroblast lines IMR90 (0.64 pmol/mg; Ref. 16) and WI38 (0.3 pmol/mg)4 and ML1 myeloid leukemia cells (0.6 pmol/mg; Ref. 19)4 are all wild type for p53 and have low levels of MGMT. Examples of cell lines containing mutant p53 protein are the colon carcinoma line GC/c1 and the Raji Blymphoblast line (19), which express high levels of MGMT (1.2 pmol/mg; Ref. 20).4 An exception is the rhabdomyosarcoma cell line Rh18, which is wild type for p53 (21) and expresses a relatively high level of 1.3 pmol/mg MGMT (20). The p53 in this line, however, is probably inactive, because Rh18 also has an amplified MDM2 gene,4 and the MDM2 protein is known to inhibit p53 function (9). Whether a true correlation exists between p53 status and MGMT expression needs to be determined by further experimentation.

Once it had been demonstrated that wild-type p53 could affect MGMT promoter function in a transient reporter-gene system, it was...
necessary to show that endogenous MGMT protein could also be down-regulated. Fig. 3 demonstrates that 48 h following IMR90 cell transduction with the p53-adenoviral vector, functional p53 protein was induced, as determined by the presence of p21. MGMT protein began to decrease at 48 h and was no longer detectable at 120 h, indicating that inhibition of promoter activity influenced endogenous protein production, as expected. It took 5 days for MGMT protein to disappear, probably because of the long half-lives of MGMT mRNA and protein, >10–12 h (22) and 15–20 h (23), respectively.

Because high-level expression of MGMT confers drug resistance (1, 3), the observation that wild-type p53 can reduce the level of MGMT in cells has important implications for cancer chemotherapy. Ionizing radiation is known to induce p53 protein levels (19); therefore, radiation in combination with a chloroethylnitrosourea antiangiogenic agent, e.g., 1,3-bis-(2-chloroethyl)-1-nitrosourea, may allow sensitization of previously drug-resistant tumors. This combination therapy is already used in the clinic for the treatment of brain tumors (24, 25) and may be improved by increasing the interval between radiation and chemotherapy, allowing the existing MGMT protein time to decay.

It has been proposed that p53 induction following DNA damage arrests cell growth, providing time for increased DNA repair prior to cell division (19). The data presented here show that the DNA repair enzyme MGMT is inhibited rather than induced on p53 overexpression, which is inconsistent with this notion, i.e., increased repair. Similarly, a recent article by Pan et al. (26) showed that p21 inhibits nucleotide excision repair by interacting with PCNA. However, at present, these data are controversial, because a previously published report by Li et al. (27) stated that p21 interacts with PCNA, inhibiting only its replication function.

The physiological function of DNA repair inhibition by p53 induction is unclear at present. Perhaps under conditions of severe DNA damage, when p53 and p21 are induced to very high levels, the cell prepares to undergo apoptosis and shuts down expression of a number of genes. However, following MGMT depletion in IMR90 cells (Fig. 3), there was no evidence of cell death, only growth inhibition; therefore, if this hypothesis is correct, down-regulation of MGMT could be an early measurable event in the apoptotic pathway.

This study demonstrates that following transient overexpression of wild-type p53 protein, MGMT transcription and expression are down-regulated. These observations could lead to the improvement of chloroethylnitrosourea cancer treatment protocols by combining them with either a p53-inducing modality, such as ionizing radiation, or p53 adoviral transduction.

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References


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