SHORT COMMUNICATION

O6-Methylguanine-DNA methyltransferase activity, p53 gene status and BCNU resistance in mouse astrocytes

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We observed previously that wild-type p53 rendered neonatal mouse astrocytes resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in a gene dose-dependent fashion. This effect of p53 appeared to be unrelated to its cell cycle regulation or apoptotic functions. Because in many cell types O6-methylguanine-DNA methyltransferase (MGMT)-mediated DNA repair is an important mechanism of resistance to nitrosoureas, we measured MGMT activity in wild-type, heterozygous and p53 knockout neonatal mouse astrocytes. Wild-type p53 astrocytes had significantly greater MGMT activity than either heterozygous or p53 knockout astrocytes: MGMT activity was ~5-fold greater in wild-type p53 astrocytes than in p53 knockout cells. However, despite successful depletion of MGMT activity in wild-type astrocytes by O6-benzylguanine (BG), resistance to BCNU persisted unchanged. Moreover, we excluded the possibility that continued resistance to BCNU at the concentrations used could be explained by a compensatory induction of MGMT triggered by exposure to either BCNU or BG. Although these studies support a role for p53 regulation of MGMT in neonatal mouse astrocytes, BCNU resistance in wild-type cells appears to be mediated by a non-MGMT mechanism. Nevertheless, regulation of DNA repair by MGMT may be another mechanism by which alterations of the p53 gene promote tumor initiation or progression.

The p53 protein protects against DNA damage by arresting cell cycle progression (1–4), allowing time for repair, and by triggering apoptosis (5–7), perhaps through induction of genes involved in the oxidative stress response (8). In addition, p53 may regulate DNA repair directly, regulate multidrug resistance or regulate other drug resistance mechanisms (9–23). Since p53 can be normal or mutated in human cancers, including brain tumors (24–28), p53 status may influence their response to DNA-damaging chemotherapies in complex ways.

The effect of p53 status on the response of human astrocytomas to cytotoxic drugs is unknown, although the tumors of elderly patients, usually with intact p53 (27), appear more resistant to chemotherapy than similar tumors in younger patients (29–31), which often harbor such mutations (27). We have hypothesized that properties intrinsic to the astrocyte together with the effects of specific genetic alterations causing cancer might influence the response of astrocytomas to chemotherapy. We began this line of inquiry by exploring the relationship between p53 gene status and drug sensitivity in astrocytes, a detoxification and barrier cell in the brain (32), and observed that wild-type p53 rendered mouse astrocytes resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a bifunctional alkylator used clinically, in a gene dose-dependent fashion. Resistance to BCNU in astrocytes could not be explained by differential cell cycle arrest nor p53-mediated apoptosis (33).

In some cells, resistance to BCNU and other alkylating agents is mediated by O6-methylguanine-DNA methyltransferase (MGMT) and depletion of MGMT activity by O6-benzylguanine (BG) reverses resistance (34–38). MGMT repairs DNA damage by removing alkyl groups from the O6 position of guanine, a critical site of alkylation by the nitrosoureas. Three recent studies have suggested that p53 may regulate MGMT activity (17,19,22) and Russell et al. (39) described a correlation between p53 mutation and sensitivity to procarbazine, another clinically useful alkylating agent. Hence, to further investigate the relationship between p53 gene status and BCNU resistance in normal astrocytes, we measured MGMT activity in wild-type (+/+), heterozygous (+/−) and p53 knockout (−/−) mouse astrocytes.

To determine the effect of p53 on MGMT activity, astrocytes were isolated from mice with distinct p53 genotypes and MGMT levels measured. Cultures of +/+, +/− and −/− astrocytes were prepared from the cerebrums of newborn TSG−/− mice (GenPharm International, Taconic, Germantown, NY; 40) using a procedure adapted from McCarthy and de Vellis (41). Cerebral hemispheres were isolated from unanesthetized neonates, dissected free of meninges and blood vessels and a single cell suspension prepared by a combination of enzymatic (0.025% trypsin) and mechanical dissociation. All cultures were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum and 50 U/ml penicillin/streptomycin. p53 genotype was determined by PCR using genomic DNA isolated from mouse tails (42). Cells of identical genotype were pooled from littermates (2–6 mice) for each independent experiment.

MGMT activity was assayed by incubation of cell extracts with [3H]methylated calf thymus DNA for 30 min at 37°C followed by acid hydrolysis and separation of free bases on HPLC as described by Dolan et al. (43,44). Activity was expressed as fmol O6-methylguanine removed per mg protein.

Abbreviations: +/+, wild-type; +/−, heterozygous; −/−, knockout; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BG, O6-benzylguanine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MGMT, O6-methylguanine-DNA methyltransferase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.
[³H]methylated DNA substrate was obtained by reaction of calf thymus DNA with 5.9 mCi/mmol N-[methyl-³H]nitrosourea (Amersham Inc., Arlington Heights, IL).

For northern blot analysis, total RNA was isolated from cultured cells using TRIzol Reagent (Gibco BRL Life Technologies, Burlington, Ontario, Canada) following the manufacturer’s instructions. Selection of mRNA was performed using the PolyATract mRNA Isolation System III (Promega Corp., Madison, WI). As described (45), samples and molecular weight markers (0.24–9.5 kb; Gibco BRL Life Technologies) were electrophoresed at 50 mA for 3–4 h in 1.1% agarose gels containing formaldehyde; mRNA was transferred to Gene Screen Plus membranes (Dupont Canada, Mississauga, Ontario, Canada) in 10× SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) by capillary action. To block non-specific binding, membranes were prehybridized in a VWR Scientific (Toronto, Ontario, Canada) Model 2710 hybridization oven at 42°C in a buffer containing 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl and 100 µg/ml herring sperm DNA. After 1–4 h, radiolabeled probe (10⁶ c.p.m./ml) was added to the prehybridization solution and incubated with constant rotation for 16–20 h at 42°C. 5’-Labeling of the MGMT probe (5’-TTTCTTACCA GCAATTTAGCA GCCTGTCGAG GCAACCCCCA AGC-3’) was carried out with T4 polynucleotide kinase and labeling of the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control cDNA probe (46,47) was performed using the Gibco Random Primers DNA Labeling System (Gibco BRL Life Technologies). Probe-specific washing procedures were followed. For the 5’-end-labeled MGMT probe, blots were washed briefly four times in 2x SSC, 0.1% SDS at room temperature, once for 30 min in 2x SSC, 0.1% SDS at 65°C, once for 5 min in 2x SSC, 0.1% SDS at room temperature and once briefly in 2x SSC at room temperature. For the random primed GAPDH probe, a more stringent protocol was used. These blots were washed twice for 5 min in 2x SSC at room temperature, twice for 30 min in 2x SSC, 1.0% SDS at 60°C and twice for 30 min in 0.1x SSC, 0.1% SDS at room temperature. Membranes were stored a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA), screens scanned by a Personal Densitometer SI Model 375A (Molecular Dynamics) and images quantitated using ImageQuant software (Molecular Dynamics). All images were below saturation and corrected for background.

p53 enhanced MGMT activity in a gene dose-dependent manner. Homozygous +/- p53 astrocytes displayed significantly greater MGMT activity than +/- astrocytes, which in turn exhibited significantly greater MGMT activity than +/- p53 astrocytes (Figure 1, left). At the level of detection afforded by the MGMT activity assay used in this study, +/- p53 astrocytes displayed MGMT levels only slightly above those exhibited by MGMT-negative cells. The effect of p53 on MGMT mRNA expression was also examined. Astrocyte cultures were prepared, mRNA isolated and northern blot analyses performed twice. In keeping with the activity assay results, +/- cells displayed higher levels of MGMT mRNA than +/- cells (see Figure 3).

In contrast to this result, Rafferty et al. (19) observed that basal levels of MGMT in whole mouse brain extracts were unaffected by p53 status. Moreover, Rafferty et al. (19) found lower levels of MGMT in brain extracts than detected by us in primary cultures of mouse astrocytes. Methodological differences may reconcile these apparently discordant results. For example, the experiments by Rafferty et al. (19) were performed on 6–8-week-old mice, whereas we measured MGMT activity in astrocyte cultures isolated from newborn mice. At early stages of differentiation, p53 expression is higher in rodent tissues, including the brain (48,49). The p53-dependent MGMT activity observed in our studies might have been difficult to detect in adult animals, where p53 expression is known to be low (48,49). In addition, p53-dependent MGMT activity may have been detected more readily in vitro because cell culture likely selects for less differentiated astrocytes with high p53 expression.

Since BCNU-resistant +/- astrocytes (33) had high levels of MGMT activity, cells were pretreated with BG to determine whether depletion of MGMT would sensitize +/- astrocytes to BCNU. First, to ensure that BCNU depleted MGMT, +/- and +/- p53 astrocytes were treated with either 25 or 50 µM BG for 2 h, after which MGMT activity was measured. BG stock solution (100 mM) was made in filter-sterilized dimethylsulfoxide, stored at -80°C and diluted to 1 mM in phosphate-buffered saline (PBS) when used; further dilutions were made with culture medium. BG significantly depleted MGMT activity in +/- p53 astrocytes (>90% reduction; Figure 1, right). Although BG appeared to slightly deplete the already low basal levels of +/- astrocytes, this difference was not statistically significant. MGMT levels found in BG-treated +/- astrocytes and the basal MGMT levels in +/- astrocytes were not significantly different (Figure 1).

Next, +/-, +/- and +/- astrocyte cultures were pretreated with 25 µM BG for 2 h, exposed to increasing concentrations...
of BCNU and cell viability tested using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (33). BCNU was dissolved in absolute alcohol, diluted to 3.3 mg/ml with sterile water and stored at −80°C; subsequent dilutions were made with culture medium. Cytotoxicity was measured using the MTT assay as modified for chemosensitivity testing by Cole (50). (Colonies formation could not be used to evaluate chemosensitivity in these non-transformed cells.) Briefly, 96-well plates were seeded and incubated at 37°C. To ensure equal cell numbers at 48 h, wild-type and heterozygous cells were seeded at 7.5×10^3/well and knockout p53 cells at 5×10^3/well. Cultures were treated with BCNU for 2 h and then incubated for 96 h. At that time, 100 µl of medium was removed, 25 µl MTT solution (2 mg/ml in PBS) added and cultures incubated for 2 h at 37°C. To solubilize formazan crystals, 0.04 M hydrochloric acid in isopropanol was added to each well and mixed thoroughly. Plates were kept at 37°C for 1 h and cell viability quantified by light absorbance (570 nm) in a Bio-Rad Model 3550 automated microplate reader (Bio-Rad Laboratories, Richmond, CA). To control for genotype-dependent growth rates, dose-response curves were normalized expressing absorbance values relative to non-treated controls. Results were analyzed using the two-tailed t-test for independent means. Despite MGMT depletion, neither +/+ nor −/− astrocytes (Figure 2) nor +/+ cells nor knockouts were sensitized to BCNU by BG. Similarly, pretreatment of astrocytes with 50 µM BG did not increase sensitivity to BCNU significantly (data not shown).

Since MGMT induction has been reported in some tissues in response to DNA damage (19,51–56) and treatment with BG could conceivably lead to MGMT induction (either or both explaining the failure of BG to reverse BCNU resistance), +/+ and −/− astrocytes were exposed to either 40 µg/ml BCNU or 25 µM BG for 2 h and samples taken at defined times for northern blot analysis and activity measurements. We did not detect MGMT mRNA induction; expression remained constant in +/+ and −/− astrocytes over 24 h (Figure 3). Likewise, there was no significant change in MGMT activity over 48 h in +/+ and −/− astrocytes in response to BCNU (Figure 4). MGMT activity decreased at 12 and 24 h in +/+ astrocytes, perhaps reflecting MGMT-mediated repair of BCNU damage, but the change was modest and not statistically significant. The absence of MGMT induction in response to BCNU, while observed following exposure to other DNA damaging agents, may simply reflect the fact that induction is an agent-specific cellular response.

In conclusion, we examined MGMT activity in wild-type, heterozygous and p53 knockout mouse astrocytes to further explore our previous observation that BCNU resistance in neonatal mouse astrocytes is p53 dependent (33). p53 enhanced MGMT activity in a gene dose-dependent manner. However,
despite successful depletion of MGMT activity in +/+ astrocytes, resistance to BCNU persisted unchanged. Moreover, we excluded the possibility that continued resistance to BCNU could be explained by a compensatory induction of MGMT triggered by exposure to either BCNU or BG. MGMT activity in astrocytes was significantly greater in the presence of p53, but MGMT was not the principal mechanism of resistance to BCNU. Our findings support a role for p53 regulation of MGMT in mouse astrocytes but p53-dependent BCNU resistance in astrocytes appears to be mediated by a non-MGMT mechanism, the nature of which remains unclear. Although we found that wild-type p53 astrocytes are protected from relatively high doses of BCNU, two recent studies have suggested that the role of MGMT in resistance to BCNU is promoted by p53 mutants.

Relatively high doses of BCNU, two recent studies have (1994) Transactivation of the human multidrug resistance (MDR1) gene by p53 promoter by p53 mutants. 

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References


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