0026-895X/98/020334-08$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, **54:**334–341 (1998).

Involvement of the Mismatch Repair System in Temozolomide-Induced Apoptosis

STEFANIA D'ATRI, LUCIO TENTORI, PEDRO MIGUEL LACAL, GRAZIA GRAZIANI, ELENA PAGANI, ELENA BENINCASA, GIOVANNA ZAMBRUNO, ENZO BONMASSAR, and JOSEF JIRICNY

Istituto Dermopatico Dell'Immacolata, Rome, Italy (S.D., P.M.L., E.P., G.Z., E.Bo.), Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Rome, Italy (L.T., G.G., E.Be.), and Institute for Medical Radiobiology, University of Zurich, Zurich, Switzerland (J.J.)

Received December 29, 1997; Accepted May 5, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

Postreplicative mismatch repair plays a major role in mediating the cytotoxicity of agents generating O^6-methylguanine in DNA. We previously showed that a methylating antitumor triazene compound, temozolomide, induces apoptosis and that the persistence of O^6-methylguanine in DNA is required to trigger the process. We wanted to test whether the latter apoptotic signal is dependent on a functional mismatch repair system. To this end, we used two human lymphoblastoid cell lines (i.e., the mismatch repair-proficient TK6 line and its mismatch repair-deficient subline MT1) that are both deficient in O^6-methylguanine repair. Temozolomide treatment of TK6 cells brought about efficient cell growth inhibition, G_2/M arrest, and apoptosis, as indicated by the results of cytofluorimetric analysis of 5-bromo-2′-deoxyuridine incorporation and DNA content and evaluation of DNA fragmentation. The drug treatment resulted also in the induction of p53 and p21/waf-1 protein expression. In contrast, MT1 cells were highly resistant to the drug and no p53 and p21/waf-1 induction was observed. Importantly, we could show that MT1 cells are not deficient in the p53-dependent apoptosis pathway; treatment with etoposide, a topoisomerase II inhibitor, resulted in p53 and p21/waf-1 protein expression and apoptosis in both cell lines. In conclusion, we demonstrate the existence of a link between a functional mismatch repair system and the trigger of apoptosis in cells exposed to clinically relevant concentrations of temozolomide. The results also suggest that p53 induction in response to O^6-guanine methylation involves the mismatch repair system.

The MRS is a DNA repair pathway dedicated to the correction of biosynthetic errors occurring during DNA replication (for reviews, see Jiricny, 1996; Modrich, 1997). Cells with a defective MRS display high rates of spontaneous mutations (Jiricny, 1996; Modrich, 1997) and microsatellite instability (Jiricny, 1996; Modrich, 1997). Germline mutations in mismatch repair genes have recently been linked to hereditary nonpolyposis colon cancer (Jiricny, 1996; Modrich, 1997).

At least five proteins [i.e., hMSH2, hMSH3, hMSH6 (also called guanine/thymine binding protein), hMLH1, and hPMS2] are involved in the initial steps of mismatch repair in human cells (Jiricny, 1996; Modrich, 1997). The protein complex hMutSα, consisting of hMSH2 and hMSH6, binds to base/base mismatches and to insertion/deletion loops of up to 14 nucleotides, whereas hMutSβ (a heterodimer of hMSH2 and hMSH3) binds to insertion/deletion loops but displays little affinity for base/base mismatches. The heterodimer of hMLH1 and PMS2, defined as hMutLα, interacts with either hMutSα or hMutSβ and thereby initiates the repair process (Modrich, 1997).

Although the MRS has evolved for the correction of replication errors, it is also implicated in the recognition of other types of DNA damage. Thus, tumor cell lines harboring mutations in the constituent proteins of hMutSα and hMutLα are resistant to the cytotoxic effects of MNNG and MNU (Kat *et al.*, 1993; Koi *et al.*, 1994; Carethers *et al.*, 1996), 6TG (Aquilina *et al.*, 1989; Hawn *et al.*, 1995; Swann *et al.*, 1996), and cisplatin, carboplatin, and doxorubicin (Anthoney *et al.*, 1996; Drummond *et al.*, 1996). These findings support the

This work was supported in part by a grant from the Istituto Superiore di Sanitá (Rome, Italy) (Italy-USA Therapy of Tumors Program) and in part by the Italian Ministry of Health. J.J. acknowledges the generous support of the Schweizerischer Nationalfonds zur Förderung der Wisseschaftlichen Forschung. Part of this work was presented at the 88th Annual Meeting of the American Association for Cancer Research (1997 April 12–16; San Diego, CA) (D'Atri *et al.*, 1997).

ABBREVIATIONS: MRS, mismatch repair system; CM, complete medium; 5-BrdU, 5-bromo-2′-deoxyuridine; FITC, fluorescein isothiocyanate; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; O^6-G, O^6-guanine; OGAT, O^6-alkylguanine-DNA alkyltransferase; O^6-MeG, O^6-methylguanine; PBS, phosphate-buffered saline; PFGE, pulsed field gel electrophoresis; PI, propidium iodide; 6TG, 6-thioguanine; TMZ, temozolomide [8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; kb, kilobase(s).

hypothesis that the MRS acts as a general sensor for genetic damage (Kat *et al.*, 1993; Hawn *et al.*, 1995) that, upon recognition of certain types of DNA modification, initiates a sequence of events resulting in cell death.

The cytotoxicity of MNU and MNNG has been attributed to the methylation of the O^6-position of guanine in DNA. Cells with high levels of the DNA-repair enzyme OGAT, which specifically removes alkyl adducts from O^6-G (Pegg, 1990), are more resistant to the cytotoxic effects of these alkylating agents than are OGAT-deficient cells (Pegg, 1990). Because a defect in the MRS rescues the OGAT$^-$ phenotype, such that MRS-deficient, OGAT-deficient cells are generally tolerant to high levels of O^6-G-methylating agents, cell death was proposed to result from the futile processing of O^6-MeG/T and O^6-MeG/C mismatches during DNA replication (Goldmacher *et al.*, 1986; Karran and Bignami, 1992). Similarly, the cytotoxicity of 6TG has been attributed to the incorporation of the base analog into DNA and its successive S^6-methylation by endogenous *S*-adenosylmethionine (Swann *et al.*, 1996). During DNA replication, S^6-methylthioguanine mispairs with thymine or cytosine, and the resulting mismatches activate the MRS.

We recently showed that treatment of human leukemic cell lines with the antitumor drug TMZ triggers apoptosis (Tentori *et al.*, 1995). TMZ is a new antitumor triazene compound that is presently undergoing phase II clinical trials (Bleehen *et al.*, 1995). Under physiological conditions, TMZ is rapidly metabolized to form the methylating species 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (Stevens *et al.*, 1987), which reacts with DNA bases to form methyl adducts, chiefly at N^7-guanine, N^3-adenine, and O^6-G. Methylation of O^6-G seems to be necessary for the triggering of apoptosis in TMZ-treated cells, because the process is prevented by high OGAT levels (Tentori *et al.*, 1995). Moreover, we have shown that transfection of OGAT cDNA into OGAT-deficient cells confers resistance to the induction of apoptosis by TMZ (Tentori *et al.*, 1997) and that pretreatment of OGAT-proficient cells with O^6-benzylguanine, a potent OGAT inhibitor (Dolan *et al.*, 1990), increases their susceptibility to TMZ-mediated apoptosis (Tentori *et al.*, 1995, 1997). Methylation of O^6-G might result in the triggering of apoptosis through the activation of the MRS. This hypothesis was substantiated by the recent observation that colon cancer lines harboring defects in the MRS are more resistant to cell growth inhibition by TMZ than are MRS-proficient lines (Liu *et al.*, 1996).

As discussed above, there is ample experimental evidence linking the cytotoxic effects of DNA-modifying agents with the MRS. However, the molecular mechanisms implicated in this process are currently unknown. In this study, we show that TMZ induces apoptosis, cell cycle arrest at the G_2/M phase, and p53 accumulation in MRS-proficient cells and that these responses are absent in MRS-deficient cells.

Materials and Methods

Cell lines. The human B lymphoblastoid cell lines TK6 and MT1 were a generous gift of W. G. Thilly (Massachusetts Institute of Technology, Cambridge, MA). The TK6 line was originally isolated as thymidine kinase heterozygous by Skopek *et al.* (1978). The MT1 line was obtained from TK6 by treatment with IRC-191, followed by selection for MNNG resistance (Goldmacher *et al.*, 1986). Both lines are OGAT-deficient and thus fail to remove alkyl adducts from O^6-G (Goldmacher *et al.*, 1986). TK6 cells are MRS-proficient (Kat *et al.*, 1993), whereas the MT1 line is MRS-deficient (Kat *et al.*, 1993), harboring different missense mutations in both alleles of the *GTBP* locus (Papadopoulos *et al.*, 1995). The cells were cultured at 37° in a 5% CO_2 humidified atmosphere and were maintained in RPMI 1640 medium (Hyclone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56°, 30 min) fetal calf serum (Hyclone), 2 mM L-glutamine, and antibiotics (Flow Laboratories, Mc Lean, VA) (referred to as CM).

Drugs and reagents. TMZ was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). Because in aqueous solution the drug readily decomposes into 5-(3-methyl-1-triazeno) imidazole-4-carboxamide (Stevens *et al.*, 1987), solutions were always prepared fresh by dissolving the drug in RPMI 1640 medium. The solutions were protected from light. Etoposide was purchased from Sigma (St. Louis, MO) and dissolved in dimethylsulfoxide. Aliquots of the stock solution were stored at −80°. Reagents for SDS-polyacrylamide gel electrophoresis were all purchased from Bio-Rad (Hercules, CA); other chemicals were obtained from Sigma.

Drug treatment and cell growth evaluation. Cells were suspended at 1×10^5 cells/ml in CM or in CM containing the appropriate amount of TMZ or etoposide and were cultured in flasks (Falcon; Becton Dickinson Labware, Frankein Lakes, NJ) at 37° in a 5% CO_2 humidified atmosphere for 72 hr. Cell growth was evaluated, in terms of viable cell counts, every 24 hr. Cells were manually counted using a hemocytometer, and cell viability was determined by the trypan blue exclusion test. All determinations were made in quadruplicate.

Assessment of apoptosis by flow cytometric analysis. Cells from cultures were harvested by centrifugation, washed with PBS, and fixed with 70% ethanol at −20° for 18 hr. The centrifuged pellets were resuspended in 1 ml of hypotonic solution containing 50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100, and 10 μg/ml RNase. The cells were incubated in the dark, at room temperature, for 30 min. Data collection was gated using forward and side light scatter, to exclude cell debris and cell aggregates. The PI fluorescence was measured on a linear scale using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Apoptotic cells are represented by a broad hypodiploid peak, which is easily distinguished from the narrow peak of cells with diploid DNA content observed in the red fluorescence channel (Nicoletti *et al.*, 1991). All data were recorded and analyzed using Lysis II software (Becton Dickinson).

Analysis of DNA fragmentation by PFGE. PFGE of high-molecular weight DNA fragments and preparation of plugs were performed according to the method of Walker *et al.* (1993). Briefly, cells (2×10^6) were harvested from cultures, washed twice with ice-cold PBS, resuspended in 1.0 ml of a buffer containing 0.15 M NaCl, 2 mM KH_2PO_4/KOH, pH 6.4, 1 mM EGTA, and 5 mM $MgCl_2$, centrifuged at 10,000 rpm for 2 min, and washed twice with 0.1 ml of the same buffer. The pellets were then resuspended in 0.05 ml of the same buffer and transferred to an Eppendorf tube containing an equal volume of melted 1.5% low-melting point agarose and 0.4 mg/ml proteinase K. The mixture was then pipetted into a 1-ml syringe and refrigerated at 4° for 15 min. The resulting agarose plugs were placed in 1 ml of 10 mM NaCl, 10 mM Tris·HCl, pH 9.5, 25 mM EDTA, 1% *N*-lauroylsarcosine, supplemented with 0.1 mg/ml proteinase K, and were incubated at 37° for 3 hr. Plugs were rinsed in three changes of 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, at 4° (10 min each), stored in 50 mM EDTA, pH 8.0, for up to 1 week, and then used for electrophoresis.

Electrophoresis was carried out using a switchback power supply (Hoefer Scientific, San Francisco, CA). The gels (1.5% agarose) were subjected to electrophoresis at 200 V at 4° for 18 hr, in 0.5× Tris-borate/EDTA buffer (45 mM Tris-borate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide, with the ramping rate changing from T_1 = 0.5 sec to T_2 = 10 sec and with a forward/backward ratio of 3. High-molecular weight DNA fragments were visualized under UV light and photographed using a Polaroid camera (Polaroid, Cambridge, MA).

Assessment of apoptosis by electron microscopy. Cell suspensions were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon resin, and then semithin and ultrathin sections were cut with an ultramicrotome (Reichert Ultracut E; Leica, Wien, Austria). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (CM100; Philips, Eindhoven, The Netherlands).

Evaluation of 5-BrdU incorporation. Cells were harvested, washed with PBS, and resuspended in 70% ethanol at −20°. Fixed cells were washed with PBS, resuspended in 2 ml of 2 M HCl, and incubated at room temperature for 30 min. They were then centrifuged, resuspended in 2 ml of 0.1 M $Na_2B_4O_7$, pH 8.5 (to neutralize acidity), and washed with PBS containing 0.5% (v/v) Tween 20 and 1% (w/v) bovine serum albumin. The cell pellets were resuspended in 0.1 ml of PBS containing 0.4 mg of anti-5-BrdU (Boehringer Mannheim, Mannheim, Germany) and were maintained at room temperature in the dark for 30 min. They were then washed and stained with 0.1 ml of diluted (1/50) FITC-labeled $(Fab')_2$ rabbit anti-mouse IgG (H+L chains) (Dako, Glostrup, Denmark). Negative controls were represented by cell samples stained with FITC-labeled $(Fab')_2$ rabbit anti-mouse IgG only. After a 30-min incubation at room temperature, the cells were washed and resuspended in 1 ml of a solution containing 50 μg/ml PI, 0.1% sodium citrate, and 10 μg/ml RNase. They were then incubated in the dark, at 37°, for an additional 30 min. Cell samples were then analyzed for green (FITC, indicating 5-BrdU incorporation) and red (PI, indicating DNA content) fluorescence, using a FACScan flow cytometer. The results were displayed as representative two-dimensional frequency contour plots of green versus red fluorescence.

Immunoblotting. Cell pellets were resuspended in a hypotonic buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.4, with freshly added 1 mM phenylmethylsulfonyl fluoride), an aliquot was saved for protein concentration determination (using the Bio-Rad protein assay solution, with bovine serum albumin as the standard), and the remainder was immediately boiled in SDS sample buffer (50 mM Tris·HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Sixty micrograms of protein per sample were analyzed in 8% (for p53 protein) or 13.5% (for p21/waf-1) SDS-polyacrylamide gels. The proteins were then transferred to supported nitrocellulose membranes (Hybond-C; Amersham Life Science, Buckinghamshire, UK), using a Transphor TE 50X unit (Hoefer Scientific). Immunodetection was carried out using the Boehringer Mannheim chemiluminescence Western blotting kit, according to the manufacturer's instructions, except that the second antibody (horseradish peroxidase-linked) was purchased from Amersham Life Science. The anti-p53 (Ab-2; Calbiochem, Cambridge, MA) and anti-p21/waf-1 (Ab-1; Calbiochem) antibodies were used at 2.5 μg/ml. Filters were exposed to X-OMAT AR autoradiographic films (Kodak, Rochester, NY) for 5–45 sec, depending on the intensity of the signal.

Results

Cell growth inhibition induced by TMZ or etoposide in TK6 and MT1 cell lines. To investigate whether cell sensitivity to TMZ was correlated with the presence of a functional MRS, the MRS-proficient TK6 and MRS-deficient MT1 cell lines, which are both unable to repair O^6-MeG (Goldmacher *et al.*, 1986), were incubated for 72 hr with graded concentrations of TMZ. Cell growth was evaluated every 24 hr. The results in Fig. 1, which refer to cell growth evaluations after 72 hr of drug exposure, show that TK6 proliferation was markedly impaired by TMZ, whereas MT1 cells were completely resistant to drug concentrations up to 50 μM. In the MT1 line, cell growth inhibition occurred at TMZ concentrations 70-fold higher than those required to obtain comparable levels of cell growth impairment in TK6 cells (data not shown). As a control, similar experiments were performed with the unrelated antitumor drug etoposide, which is known to exert its cytotoxic effects through the inhibition of topoisomerase II (Bender *et al.*, 1990). In this case, no substantial differences in cell growth inhibition were observed between the two lines (Fig. 1).

Time course analysis of cell growth impairment induced by selected concentrations of TMZ or etoposide in TK6 cells is shown in Fig. 2. The data indicate that, during the first 24 hr of cell culture, both control and TMZ-treated cells doubled in number, whereas cell growth was arrested after 48 hr in the drug-treated cultures. At the highest TMZ concentration, the cell number began to decrease. In etoposide-treated cells, the impairment of proliferation was already evident after 24 hr of drug exposure, and cells did not increase their number.

Induction of apoptosis in TK6 and MT1 cells treated with TMZ or etoposide. The TK6 and MT1 cells were incubated with TMZ at 12.5 or 25 μM for 72 hr, and apoptosis was evaluated by flow cytometry after 6, 24, 48, and 72 hr of drug exposure. The cells were also treated with 250 nM etoposide, which was expected to induce apoptosis indepen-

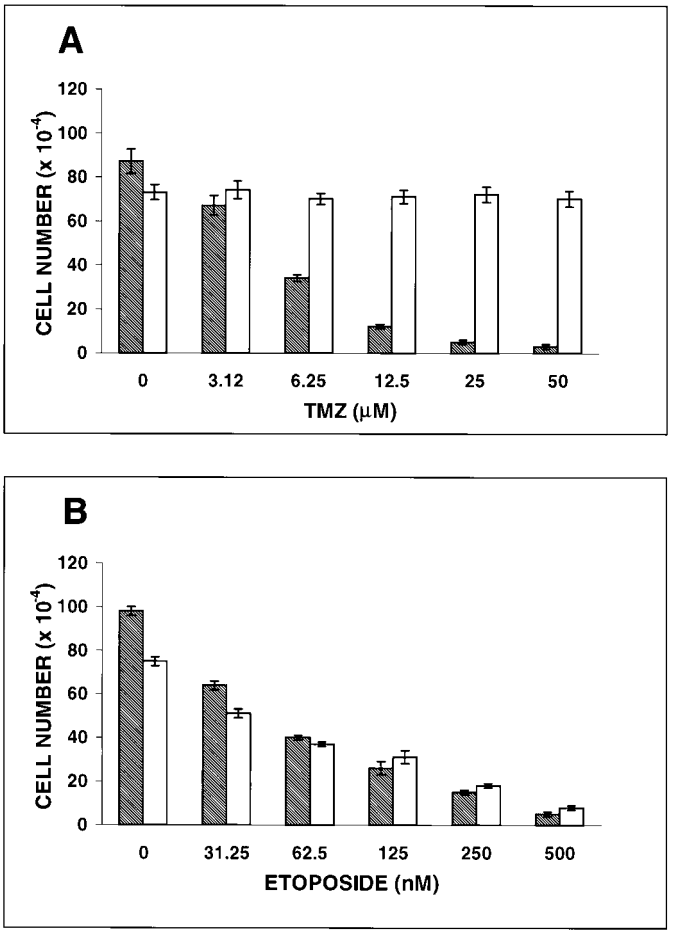

Fig. 1. Inhibition of cell growth induced by TMZ or etoposide in TK6 and MT1 cell lines. TK6 (▨) and MT1 (□) cells (1×10^5 cells/ml) were incubated in medium alone or in medium containing the indicated concentrations of TMZ (A) or etoposide (B), and cell growth was evaluated after 72 hr. Each value represents the mean of two independent experiments, with error bars indicating standard errors. The figure shows that only the MRS-proficient line TK6 was affected by increasing concentrations of TMZ, whereas both lines were inhibited by etoposide.

dently of the MRS status of the cells. The results illustrated in Table 1 show that TMZ induced apoptosis only in TK6 cells, whereas the effects of etoposide were comparable in the two lines. The percentage of apoptotic cells in TMZ-treated cultures increased substantially after 48 and 72 hr of drug exposure, whereas only a slight increase was observed after 24 hr. On the other hand, in etoposide-treated cultures a significant induction of apoptosis was evident by 24 hr after drug exposure.

Fig. 3 illustrates the flow cytometric analysis of a representative experiment, showing apoptosis induced in the TK6 and MT1 cell lines by a 48-hr treatment with TMZ (12.5 μM) or etoposide (250 nM). Drug-induced apoptosis is indicated by the appearance of a cell population with hypodiploid DNA contents.

Apoptosis was further confirmed by the analysis of DNA fragmentation in control and drug-treated cells. During the process of apoptosis, genomic DNA is first degraded to 300- and 50-kb fragments, and these are subsequently digested to oligonucleosomal fragments (Walker *et al.*, 1995). However, in many lines, including TK6 and MT1, apoptotic degradation of DNA is arrested at the stage of 50-kb fragments (Oberhammer *et al.*, 1993; Walker *et al.*, 1995). In these cases, conventional agarose gel electrophoresis fails to show the ladder-like pattern characteristic of internucleosomal DNA degradation, whereas PFGE reveals the presence of the 300- and 50-kb fragments in apoptotic cells. Fig. 4 illustrates the results of a representative experiment in which TK6 and MT1 cells were exposed to TMZ (12.5 or 25 μM) or etoposide (125 or 250 nM) for 72 hr and apoptotic DNA degradation was analyzed by PFGE. The data show that etoposide caused DNA fragmentation in both lines, whereas TMZ treatment was effective only in TK6 cells.

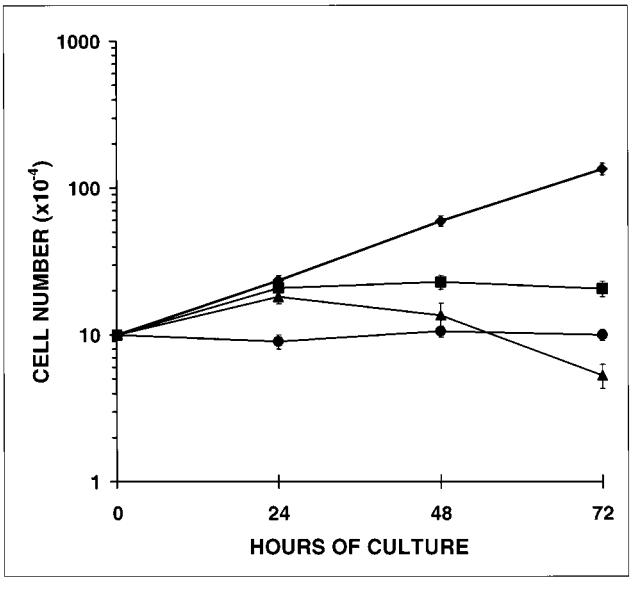

Fig. 2. Growth arrest in TK6 cells treated with TMZ or etoposide. TK6 cells (1×10^5 cells/ml) were incubated either in medium alone (♦) or in medium containing 12.5 μM TMZ (■), 25 μM TMZ (▲), or 250 nM etoposide (●) and were counted at 24-hr intervals for 72 hr. Each data point represents the mean of four independent experiments, with errors bars indicating standard errors. The control untreated cells doubled every 20–24 hr, whereas TK6 cells treated with etoposide failed to undergo cell division. Cells treated with TMZ appeared to undergo one doubling, and their numbers either stabilized (12.5 μM TMZ) or declined (25 μM TMZ) at later times.

Apoptosis induction in TK6 cells exposed to TMZ was also confirmed by electron microscopy. Untreated TK6 cells displayed a large nucleus and an irregular outline, with numerous microvilli and surface projections. After 48-hr treatment with 12.5 μM TMZ, cells exhibited the characteristic features of apoptosis, including cell shrinkage with loss of microvilli, peripheral cytoplasmic vacuole formation, chromatin condensation and margination, and micronuclei formation (Fig. 5).

Cell cycle perturbations induced in TK6 and MT1 cells by TMZ or etoposide. The TK6 and MT1 cells were incubated with TMZ (12.5 μM) or etoposide (250 nM) for 72 hr, and the percentage of cells in each phase of the cell cycle was evaluated by bivariate analysis of 5-BrdU incorporation and DNA content after 24, 48, and 72 hr of drug exposure. 5-BrdU (10 μM) was added to the cultures 1 hr before harvesting. The results illustrated in Table 2 show that, in TK6 cells treated with TMZ, only a slight increase in the G_2/M fraction occurred during the first 24 hr of drug exposure, whereas after 48 hr about one third of the cells appeared to be arrested at the G_2/M phase of the cell cycle. Moreover, a significant fraction (30%) of the cells showed hypodiploid DNA contents at this point and were therefore considered to be undergoing apoptosis. A decrease in the G_2/M fraction associated with a further increase in the sub-G_1 cell fraction was observed at 72 hr (Table 2). On the other hand, the MT1 line exposed to TMZ failed to show any alteration of the cell cycle (Table 2).

Upon exposure to etoposide, both TK6 and MT1 cells accumulated in the G_2/M phase of the cell cycle during the first 24 hr of drug treatment (Table 2). From 48 hr onwards, the G_2/M fraction decreased and a large proportion of the cells showed sub-G_1 DNA contents (Table 2). These results indicate that both TK6 and MT1 cells, when exposed to etoposide, are rapidly arrested at the G_2/M phase of the cell cycle and undergo apoptosis.

p53 and p21/waf-1 accumulation in TK6 and MT1 cells after treatment with TMZ or etoposide. It is well established that p53 plays a crucial role in the regulation of the cell cycle and in the induction of apoptosis in response to

TABLE 1

Time course analysis of apoptosis induced by TMZ or etoposide in TK6 and MT1 cell lines

The percentage of apoptotic cells was evaluated by flow cytometric analysis of DNA content. Data represent the values obtained in two independent experiments. Cells were cultured in the presence of the indicated concentrations of TMZ or etoposide (ETO) for 6, 24, 48, or 72 hr and were then processed for apoptosis evaluation.

Drug	Time	Fraction of apoptotic cells	
		TK6	MT1
	hr	*%*	
None	6	7, 5	5, 4
TMZ, 12.5 μM	6	7, 8	2, 6
TMZ, 25 μM	6	7, 2	2, 1
ETO, 250 nM	6	11, 9	3, 6
None	24	6, 5	2, 3
TMZ, 12.5 μM	24	8, 10	4, 3
TMZ, 25 μM	24	14, 11	4, 6
ETO, 250 nM	24	21, 27	21, 13
None	48	2, 3	4, 3
TMZ, 12.5 μM	48	24, 27	4, 3
TMZ, 25 μM	48	32, 23	4, 5
ETO, 250 nM	48	35, 49	39, 37
None	72	2, 1	2, 2
TMZ, 12.5 μM	72	28, 35	2, 1
TMZ, 25 μM	72	45, 35	2, 8
ETO, 250 nM	72	44, 50	37, 44

DNA-damaging agents (for reviews, see Leonard *et al.*, 1995; Harris, 1996). We therefore investigated whether there was an alteration in p53 protein expression in TMZ-treated cells and whether a correlation existed between p53 induction and MRS activity. In addition, we evaluated the level of the cyclin-dependent kinase inhibitor p21/waf-1 (Xiong *et al.*, 1993), which is known to increase as a result of p53-mediated transcriptional activation (El-Deiry *et al.*, 1994). To this end, TK6 and MT1 cells were incubated with TMZ (12.5 μM) or etoposide (250 nM) for 72 hr and the p53 and p21/waf-1 levels, in control and drug-treated cells, were evaluated by Western blot analysis after 6, 24, 48, and 72 hr of drug exposure. The results presented in Fig. 6 show that TMZ treatment of TK6 cells induced a progressive increase in the amount of p53, which was evident, although at low levels, after 6 hr of drug treatment. The induction of p53 was accompanied by a parallel increase in the amount of p21/waf-1 protein. Neither p53 nor p21/waf-1 protein levels increased upon TMZ treatment in the MT1 line, whereas exposure to etoposide resulted in increases in p53 and p21/waf-1 protein levels in both TK6 and MT1 cells.

Discussion

DNA mismatch repair plays a critical role in the maintenance of genomic stability, as demonstrated by the observations that mutations in mismatch repair genes bring about a strong mutator phenotype and microsatellite instability and that the inheritance of MRS gene mutations is linked to hereditary colon cancer (for review, see Jiricny, 1996). Defects in the MRS are also associated with tumor cell resistance to the cytotoxic activities of MNNG, MNU, 6TG, cisplatin, doxorubicin, and TMZ, suggesting that cell death induced by these agents occurs through an active process that requires the participation of the MRS, although the molecular events involved in the process have not yet been elucidated.

We recently showed that TMZ exerts its cytotoxic effects mainly through the induction of apoptosis and that the persistence of O^6-MeG in DNA is necessary for the triggering of the programmed cell death process (Tentori *et al.*, 1995, 1997). In this article, we demonstrate that cell susceptibility to TMZ-induced apoptosis is correlated with the activity of the MRS.

The MRS-proficient cell line TK6 and its MRS-deficient subline MT1, both lacking OGAT and therefore being incapable of removing methyl groups from O^6-G, were, indeed, differentially susceptible to TMZ, in terms of cell growth inhibition (Fig. 1) and apoptosis induction (Table 1 and Figs. 3 and 4). The growth of TK6 cells was readily inhibited by TMZ; the MT1 line was markedly more resistant to this agent, and apoptosis could not be induced even with TMZ concentrations up to 800 μM (Table 1, Fig. 4, and data not shown). It could be argued that the resistance of MT1 cells to TMZ is the result of a defect in one or more pathways that induce apoptosis after DNA damage. However, the fact that the two lines showed comparable susceptibility to cell growth inhibition (Fig. 1) and apoptosis induction (Table 1 and Figs. 3 and 4) in response to treatment with etoposide, a topoisomerase II inhibitor, eliminates this possibility and implies that the apoptotic response in the TMZ-treated TK6 cells is dependent on a functional MRS. The correlation between induction of apoptosis by TMZ and the activity of the MRS is further strengthened by the results presented in Table 2, which show that, upon treatment with TMZ, the MRS-proficient TK6 cells but not the MRS-deficient MT1 cells were arrested preferentially at the G_2/M phase of the cell cycle and

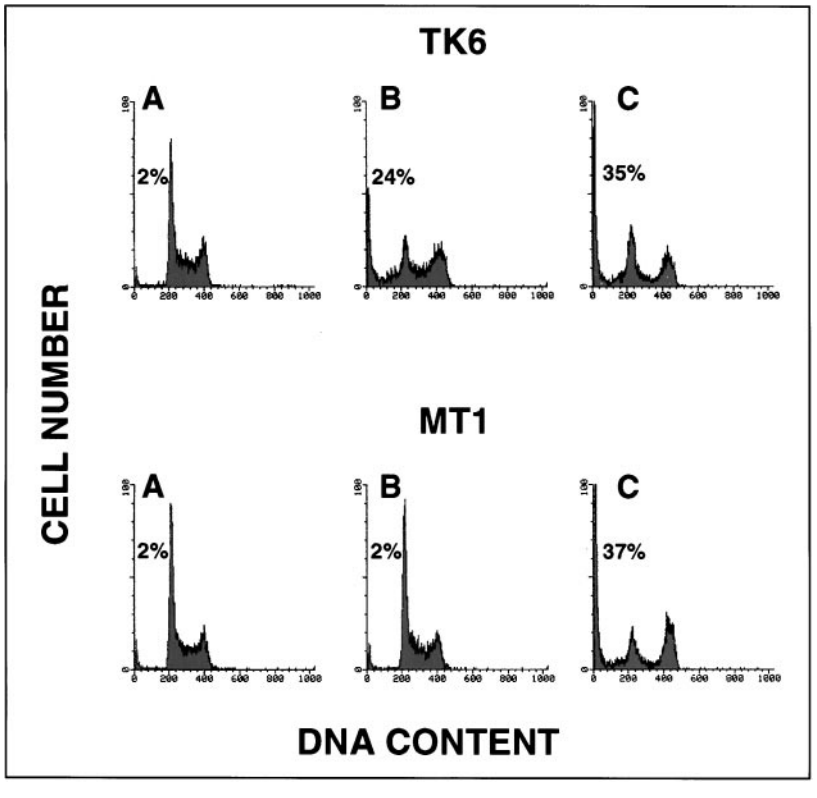

Fig. 3. Induction of apoptosis in TK6 and MT1 cells treated with TMZ or etoposide. TK6 and MT1 cells (1×10^5 cells/ml) were incubated in medium alone (A) or in medium containing 12.5 μM TMZ (B) or 250 nM etoposide (C). After 48 hr of culture, the cells were harvested and apoptosis was evaluated by flow cytometric analysis of DNA content. Data collection was gated using forward and side light scatter, to exclude cell debris and cell aggregates. The PI fluorescence was measured on a linear scale. The percentage of apoptotic cells exhibiting hypodiploid DNA content is also shown. The FACScan profiles show that, whereas apoptosis was induced by both TMZ and etoposide treatments in TK6 cells, only the latter drug seems to induce apoptosis in MT1 cells.

underwent apoptosis, whereas the two lines exhibited comparable levels of G_2/M accumulation and apoptosis after treatment with etoposide.

Time course analysis of cell growth inhibition, apoptosis induction, and cell cycle perturbation in drug-treated cells showed that the effects of TMZ on TK6 cells became particularly evident after 48 hr of drug exposure (Fig. 2 and Tables 1 and 2). Our findings agree with the results of previous studies, performed with unsynchronized TK6 cells (Goldmacher *et al.*, 1986) or other cell lines (Plant and Roberts, 1971; Black *et al.*, 1989), which suggested that, after methylation of O^6-G by MNNG or MNU, the cells proceed through a single cell division and are arrested at the G_2/M phase of the second cell cycle (Karran and Bignami, 1992). Although these results apparently differ from the findings of Carethers *et al.* (1996), who showed that several MRS-proficient cell lines treated with MNNG were arrested at the G_2/M phase of the first cell cycle, it is conceivable that the cells used in the

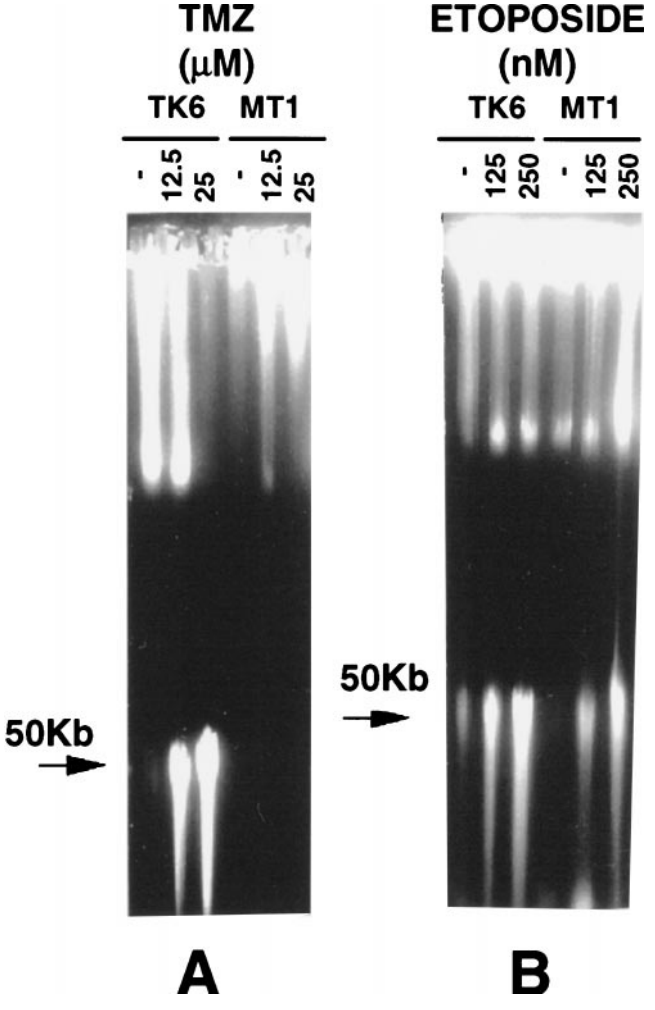

Fig. 4. Apoptosis-associated DNA degradation in TK6 and MT1 cells treated with TMZ or etoposide. TK6 and MT1 cells were incubated (1×10^5 cells/ml) in medium alone or in medium containing the indicated concentrations of TMZ (A) or etoposide (B). High-molecular weight DNA was extracted from untreated or drug-treated cells, and apoptotic fragmentation was analyzed by PFGE. The sizes of the DNA fragments were estimated using molecular weight markers. The figure is a photograph of an agarose gel stained with ethidium bromide, and it shows that DNA of both cell lines was degraded after etoposide treatment, whereas treatment with TMZ resulted in the degradation of genomic DNA only in the TK6 cells.

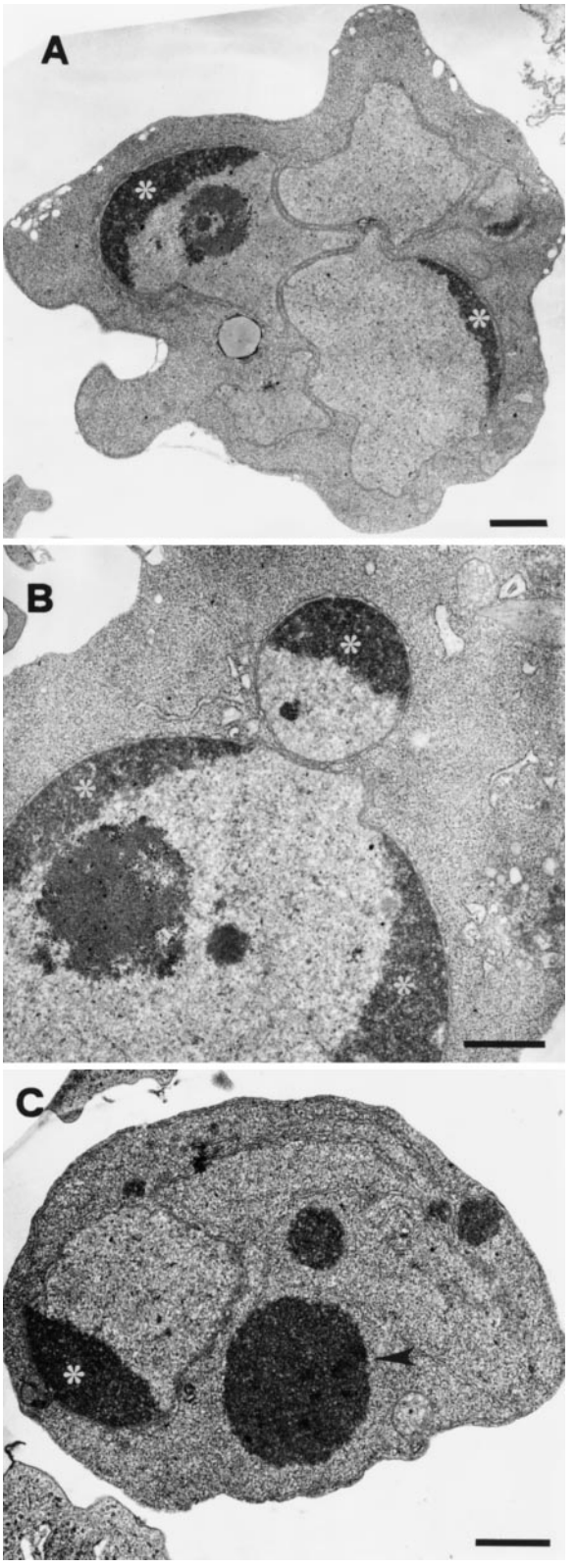

Fig. 5. Apoptotic TK6 cells after treatment with 12.5 μM TMZ for 48 hr. The cell surfaces appear completely devoid of microvilli (A and C), and several small vacuoles are distributed at the periphery of relatively well preserved cytoplasm (A). Typical chromatin condensation and margination are present in each cell (*asterisks*). A single micronucleus is visible in one cell (B), and several micronuclei, each surrounded by a double membrane, are evident in another (A). Electron-dense nuclear fragments free of membrane (*arrowhead*) are present in the cytoplasm of a cell in a later stage of apoptosis (C). *Bars*, 1 μm.

latter study were arrested at the first G_2/M phase because of the level of DNA damage induced by drug concentrations significantly higher than those in the other series of experiments. The hypothesis that cell cycle arrest after treatment with TMZ occurs mainly at the G_2/M phase of the second cell cycle seems to be supported also by the different kinetics of cell growth inhibition, G_2/M arrest, and apoptosis induction observed in TK6 cells exposed to etoposide. In those cells, G_2/M arrest and apoptosis were evident after 24 hr of drug exposure (Tables 1 and 2), and cell numbers did not increase (Fig. 2). Moreover, if TK6 and MT1 lines were exposed to concentrations of etoposide that allowed cells to replicate during the first 24 hr of drug treatment (i.e., concentrations ranging between 15.62 and 62.5 nM), no substantial induction of apoptosis was observed (data not shown).

TABLE 2

Time course analysis of cell cycle perturbations induced by TMZ or etoposide in TK6 and MT1 cell lines

Cells were cultured in the presence of the indicated concentrations of TMZ or etoposide (ETO) for 24, 48, or 72 hr and were then processed for cell cycle analysis. 5-BrdU (10 μM) was added to the cultures 1 hr before harvesting. The percentage of cells in each phase of the cell cycle or in apoptosis was evaluated by flow cytometric analysis of 5-BrdU incorporation and DNA content. Data represent the means of two independent experiments, with standard errors not exceeding 10%.

Drug	Time	Fraction of cells							
		TK6				MT1			
		Apoptosis	G_1/G_0	S	G_2/M	Apoptosis	G_1/G_0	S	G_2/M
	hr	%							
None	24	8	30	55	7	3	37	55	5
TMZ, 12.5 μM	24	12	24	54	10	3	38	52	7
ETO, 250 nM	24	22	25	17	36	17	23	15	45
None	48	8	39	43	10	2	39	51	8
TMZ, 12.5 μM	48	30	31	10	29	2	38	53	7
ETO, 250 nM	48	46	28	10	16	38	31	7	24
None	72	7	57	22	14	3	59	31	7
TMZ, 12.5 μM	72	39	30	16	15	4	57	30	9
ETO, 250 nM	72	53	24	12	11	49	28	4	19

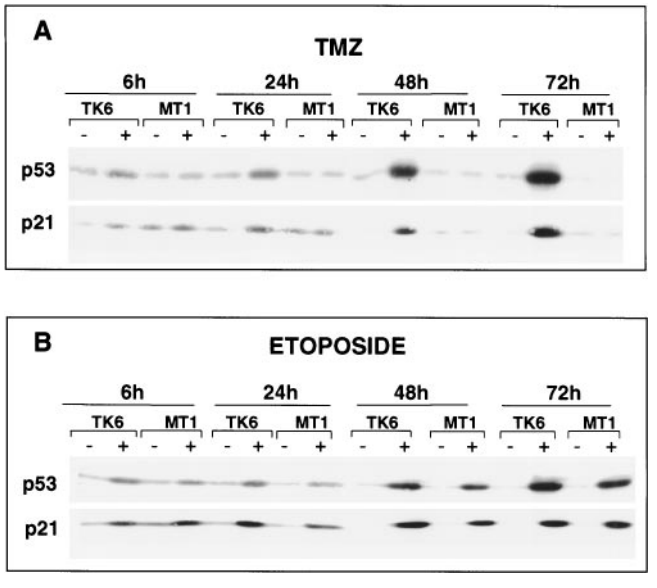

Fig. 6. p53 and p21/waf-1 accumulation in TK6 and MT1 cells after treatment with TMZ or etoposide. TK6 and MT1 cells were incubated (1×10^5 cells/ml) in medium alone (−) or in medium containing 12.5 μM TMZ (A) or 250 nM etoposide (B) (+). The amounts of p53 and p21/waf-1 proteins were evaluated at the indicated times by immunoblotting. The figure shows that induction of p53 and p21/waf-1 protein expression in the MRS-proficient line TK6 occurred after treatment with either TMZ or etoposide. In contrast, only the latter drug induced these proteins in the MRS-deficient line MT1.

p53 is a critical participant in the signal transduction pathway that mediates apoptosis in response to DNA damage (Leonard *et al.*, 1995; Harris, 1996). Induction of apoptosis by p53 may occur through up-regulation of Bax (Miyashita *et al.*, 1994) and/or Fas/Apo-1 protein expression (Owen-Schaub *et al.*, 1995), through activation of caspase 3 (Fuchs *et al.*, 1997), or through down-modulation of Bcl-2 (Miyashita *et al.*, 1994).

The results illustrated in Fig. 6 show that, in the MRS-proficient TK6 cell line, p53 was induced in response to TMZ and this increase was accompanied by up-regulation of p21/waf-1 protein levels. In contrast, neither p53 nor p21/waf-1 levels were increased in the MRS-deficient MT1 cells after TMZ treatment, although both TK6 and MT1 cells were capable of inducing p53 and p21/waf-1 expression in response to etoposide treatment (Fig. 6) or X-irradiation (data not shown). Therefore, a functional MRS seemed to be required for up-regulation of p53 in cells treated with TMZ, at least at the drug concentrations used, which are in the range of those achievable clinically (Newlands *et al.*, 1992).

As mentioned above, the MRS seems to be involved in triggering the cytotoxic response to cisplatin. Indeed, several ovarian tumor cell lines isolated *in vitro* by single-step selection for resistance to the drug were found to be defective in the expression of hMLH1 (Drummond *et al.*, 1996). Cisplatin resistance in these MRS-deficient lines has been associated with a reduced ability to activate a p53-dependent response, as indicated by absent or reduced up-regulation of p21/waf-1 mRNA, and to undergo apoptosis upon treatment with the drug (Anthoney *et al.*, 1996). Although these results resemble our data in TK6 and MT1 cells, it must pointed out that the cisplatin-resistant sublines showed impaired p53 function (with no, or markedly reduced, G_1 arrest and transcription of the p21/waf-1 gene) also in response to ionizing radiation, whose cytotoxic activity does not depend on a functional MRS. Moreover, the cells were cross-resistant to multiple cytotoxic agents (Hamaguchi *et al.*, 1993), suggesting a defect in the engagement of apoptosis after any DNA damage. Therefore, the reduced ability of the cisplatin-resistant lines to undergo apoptosis upon treatment with the drug is attributable not only to a deficiency in mismatch repair but also to a loss of p53 function, which was selected for during the generation of the resistant lines. Our results, showing that p53 and p21/waf-1 are induced in MRS-proficient and MRS-deficient cells upon treatment with etoposide (Fig. 6) or X-irradiation (data not shown) but not with TMZ (Fig. 6), represent the first experimental evidence that MRS-deficient cells are incapable of generating the signal leading to p53 induction in response to an O^6-G-methylating agent, whereas they retain the ability to induce p53 after DNA damage not involving methylation of O^6-G.

We previously showed that TMZ-mediated apoptosis can occur also in p53-null cells (Tentori *et al.*, 1995, 1997). However, expression of wild-type p53 in p53-null HL-60 cells increased their susceptibility to TMZ-mediated apoptosis (Tentori L, Lacal PM, Benincasa E, Franco D, Faraoni I, Bonmassar E, Graziani G, manuscript submitted for publication). It is therefore possible to speculate that in MRS-proficient lines TMZ might activate both p53-dependent and p53-independent apoptosis and the two pathways might interact with each other, as described in other models (Peled *et al.*, 1996). In cells expressing

wild-type p53, the triggering of apoptosis might occur at lower drug concentrations or with faster kinetics. For instance, it has already been shown in a murine model that the p53 status of cells affects the rate of the onset, but not the overall extent, of apoptosis induced by doxorubicin (Han *et al.*, 1997).

Further studies are required to establish whether apoptosis in TK6 cells occurs through a p53-dependent or p53-independent pathway. In fact, p53 has not been sequenced in this line and, although the protein shows an ability to *trans*-activate p21/waf-1, we cannot assume that the apoptotic function of p53 is intact in TK6 cells. Indeed, mutant forms of p53 that are capable of *trans*-activating p21/waf-1 but show impaired apoptotic function have already been described (Ludwig *et al.*, 1996). Moreover, in TMZ-treated TK6 cells no changes in the levels of Bax, Fas/Apo-1, or Bcl-2 proteins have been observed (data not shown).

In conclusion, we demonstrated the existence of a link between a functional MRS and the trigger of apoptosis in cells exposed to clinically achievable concentrations of TMZ. We also showed that p53 induction in response to the methylation of O^6-G is associated with a functional MRS. Studies designed to clarify these mechanisms, in particular to establish to what extent p53 and downstream effectors are involved in cell cycle arrest and apoptosis induction in TMZ-treated cells, are currently in progress.

Acknowledgments

We thank Dr. Giancarlo Marra for critical comments on the manuscript. We also thank Cesare Secci for the excellent artwork, Dr. Federica Pochesci and Dr. Giuseppe Aleo for secretarial assistance, and Anna Bucci for technical assistance.

References

Anthoney DA, MacIlwrath AJ, Gallagher WM, Edlin ARM, and Brown R (1996) Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. *Cancer Res* **56:**1374–1381.

Aquilina G, Zijno A, Moscufo N, Dogliotti E, and Bignami M (1989) Tolerance to methylnitrosourea-induced DNA damage is associated with 6-thioguanine resistance in CHO cells. *Carcinogenesis (Lond)* **10:**1219–1223.

Bender RA, Hamel E, and Handle KR (1990) The plant alkaloids, in *Cancer Chemotherapy: Principles and Practice* (Chabner BA and Collins JM, eds) pp 253–275, JB Lippincott, Philadelphia.

Black KA, McFarland RD, Grisham JW, and Smith GJ (1989) Cell cycle perturbation and cell death after exposure of a human lymphoblastoid cell strain to *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine. *Am J Pathol* **134:**53–61.

Bleehen NM, Newlands ES, Lee SM, Thatcher N, Selby P, Calvert AH, Rustin GJ, Brampton M, and Stevens MF (1995) Cancer Research Campaign phase II trial of temozolomide in metastatic melanoma. *J Clin Oncol* **13:**910–913.

Carethers JM, Hawn MT, Chauhan DP, Luce MC, Marra G, Koi M, and Boland CR (1996) Competency in mismatch repair prohibits clonal expansion of cancer cells treated with *N*-methyl-*N′*-nitro-*N*-nitrosoguanide. *J Clin Invest* **98:**199–206.

D'Atri S, Graziani G, Lacal PM, Tentori L, Pagani E, and Jiricny J (1997) Role of mismatch repair system in apoptosis induced by methyltriazenes, in *Proceedings of the 88th Annual Meeting of the American Association for Cancer Research*, p1, Cadmus Journal Services, Linthicum, MD.

Dolan EM, Moshel RC, and Pegg AE (1990) Depletion of O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc Natl Acad Sci USA* **87:**5368–5372.

Drummond JT, Anthoney A, Brown R, and Modrich P (1996) Cisplatin and Adriamycin resistance are associated with MutLα and mismatch repair deficiency in an ovarian tumor cell line. *J Biol Chem* **271:**19645–19648.

El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrel M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, and Vogelstein B (1994) WAF-1/CIP-1 is induced in p53-mediated G_1 arrest and apoptosis. *Cancer Res* **54:**1169–1174.

Fuchs EJ, McKenna KA, and Bedi A (1997) p53-dependent DNA damage-induced apoptosis requires Fas/APO-1-independent activation of CPP32β^1. *Cancer Res* **57:**2550–2554.

Goldmacher VS, Cuzick RA, and Thilly WG (1986) Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine. *J Biol Chem* **261:**12462–12471.

Hamaguchi K, Godwin AK, Yakushiji M, O'Dwyer PJ, Ozols RF, and Hamilton TC (1993) Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. *Cancer Res* **53:**5225–5232.

Han J, Dionne CA, Kedersha NL, and Goldmacher VS (1997) p53 status affects the rate of the onset but not the overall extent of doxorubicin-induced cell death in Rat-1 fibroblasts constitutively expressing c-Myc. *Cancer Res* **57:**176–182.

Harris CC (1996) Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst* **88:**1442–1455.

Hawn MT, Umar A, Carethers JM, Marra G, Kunkel TA, Boland CR, and Koi M (1995) Evidence for a connection between mismatch repair system and the G_2 cell cycle checkpoint. *Cancer Res* **55:**3721–3725.

Jiricny J (1996) Mismatch repair and cancer, in *Genetic Instability in Cancer* (Lindahl T, ed) pp 47–68, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Karran P and Bignami M (1992) Self-destruction and tolerance in resistance of mammalian cells to alkylation damage. *Nucleic Acids Res* **20:**2933–2940.

Kat A, Thilly WG, Fang WH, Longley MJ, Li GM, and Modrich P (1993) An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. *Proc Natl Acad Sci USA* **90:**6424–6428.

Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, and Boland CR (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. *Cancer Res* **54:**4308–4312.

Leonard CJ, Canman CE, and Kastan MB (1995) The role of p53 in cell-cycle control and apoptosis: implications for cancer, in *Important Advances in Oncology* (De Vita VT, Hellman S, and Rosenberg SA, eds) pp 33–42, J. B. Lippincott, Philadelphia, PA.

Liu L, Markowitz S, and Gerson SL (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Res* **56:**5375–5379.

Ludwig RL, Bates S, and Vousden KH (1996) Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. *Mol Cell Biol* **16:**4952–4960.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, and Reed JC (1994) Tumor suppressor p53 is a regulator of *bcl*-2 and *bax* gene expression *in vitro* and *in vivo*. *Oncogene* **9:**1799–1805.

Modrich P (1997) Strand-specific mismatch repair in mammalian cells. *J Biol Chem* **272:**24727–24730.

Newlands ES, Blackledge GRP, Slack JA, Rustin GJS, Smith DB, Stuart NSA, Quarterman CP, Hoffman R, Stevens MFG, Brampton MH, and Gibson AC (1992) Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). *Br J Cancer* **65:**287–291.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, and Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139:**271–279.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, and Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO (Eur Mol Biol Organ) J* **12:**3679–3684.

Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara I, Roth JA, Deisseroth AB, Zhang WW, Kruzel E, and Radinsky R (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol* **15:**3032–3040.

Papadopoulos N, Nicolaides NC, Liu B, Parsons R, Lengauer C, Palombo F, D'Arrigo A, Markowitz S, Willson JKV, Kinzler KV, Jiricny J, and Vogelstein B (1995) Mutation of GTBP in genetically unstable cells. *Science (Washington DC)* **268:**1915–1917.

Pegg AE (1990) Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* **50:**6119–6129.

Peled A, Zipori D, and Rotter V (1996) Cooperation between p53-dependent and p53-independent apoptotic pathways in myeloid cells. *Cancer Res* **56:**2148–2156.

Plant JE and Roberts JJ (1971) A novel mechanism for the inhibition of DNA synthesis following methylation: the effect of *N*-methyl-*N*-nitrosourea on HeLa cells. *Chem Biol Interact* **3:**337–342.

Skopek TR, Liber HL, Penman BW, and Thilly WG (1978) Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: possibility for a rapid human cell mutation assay. *Biochem Biophys Res Commun* **84:**411–416.

Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, and Slack JA (1987) Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M&B 39831), a novel drug with potential as an alternative to dacarbazine. *Cancer Res* **47:**5846–5852.

Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, Edwards M, and Mace R (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. *Science (Washington DC)* **273:**1109–1111.

Tentori L, Graziani G, Gilberti S, Lacal PM, Bonmassar E, and D'Atri S (1995) Triazene compounds induce apoptosis in O^6-alkylguanine-DNA alkyltransferase deficient leukemia cell lines. *Leukemia* **9:**1888–1895.

Tentori L, Orlando L, Lacal PM, Benincasa E, Faraoni I, Bonmassar E, D'Atri S, and Graziani G (1997) Inhibition of O^6-alkylguanine DNA-alkyltransferase or poly-(ADP-ribose) polymerase increases susceptibility of leukemic cells to apoptosis induced by temozolomide. *Mol Pharmacol* **52:**249–258.

Walker PR, Kokileva L, LeBlanc J, and Sikorska M (1993) Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques* **15:**1032–1040.

Walker PR, Pandey S, and Sikorska M (1995) Degradation of chromatin in apoptotic cells. *Cell Death Differ* **2:**97–104.

Xiong Y, Hannon GJ, Zhang H, Casso D, and Kobayashi R (1993) p21 is a universal inhibitor of cyclin kinases. *Nature (Lond)* **366:**701–704.

Send reprint requests to: Dr. Stefania D'Atri, IDI-IRCCS, Laboratory of Clinical Pharmacology, Via dei Monti di Creta 104, 00167 Rome, Italy. E-mail: s.datri@idi.it