

# The Apurinic/Apyrimidinic Endonuclease Activity of Ape1/Ref-1 Contributes to Human Glioma Cell Resistance to Alkylating Agents and Is Elevated by Oxidative Stress<sup>1</sup>

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## ABSTRACT

Alkylating agents are standard components of adjuvant chemotherapy for gliomas. We provide evidence here that Ape1/Ref-1, the major mammalian apurinic/apyrimidinic endonuclease (Ap endo), contributes to alkylating agent resistance in human glioma cells by incising DNA at abasic sites. We show that antisense oligonucleotides directed against Ape1/Ref-1 in SNB19, a human glioma cell line lacking O<sup>6</sup>-methylguanine-DNA-methyltransferase, mediate both reduction in Ape1/Ref-1 protein and Ap endo activity and concurrent reduction in resistance to methyl methanesulfonate and the clinical alkylators temozolomide and 1,3-(2-chloroethyl)-1-nitrosourea. An accompanying increase in the level of abasic sites indicates that the DNA repair activity of Ape1/Ref-1 contributes to resistance. Conversely, we also show that exposure of SNB19 cells to HOCl, a generator of reactive oxygen species (ROS), results in elevated Ape1/Ref-1 protein and Ap endo activity, enhanced alkylator resistance, and reduced levels of abasic sites. Given current evidence that heightened oxidative stress prevails within brain tumors, the finding that ROS increase resistance to clinical alkylators in glioma cells may have significance for the response of gliomas to alkylating agent-based chemotherapy. Our results may also be relevant to the design of therapeutic regimens using concurrent ionizing radiation (a generator of ROS) and alkylating agent-based chemotherapy.

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## INTRODUCTION

Despite advances in surgery and radiotherapy, the prognosis for malignant gliomas remains poor; the overall 2-year survival rate is <20% (1). Alkylating agent-based chemotherapy has been shown to modestly increase response rates and survival times when used as an adjuvant to surgery and radiation (2, 3). The chloroethylating agents BCNU<sup>3</sup> (carmustine) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, lomustine), and the methylating agents procarbazine and TMZ, used alone or in combination regimens, are the most commonly used tumoricidal drugs for malignant glioma (3, 4). However, alkylating agents provide no benefit to ~50% of patients and seldom produce long-term remission; moreover, the modest benefit that they confer depends greatly on patient age and tumor histology (3).

DNA repair is one likely factor limiting the clinical efficacy of alkylators. We (5–7) and others (*e.g.*, Refs. 8–10) have documented the contribution of MGMT, a protein that removes cytotoxic O<sup>6</sup>-alkylguanine DNA adducts (11), to the resistance of human glioma cell lines and xenografts to clinically relevant alkylators. Consistent with these findings, we have observed that glioma cells lacking MGMT appear to be selectively killed *in situ* by alkylating agent-based chemotherapy (12). However, our analysis of nine glioma cell lines indicates that MGMT is not the only mechanism or necessarily the major mechanism of alkylator resistance (5–7).

Fundamental chemical, biochemical, and genetic considerations support the premise that DNA repair activities, in addition to MGMT, contribute to glioma alkylator resistance. Ap endo is prominent among such activities for the following reasons. Methylating and chloroethylating agents produce a diversity of *N*-alkyl purines and pyrimidines in DNA (13, 14); together, these alkylated bases constitute >50 to 80% of chloroethylator- and methylator-induced lesions, respectively. *N*-Alkylation promotes formation of abasic sites by destabilizing the glycosylic bond between the base and deoxyribose as much as four orders of magnitude (15) and by creating substrates for DNA glycosylases that cleave the glycosylic linkage in damaged nucleotides (16). These observations indicate that the predominant base adducts produced by clinically used alkylating agents are precursors of abasic sites. Abasic sites impede DNA synthesis by replicative DNA polymerases, and blockage of DNA replication is an apparent source of cytotoxicity (15, 17, 18). Consistent with the foregoing considerations, mutants of bacteria (19) and

<sup>3</sup> The abbreviations used are: BCNU, 1,3-(2-chloroethyl)-1-nitrosourea; TMZ, temozolomide; Ap endo, apurinic/apyrimidinic endonuclease; MGMT, O<sup>6</sup>-methylguanine-DNA methyltransferase; ASO, antisense oligonucleotide; SO, sense oligonucleotide; MMS, methyl methanesulfonate; ARP, aldehyde-reactive probe; ROS, reactive oxygen species; Mer, methyl repair.

yeast (20) that are genetically deficient in Ap endo activity are hypersensitive to killing by alkylating agents.

Ape1/Ref-1 (also known Hap-1 and Apex) is the major Ap endo activity in human cells and is a key enzyme in base excision repair pathways that remove alkylation-induced abasic sites (19, 21, 22). Ape1/Ref-1 is a multifunctional  $M_r$  35,500 protein that possesses a strong,  $Mg^{+2}$ -dependent endonuclease activity that hydrolyzes the phosphodiester bond 5' to abasic sites, leaving a 5' deoxyribose phosphate and a 3'-hydroxyl terminus for DNA repair synthesis (19, 21, 22). Mammalian Ape1/Ref-1 also has a 3'-phosphodiesterase activity that excises deoxyribose fragments and phosphate groups from the 3'-terminus of single-strand breaks, yielding a 3'-hydroxyl group (23). Current evidence indicates that Ape1/Ref-1 and Ap endo activity in HeLa (24) and rodent (25) cells are transiently elevated in response to treatment with subtoxic levels of agents that produce ROS such as  $\gamma$ -rays, HOCl, and  $H_2O_2$ . Importantly, greater resistance to alkylation-induced killing accompanies the elevation of activity (24).

Ape1/Ref-1 is abundant in mammalian cells ( $\sim 10^5$ - $10^6$  molecules/cell) and accounts for nearly all of the abasic site incision activity observed in cell extracts (22, 26). The abundance of Ape1/Ref-1 may reflect its function as a reduction-oxidation protein that participates in other crucial cellular processes, including the response to oxidative stress, regulation of transcription factors, cell cycle control, and apoptosis (21). For example, Ape1/Ref-1 promotes signal transduction in response to oxidative stress by reducing a conserved cysteine residue in Jun/Fos family members, facilitating formation of hetero- and homodimers that bind to transcriptional regulatory elements containing activator protein-1 and cyclic AMP motifs. Ape1/Ref-1 has also been implicated in regulating the transactivation and proapoptotic activities of p53 (27). The biological importance of Ape1/Ref-1 is evidenced by its essentiality for early embryonic development; homozygous null mice die shortly after blastocyst formation (28). Moreover, mice heterozygous for Ape1/Ref-1 display hypersensitivity to oxidative stress *in vivo*, and embryonic fibroblasts display diminished survival in response to agents that cause oxidative damage to DNA (29).

We have recently found that substantial elevation of Ap endo activity (13-fold, on average), as well as Ape1/Ref-1 levels, is characteristic of adult glial tumors (30). A clinically important consequence of the increased Ap endo activity in malignant gliomas may be enhanced resistance to alkylating agent-based chemotherapy. In support of this possibility, we show here that antisense suppression of Ape1/Ref-1 protein and Ap endo activity reduces the resistance of the human glioma-derived cell line SNB19 to TMZ and BCNU. Conversely, we show that oxidative stress induces elevation of Ape1/Ref-1 protein and Ap endo activity and is accompanied by increased resistance to these alkylators. Importantly, reduced resistance is accompanied by increased levels of abasic sites, and enhanced resistance is accompanied by decreased abundance of abasic sites, indicating that the apurinic/apyrimidinic site incision activity of Ape1/Ref-1 is at least partially responsible for resistance. Because heightened oxidative stress may be common in brain tumors (31), our demonstration of the effects of ROS on alkylating agent resistance in human glioma cells may have implications for adjuvant alkylating agent-based chemotherapy of gliomas. The interaction of oxidative stress and alkylator

resistance may also have implications for concurrent treatment with ionizing radiation and alkylating agents.

## MATERIALS AND METHODS

**Cell Culture.** The human glioblastoma-derived cell line SNB19 (32) was grown as adherent cultures at 37°C in 95% humidified air/5%  $CO_2$  in DMEM/F12 supplemented with 2% iron-supplemented bovine calf serum (HyClone), 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (Life Technologies, Inc.).

**Antisense Suppression of Ape1/Ref-1.** Cationic transfection was used to introduce two ASOs (Operon) directed against Ape1/Ref-1 mRNA into SNB19 cells. One sequence was complementary to the translation start site (5'-TTTC-CCACGCTTCGGATTCC-3') and the other to the second exon-intron junction (5'-TTCCCTTACCTGTCCTGA-3'). These sequences were chosen because of their low potential for self-association, and the presence of a TCCC motif, which occurs in half of ASOs that suppress expression of tumor necrosis factor  $\beta$  (33). As a control for specificity, cells were concurrently transfected with oligomers complementary to the ASOs (*i.e.*, SOs).

To prepare oligonucleotides for transfection, cationic lipid (Lipofectin; Life Technologies, Inc.) was gently mixed with 10 mM sodium citrate (pH 8.0) [1:8 vol/vol] and held at room temperature for 1 h. An equimolar mixture of the two ASOs was added so that the final oligonucleotide concentration was 200 nM. After standing at room temperature for 1 h, the mixture was held at 4°C overnight. Ten volumes of prewarmed serum- and antibiotic-free medium were added with gentle mixing. Subconfluent cultures were washed twice with prewarmed PBS and incubated for 5 h at 37°C with the oligonucleotides. The cells were then returned to oligonucleotide-free, complete medium and incubated for 19 h. This cycle was repeated three times. When ASO treatment preceded exposure to an alkylating agent, the final cycle was as follows. After removal of oligonucleotides, cells were returned to complete medium for 4 h and then inoculated into 6-well trays (1000–2000 cells in 2 ml of complete medium/well); the trays were incubated for an additional 15 h to permit attachment and resumption of proliferation before addition of alkylator.

**Induction of Oxidative Stress with HOCl.** Stock HOCl (Aldrich) was diluted 1:1000 into sterile 100 mM Tris-HCl (pH 9.0), and the concentration determined by absorbance at  $A^{292}$  ( $\epsilon = 350$ ). To induce oxidative stress, three 100-mm dishes of cells at  $\sim 70\%$  confluence were washed twice with 7 ml of PBS and incubated in 5 ml of unsupplemented medium containing either HOCl or an equal volume of 100 mM Tris-HCl (pH 9.0) for 1 h at 37°C. The cells were then returned to complete medium and incubation continued for a total of 24 h before determination of Ap endo activity/Ape1-Ref-1 level or alkylator sensitivity.

**Alkylating Agent Survival.** MMS (Sigma) was diluted, and TMZ (a generous gift from Dr. Joseph J. Catino of Schering-Plough Research Institute) was dissolved in DMSO at a concentration of 0.15 M and stored as single-use aliquots at  $-80^\circ C$ . BCNU (Bristol-Myers Squibb) was dissolved in absolute ethanol at a concentration of 1 M and stored as single use

aliquots at  $-80^{\circ}\text{C}$ . All drugs were diluted in the appropriate solvent immediately before use so that a constant volume was added for all doses. No-drug controls received an equivalent volume of solvent. The final concentration of DMSO or ethanol was 1%.

Alkylating agent survival was carried out as we have described previously in detail (5). Six-well trays (35 mm) were inoculated with 2 ml of supplemented DMEM/F12 containing 1000–2000 cells harvested by trypsinization. The trays were incubated for 6–8 h to allow cells to attach and resume proliferation before addition of alkylating agent in triplicate for each dose and additional incubation for 1 h. In cases where HOCl treatment preceded alkylator treatment, cells were harvested 16 to 18 h after exposure to HOCl, and the trays were incubated for 6 to 8 h before addition of alkylating agent (*i.e.*, 24 h after HOCl exposure). The cells were then washed free of residual alkylator and incubated in fresh, supplemented medium for 5 to 7 days to allow formation of colonies. The colonies were stained with 0.5% methylene blue in 1:1 methanol/ $\text{H}_2\text{O}$  (vol/vol) to aid visualization during counting by light microscopy at  $\times 40$ . Only colonies containing  $\geq 50$  cells were counted. The colony-forming efficiency of untreated SNB19 was  $\sim 25\%$ .

Drug sensitivity was determined by analysis of survival curves (log surviving fraction *versus* dose) using standard methods (34), as we have described previously in detail (especially Fig. 1; Ref. 5). Unless otherwise stated, each survival curve was determined from four independent experiments in which drug dose was assayed in triplicate (*i.e.*, 12 determinations/drug concentration).

**Ap endo Assay.** Cells were harvested by trypsinization, washed twice with PBS, flash-frozen in liquid  $\text{N}_2$ , and stored at  $-80^{\circ}\text{C}$ . The cell pellets were suspended in 1.0 ml of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 1  $\mu\text{g}/\text{ml}$  each of aprotinin, leupeptin, and pepstatin, and the cells were disrupted by sonication on ice for four 15 s intervals. Debris was pelleted by centrifugation at  $10,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The pellet was re-extracted in 0.5 ml of extraction buffer, and the supernatants were combined. Multiple small aliquots (25–50  $\mu\text{l}$ ) were flash-frozen in liquid  $\text{N}_2$  and stored at  $-80^{\circ}\text{C}$ ; aliquots were thawed only once.

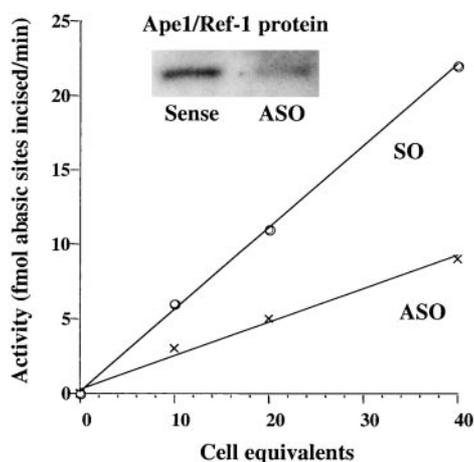
Ap endo activity was quantitated by using a standard, highly sensitive assay that measures the conversion of plasmid DNA from supercoiled to relaxed form caused by incision at an abasic site (26). We have previously used this assay to measure activity in extracts of human gliomas and normal brain (30, 35). Each determination comprised assay of increasing amounts of sample and yielded activity (fmol abasic sites incised/cell/min, abbreviated to fmol/cell/min) calculated by regression analysis of points on the initial linear portion of the curve; representative determinations illustrating linearity of activity with added extract are shown in Figs. 1 and 3A. Assay mixtures (30  $\mu\text{l}$ ) contained 0.033  $\mu\text{g}/\text{ml}$  depurinated pKT100 plasmid DNA, 50 mM HEPES (pH 7.5), 150 mM KCl, 5 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CoCl}_2$ , 100  $\mu\text{g}/\text{ml}$  BSA, and extract equivalent to 5–40 cells. After incubation for 15 min at  $37^{\circ}\text{C}$ , reaction products were resolved on a 0.8% agarose gel in 40 mM Tris-acetate and 2 mM EDTA. The gel was stained with ethidium bromide to visualize supercoiled and nicked, relaxed plasmid DNA and photographed with

a Kodak Digital DC120 camera. Band density of the scanned image was quantitated by using NIH Image version 1.68, with *HindIII* linearized pKT100 as a standard. DNA substrate was prepared as we have described (30) by using a modification of a previously reported protocol (26).

**Immunoblotting.** Extract proteins were resolved by electrophoresis at 140 V for 50 min in a 12% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid containing 10% methanol at 140 V for 90 min. The blot was incubated with a 1:1000 dilution of rabbit antihuman Ape1 antibody (Novus) or a 1:2000 dilution of rabbit antihuman Hsp72 (StressGen) for 60 min and then with a goat-antirabbit secondary antibody conjugated with alkaline phosphatase. Antibody binding was detected by chemiluminescence. Images of bands on X-ray film were photographed with a Kodak Digital DC120 camera, and band density was quantitated by using NIH Image version 1.68.

**Quantitation of Abasic Sites.** Abasic sites were quantitated by using a slot-blot technique (36, 37). DNA (5–10  $\mu\text{g}$ ), isolated as described previously (36, 37), in 150  $\mu\text{l}$  of PBS was incubated at  $37^{\circ}\text{C}$  for 10 min with 1 mM  $N'$ -aminooxy-methyl-carbonylhydrazino-D-biotin (ARP; Dojindo Laboratories). The treated DNA was ethanol precipitated, repeatedly washed with 70% ethanol, and resuspended at 30  $\mu\text{g}/\text{ml}$  in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. After heating at  $100^{\circ}\text{C}$  for 5 min followed by cooling in ice water, 0.01–0.3  $\mu\text{g}$  of cellular DNA was brought to a total of 0.3  $\mu\text{g}$  by addition of carrier calf thymus DNA pretreated with methoxyamine to eliminate abasic sites, as described below; at least two different amounts of cellular DNA were analyzed in every experiment. DNA samples were mixed with an equal volume of 2 M  $\text{NH}_4$  acetate and loaded onto nitrocellulose filters saturated with 1 M  $\text{NH}_4$  acetate by using a vacuum manifold containing 96 slots (0.75  $\times$  8.0 mm). The nitrocellulose filters were then soaked in  $5 \times \text{SSC}$  (0.75 M NaCl and 0.075 M trisodium citrate) for 5 min, air dried, and baked for 2 h at  $80^{\circ}\text{C}$  in a vacuum oven. After soaking for 1 h at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.5% casein, 0.25% BSA, and 0.1% Tween 20, the membrane was incubated with streptavidin-conjugated horseradish peroxidase at room temperature for 30 min. The membrane was rinsed for 15 min in 20 mM Tris-HCl (pH 7.5), 0.26 M NaCl, 1 mM EDTA, and 0.1% Tween 20, and the bound peroxidase activity visualized by enhanced chemiluminescence. Images on X-ray film were photographed with a Kodak Digital DC120 camera, and band (pixel) density was quantitated by using NIH Image version 1.68.

Abasic site levels in cellular DNA were determined by comparison with a standard curve constructed with reference DNA containing 1080 abasic sites/ $10^6$  nucleotides. To prepare reference DNA, calf thymus DNA was first incubated with 2 mM methoxyamine in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 2 h at  $37^{\circ}\text{C}$  to eliminate pre-existing ARP-reactive sites. After ethanol precipitation and repeated washes with 70% ethanol, abasic sites were introduced by resuspending the DNA in 100 mM sodium citrate (pH 5.0) and 100 mM NaCl and heating at  $70^{\circ}\text{C}$  for 80 min. After ethanol precipitation, the DNA was dissolved in PBS and the concentration determined by absorbance. The depurinated reference DNA contained 1080



**Fig. 1** ASOs directed against Ape1/Ref-1 suppress Ap endo activity in human glioma cells. Subconfluent SNB19 cells were treated with ASO (×) or SO (○) as described in “Materials and Methods.” At the end of the final cycle of oligonucleotide treatment, Ap endo activity was measured in a plasmid-nicking assay that monitors conversion from supercoiled to relaxed DNA (26, 30). Ape1/Ref-1 protein (*inset*) was assessed by immunoblotting with chemiluminescence detection.

abasic sites/ $10^6$  nucleotides, as determined in a commercially available ELISA assay (Dojindo Laboratories). Reference DNA was mixed with carrier, methoxyamine-treated DNA to yield standards containing 0–540 abasic sites/ $10^6$  nucleotides. Standards (0.3  $\mu$ g) were incubated with ARP and processed simultaneously on the same nitrocellulose filter with cellular DNA samples. In our experience, band density of standards is linear within the range of 0.8 to 35 (or up to 200 with shorter exposure times) abasic sites/ $10^6$  nucleotides when 0.3  $\mu$ g of DNA is blotted (see standard curve in Fig. 6B). DNA concentrations were determined by quantitating deoxyribose as described by Burton (38) with dAMP as a standard.

**Statistical Analysis.** Standard statistical procedures (39) were applied by using Microsoft Excel (Microsoft, Redmond, WA). Comparison of means was by Student’s *t* test assuming unequal variances. Results are reported as two-tailed *P*s. Statistically significant relationships were determined at the 95% confidence level.

## RESULTS

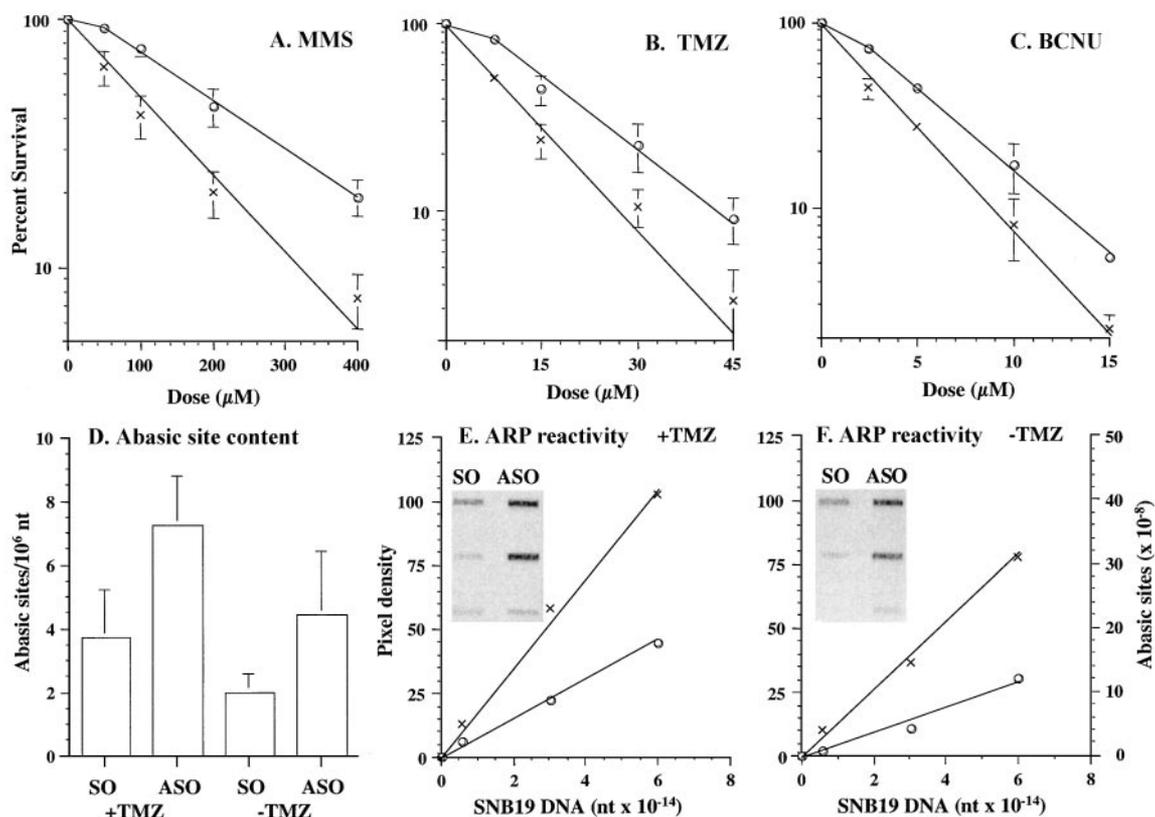
**ASOs Suppress Ape1/Ref-1-catalyzed Ap endo Activity in Human Glioma Cells.** Ape1/Ref-1 catalyzes almost all Ap endo activity in mammalian cells (21, 22). We used two ASOs, one complementary to the translation start site and the other complementary to the second exon-intron junction, to suppress expression of Ape1/Ref-1 in the human glioma-derived line SNB19. As illustrated in Fig. 1, Ap endo activity was reduced in extracts of ASO-treated cells relative to extracts of cells transfected with SO. The data show that activity in our plasmid incision assay is a linear function of added extract for both ASO- and SO-treated cells. In four independent experiments, ASO treatment reduced activity  $2.4 \pm 0.5$ -fold from  $0.51 \pm 0.14$  to  $0.23 \pm 0.07$  fmol abasic sites incised/cell/min ( $P \leq 0.01$ ). A comparable reduction in Ape1/Ref-1 protein level was

observed by Western blotting (Fig. 1, *inset*). The activity of SO-transfected cells was comparable with that of untransfected SNB19 ( $0.44 \pm 0.10$  fmol/cell/min, the average of four experiments).

**Suppression of Ape1/Ref-1 Reduces Resistance to Alkylating Agents and Increases the Level of Abasic Sites.** We have previously quantitated the resistance of SNB19 and other human glioma cell lines to mono- and bifunctional alkylating agents (5–7). These alkylators include the methylating agent TMZ, used in the adjuvant therapy of gliomas (4), and the bifunctional chloroethylating agent BCNU, a mainstay of alkylating agent-based glioma chemotherapy (1–3). As shown in Fig. 2A, ASO treatment reduced resistance to MMS, a laboratory methylator, in accord with earlier results for HeLa and rat glioma cells (40, 41). Resistance to TMZ (Fig. 2B) and BCNU (Fig. 2C) was reduced as well. Resistance was reduced uniformly in the ASO-treated cells, as indicated by the constant slope of the survival curves, suggesting that all cells were similarly affected. The sensitivity of SO-transfected cells to MMS, TMZ, and BCNU was the same as that of cells carried through the transfection protocol without oligonucleotides (data not shown), demonstrating that the increased cytotoxicity was specific to ASOs. ASO- and SO-treated cells did not differ in growth rate or plating efficiency, abundance of heat shock protein 72 (Hsp72) as assessed by immunoblotting, and  $N^3$ -methyladenine-DNA glycosylase activity (data not shown). These controls indicate that the diminished alkylator resistance of ASO-treated cells did not reflect nonspecific effects of ASOs on cell physiology or DNA repair capacity.

As summarized in Table 1, ASOs diminished survival of alkylator exposure, measured as  $LD_{10}$ , by two mechanisms. First, the dose tolerated without killing (*i.e.*,  $D_T$ , the threshold dose indicated by a shoulder on the survival curve) was eliminated or greatly reduced, indicating that Ape1/Ref-1 contributed to tolerance of low alkylator doses. This tolerance may be particularly important in clinical settings; the 12-fold decrease in the  $D_T$  for TMZ is noteworthy in this regard. Second, the rate of killing on the linear portion of the survival curve was increased, as indicated by lower  $D_{37}$ . The reduction in survival parameters was statistically significant, except in the case of the very low  $D_T$  for BCNU. We conclude that Ape1/Ref-1 contributes to the resistance of SNB19 cells to clinically important methylating and chloroethylating agents.

ASO treatment was accompanied by elevated levels of abasic sites measured in a slot-blot protocol using an ARP that reacts with abasic sites (36). As shown in Fig. 2D, abasic site content was increased 2-fold, both in cells exposed to TMZ ( $7.2 \pm 1.6$  versus  $3.7 \pm 1.5$  abasic sites/ $10^6$  nucleotides;  $P \leq 0.04$ ) and in control cells that were not exposed to alkylator ( $4.4 \pm 2.0$  versus  $2.0 \pm 0.6$  abasic sites/ $10^6$  nucleotides;  $P \leq 0.05$ ); the values are the means of six determinations in three independent experiments. Primary data documenting the linearity of ARP reactivity (chemiluminescence of bands, measured in pixel density) with increasing amounts of cellular DNA are illustrated in Fig. 2, E and F, respectively; a standard curve relating pixel density to known numbers of abasic sites is included in Fig. 6. Our data indicate that Ape1/Ref-1-catalyzed Ap endo activity reduces the level of abasic sites arising from both exposure to exogenous alkylators and from endogenous



**Fig. 2** Antisense suppression of Ape1/Ref-1 reduces resistance to MMS, TMZ, and BCNU and increases abasic site abundance. *A–C*, alkylating agent resistance. Subconfluent SNB19 cells were treated with ASO ( $\times$ ) or SO ( $\circ$ ) and then exposed to either MMS, TMZ, or BCNU for 1 h. Alkylator-treated cells were washed free of drug and incubated for 5–7 days before counting of colonies for determination of clonogenic survival. Each point is the mean  $\pm$  SD of triplicate assays in at least four independent experiments. Curves were constructed by linear regression analysis of points on the linear portion of the curves. *D*, abasic site levels. The abasic site content of ASO- and SO-treated cells was determined immediately after exposure to 45  $\mu\text{M}$  TMZ or its carrier DMSO. The mean  $\pm$  SD of seven determinations in three experiments is shown. Abasic site abundance was determined by using chemiluminescence detection of an ARP bound to abasic sites in sample DNA and in DNA standards, immobilized on a nitrocellulose membrane (36), as described in “Materials and Methods.” *E* and *F*, primary data illustrating the linear relationship between chemiluminescence (pixel density) and amount of SNB19 DNA loaded on blots determined by linear regression analysis. *Insets* show blots of 0.01, 0.05, and 0.1  $\mu\text{g}$  of DNA ( $0.6$  to  $6 \times 10^{14}$  nucleotides) isolated from TMZ-treated (*E*) and -untreated cells (*F*). See also Fig. 6*B* for a standard curve relating chemiluminescence to known numbers of abasic sites.

processes. Taken together, the results in Figs. 1 and 2 and Table 1 indicate that the DNA repair activity of Ape1/Ref-1, *i.e.*, the Ape1/Ref-1-mediated reduction in cytotoxic abasic sites, is at least partially responsible for alkylating agent resistance. This inference is consistent with the alkylator hypersensitivity of prokaryotic (19) and lower eukaryotic (20) mutants deficient in Ap endo activity. Our findings do not, of course, bear on other possible roles of the multifunctional Ape1/Ref-1 protein in resistance.

**Oxidative Stress Elevates Ape1/Ref-1-catalyzed Ap endo Activity.** Previous reports have documented a transient, modest elevation of Ape1/Ref-1 protein and activity in rodent and human cells exposed to minimally cytotoxic doses of ROS (24, 25). As illustrated in Fig. 3*A*, treatment of SNB19 cells with 26  $\mu\text{M}$  HOCl, an ROS generator (42), resulted in a 2.5-fold increase in Ap endo activity 24 h after exposure; concomitant cell killing was  $\leq 25\%$ , assessed by clonogenic assay (data not shown). Increased activity was not evident until after the first 12 h following HOCl treatment; activity peaked between 18 and

24 h and returned to untreated levels by 30 h (data not shown). As illustrated in Fig. 3*B*, a dose of 18  $\mu\text{M}$  HOCl ( $\sim 20\%$  killing) elicited the greatest elevation ( $\sim 3.5$ -fold), observed at 24 h after exposure. Western analysis revealed that the increase in activity was accompanied by an increase in the level of Ape1/Ref-1 protein (Fig. 3*B*, *inset*). In five independent experiments, mean Ape1/Ref-1 level, estimated by digital image analysis of immunoblots, was 2.1- to 6.4-fold (mean  $\pm$  SD =  $3.7 \pm 1.7$ ) greater in treated cells at 24 h after exposure to HOCl than in control cells not exposed to HOCl. These results are in accord with the 2- to 3-fold increase observed in other mammalian cells (24, 25). To verify that the elevation of Ape1/Ref-1 protein was attributable to ROS, treatment with HOCl was carried out in the presence of *N*-acetylcysteine, a potent free radical scavenger. As shown in Fig. 3*C*, cells treated with HOCl alone showed a 3.5-fold increase in Ape1/Ref-1 protein level 24 h after exposure, whereas cells exposed to HOCl in the presence of *N*-acetylcysteine exhibited no elevation.

To further document the oxidative stress response, we

Table 1 Antisense oligonucleotides against Ape1/Ref-1 decrease resistance to clinical alkylators in human glioma cells<sup>a</sup>

	SO	ASO	SO/ASO	P≤
MMS				
D <sub>T</sub>	54 ± 40	0		0.008
D <sub>37</sub>	260 ± 31	157 ± 16	1.7	0.002
LD <sub>10</sub>	556 ± 35	363 ± 37	1.5	0.0003
TMZ				
D <sub>T</sub>	3.7 ± 1.6	0.3 ± 0.5	12	0.02
D <sub>37</sub>	19 ± 3.2	12 ± 2.6	1.6	0.02
LD <sub>10</sub>	41 ± 6.2	29 ± 6.0	1.4	0.03
BCNU				
D <sub>T</sub>	0.4 ± 0.3	0		0.12
D <sub>37</sub>	5.3 ± 0.4	3.9 ± 0.5	1.4	0.02
LD <sub>10</sub>	12 ± 0.8	8.9 ± 1.2	1.3	0.04

<sup>a</sup> SNB19 cells were treated with ASO or SO, exposed to MMS, TMZ, or BCNU for 1 h, and then incubated to permit growth of colonies. Alkylator survival was determined by clonogenic assay as described in "Materials and Methods." Survival was quantitated by three parameters derived from survival curves such as shown in Fig. 2: D<sub>T</sub>, the threshold dose below which cells are not killed; D<sub>37</sub>, a measure of the rate of killing on the linear portion of the survival curve; and LD<sub>10</sub>, the dose required to reduce overall survival to 10% (5, 34). Values (drug dose, μM) are the mean ± SD of 12 determinations (triplicate assays in four independent experiments). *P*s were calculated by the *t* test assuming unequal variances.

examined the level of Hsp72, an inducible member of the heat shock protein 70 family (43). Hsp72 levels are increased as part of a protective response to a number of environmental insults, including oxidative stress. As shown in Fig. 3C, treatment with HOCl alone, but not with HOCl and *N*-acetylcysteine, elicited an ~5-fold elevation in the level of Hsp72 at 24 h after exposure consistent with our observations for Ape1/Ref-1. These results are in agreement with those of Matsumoto *et al.* (44) who reported elevation of Hsp72 in glioma cell lines in response to ROS.

**Oxidative Stress Increases Resistance to MMS, TMZ, and BCNU.** It is a reasonable expectation that the HOCl-induced elevation of Ap endo activity and Ape1/Ref-1 protein would be accompanied by enhanced MMS survival because such a result has been reported for HeLa cells (24). An initial examination of SNB19 confirmed this expectation. After treatment with HOCl and subsequent exposure to MMS, clonogenic survival was increased (Fig. 4A). The HOCl dependence of survival paralleled the HOCl dependence of Ap endo activity illustrated in Fig. 3B, exhibiting a maximum at 18 μM HOCl. As shown in Fig. 4B, *N*-acetylcysteine abrogated the increase in survival, indicating that ROS are responsible for the enhanced resistance.

Treatment with HOCl also increased resistance to TMZ and BCNU (Fig. 5). In these experiments, survival of cells exposed to MMS, TMZ, and BCNU was determined concurrently, allowing direct, quantitative comparison of the effects on resistance. As documented in Table 2, substantial increases in D<sub>T</sub> (3- to 16-fold) and greater D<sub>37</sub>s resulted in ~2-fold increases in LD<sub>10</sub> for all three agents; all increments in survival parameters were statistically significant. We conclude that oxidative stress enhances resistance to clinically relevant alkylators in SNB19 cells.

### The DNA Repair Activity of Ape1/Ref1 Contributes to the Enhanced Alkylator Resistance Induced by HOCl.

To determine whether the abasic site-specific endonuclease activity of Ape1/Ref1 contributes to the elevated alkylating agent resistance in SNB19 cells, we measured the steady-state level of abasic sites in HOCl-treated and -untreated cells after exposure to TMZ. As previously noted, abasic sites were measured by using a slot-blot method using an ARP that reacts with abasic sites (36, 37). As shown in Fig. 6A, DNA from cells treated with 18 μM HOCl before exposure to 45 μM TMZ displayed a 1.5-fold lower abundance of abasic sites (3.8 ± 0.6 versus 5.5 ± 1.5 abasic sites/10<sup>6</sup> nucleotides; *P* ≤ 0.035) based on five measurements of DNA isolated in two independent experiments; at this TMZ dose, survival of cells treated with or without HOCl was 13.7 ± 3.6 and 3.7 ± 1.0%, respectively, as shown in Fig. 5. HOCl treatment also resulted in reduced abasic site abundance in the absence of exogenous alkylator (1.9 ± 0.2 versus 3.8 ± 0.6 abasic sites/10<sup>6</sup> nucleotides; *P* ≤ 0.0025). The abasic site level observed in untreated cells, 3.8 ± 0.6/10<sup>6</sup> nucleotides, was similar to that reported by Nakamura *et al.* (36) (~4/10<sup>6</sup> nucleotides) for HeLa cells grown in suspension. Taken together, the foregoing results indicate that increased Ape1/Ref1-mediated incision at abasic sites contributes to the enhanced resistance of HOCl-treated cells to TMZ.

## DISCUSSION

Alkylating agents are the cornerstone of adjuvant chemotherapy for malignant gliomas (1–4). Yet, half of the tumors do not respond, and for those that do, response is generally short-lived (2, 3). As a result, malignant gliomas remain one of the most lethal cancers with a median survival of 12 months for the most common diagnostic types (1–3). These considerations emphasize the need to document mechanisms of resistance to clinically effective alkylating agents to devise antiresistance strategies. Repair of DNA damage caused by methylating and chloroethylating agents is one source of resistance. We have previously provided evidence that the DNA repair protein MGMT reduces alkylator cytotoxicity in glioma cells both *in vitro* (5–7) and *in vivo* (12). Our results also demonstrate that a resistance mechanism(s), in addition to the MGMT-mediated removal of cytotoxic O<sup>6</sup>-alkylguanine adducts, is important.

Ap endo is required for wild-type resistance to alkylating agents in prokaryotic (19) and lower eukaryotic cells (20). In accord, antisense suppression of the major mammalian Ap endo Ape1/Ref-1 (19, 21, 22) increases sensitivity to MMS in HeLa (40) and rat glioma (41) cells. Here, we show that Ape1/Ref-1 contributes to resistance to MMS and to the clinically used alkylators TMZ and BCNU in a human glioma cell line. This demonstration rests on two complementary approaches. One approach is antisense suppression, which decreased Ape1/Ref-1 protein levels and Ap endo activity, and concomitantly decreased resistance. The other approach is exposure to ROS, which increased Ape1/Ref-1 protein levels and Ap endo activity, and increased resistance. Analysis of abasic site abundance indicated that the DNA repair activity of Ape1/Ref-1 is at least partially responsible for resistance. This latter finding is not surprising because the Ap endos of bacteria (the *Xth* and *Nfo* gene products) and yeast (the *ETH1/APE2* and *APE1* gene

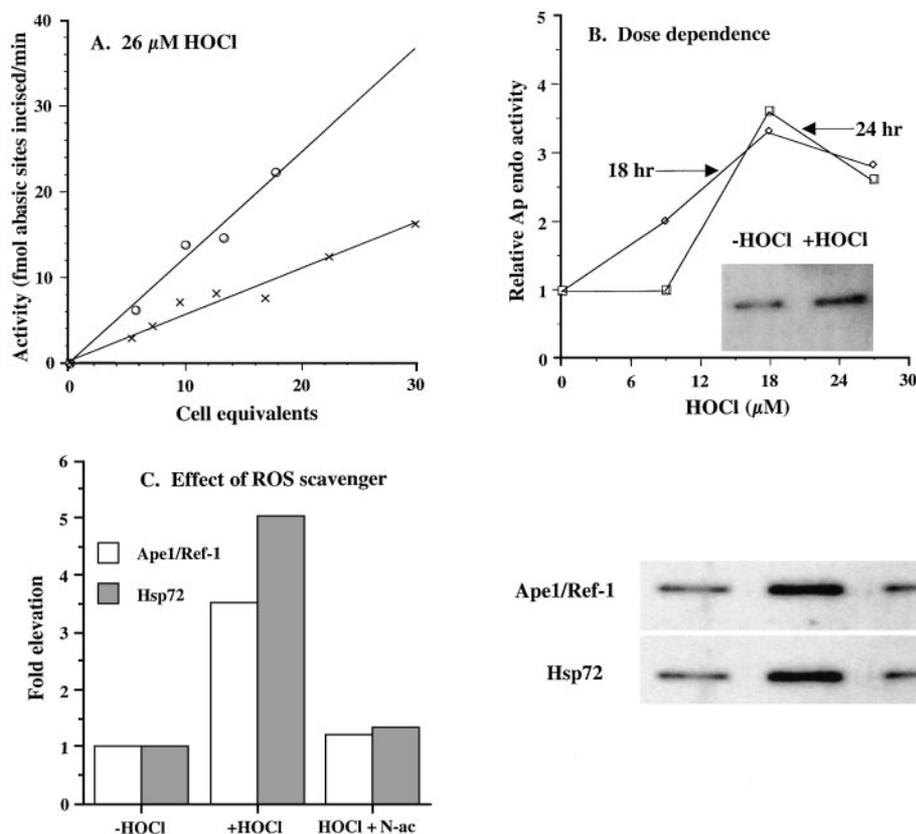
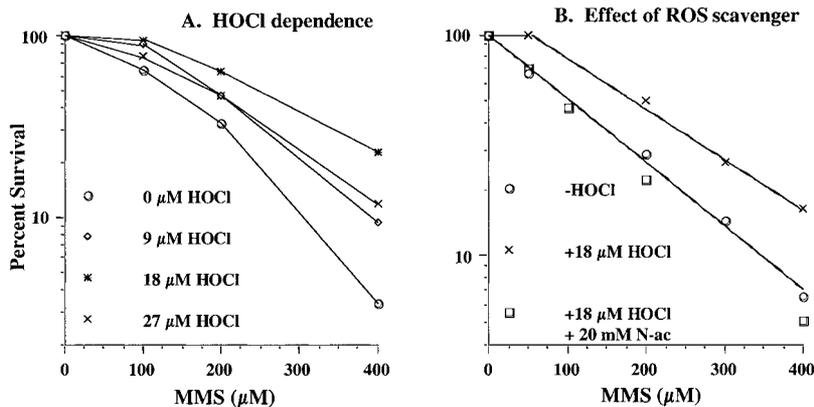


Fig. 3 Oxidative stress elevates Ap endo activity and the levels of Ape1/Ref-1 and Hsp72. SNB19 cells were grown to ~70% confluence, washed free of medium, and exposed to HOCl in serum-free medium for 1 h. Cells were then returned to supplemented medium and incubated for the time specified before assay of Ap endo activity or Ape1/Ref-1 content. A, Ap endo activity in extracts of cells prepared 0 or 24 h after exposure to 26  $\mu\text{M}$  HOCl. B, dose dependence of Ap endo activity assayed 18 ( $\square$ ) or 24 h ( $\diamond$ ) after exposure to HOCl. The inset shows Western detection of Ape1/Ref-1 at 24 h after treatment with 18  $\mu\text{M}$  HOCl. C, immunodetection of Ape1/Ref-1 ( $\square$ ) and Hsp72 ( $\blacksquare$ ) at 24 h after exposure to a buffer control, or 18  $\mu\text{M}$  HOCl, or 18  $\mu\text{M}$  HOCl and 20 mM *N*-acetylcysteine (N-ac), a scavenger of ROS. Quantitation was by digital image analysis of the immunoblot beside the graph.

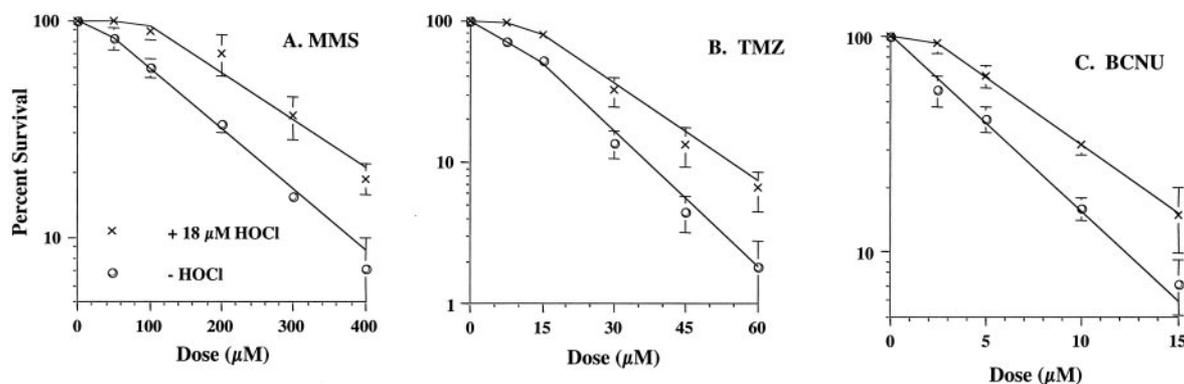
Fig. 4 Oxidative stress increases resistance to MMS. The MMS sensitivity of SNB19 cells was determined by clonogenic assay 24 h after exposure to HOCl. For these experiments, cells were treated with HOCl for 1 h at the specified dose, returned to complete medium for 24 h, then treated with MMS and assayed for proliferative survival, as described in detail in "Materials and Methods." A, HOCl dependence of enhancement in MMS survival. B, effect of simultaneous treatment with HOCl and *N*-acetylcysteine (N-ac), a scavenger of ROS. Treatment with *N*-acetylcysteine alone had no detectable effect on survival (data not shown). All points are the average of triplicate determinations.



products) that are essential for wild-type alkylator resistance, unlike Ape1/Ref-1, have no recognized functions other than DNA repair (19, 20). Our results do not address other possible roles of Ape1/Ref-1 in resistance, such as involvement of the redox function in alkylation-induced signal transduction. Because alkylation of lower eukaryotic cells alters the expression of ~400 genes (45), it is reasonable to suppose that a comparable phenomenon, perhaps involving Ape1/Ref-1, occurs in glioma cells.

The human glioma cell line used in this work, SNB19, is Mer<sup>-</sup>, i.e., it lacks MGMT and is therefore unable to remove

cytotoxic O<sup>6</sup>-alkylguanine monoadducts and to prevent the formation of lethal interstrand cross-links from O<sup>6</sup>-chloroethylguanine precursors (11). Given the role of MGMT in alkylator resistance (5–11), it might be presumed that the observed killing of SNB19 cells is due primarily to lethal amounts of O<sup>6</sup>-alkylguanine and its derivative lesions. Yet, our results show that Ap endo activity is important in reducing alkylator cytotoxicity, even in the presence of presumptively high levels of unrepaired O<sup>6</sup>-alkylguanine adducts. The data for TMZ are particularly relevant in this regard because this agent yields a relatively large proportion of O<sup>6</sup>-methylguanine (46), which we



**Fig. 5** Oxidative stress increases resistance to the clinically used alkylators TMZ and BCNU, as well as to MMS. The survival of SNB19 cells was determined by clonogenic assay 24 h after exposure to 18  $\mu\text{M}$  HOCl. For these experiments, cells were treated with 18  $\mu\text{M}$  HOCl for 1 h, returned to complete medium for 24 h, then treated with alkylator, and assayed for proliferative survival, as described in detail in "Materials and Methods." Each point is the mean  $\pm$  SD of triplicate assays in four independent experiments in which survival of all alkylators was analyzed concurrently. Curves were determined by linear regression analysis.

**Table 2** HOCl treatment increases resistance to MMS, TMZ, and BCNU in human glioma cells<sup>a</sup>

	+HOCl	-HOCl	+HOCl/-HOCl	<i>P</i> ≤
<b>MMS</b>				
$D_T$	90 $\pm$ 50	11 $\pm$ 7	8.2	0.02
$D_{37}$	308 $\pm$ 64	179 $\pm$ 21	1.7	0.004
$LD_{10}$	650 $\pm$ 151	393 $\pm$ 35	1.7	0.007
<b>TMZ</b>				
$D_T$	9.5 $\pm$ 3.9	2.8 $\pm$ 2.7	3.4	0.003
$D_{37}$	27 $\pm$ 3.9	18 $\pm$ 2.5	1.5	0.0004
$LD_{10}$	57 $\pm$ 9.7	39 $\pm$ 5.6	1.5	0.002
<b>BCNU</b>				
$D_T$	1.6 $\pm$ 1.4	0.1 $\pm$ 0.3	16	0.04
$D_{37}$	9.9 $\pm$ 3.5	5.1 $\pm$ 0.6	1.9	0.02
$LD_{10}$	19 $\pm$ 3	12 $\pm$ 2	1.6	0.04

<sup>a</sup> SNB19 cells were treated with alkylator 24 h after exposure to 18  $\mu\text{M}$  HOCl or its carrier 100 mM Tris-HCl (pH 9.0), and proliferative survival was determined by clonogenic assay. Survival was quantitated by three parameters derived from survival curves such as shown in Fig. 5 (see footnote to Table 1 for additional details). Values (drug dose,  $\mu\text{M}$ ) are the mean  $\pm$  SD of 12 determinations (triplicate assays in four independent experiments). *P*s were calculated by the *t* test assuming unequal variances.

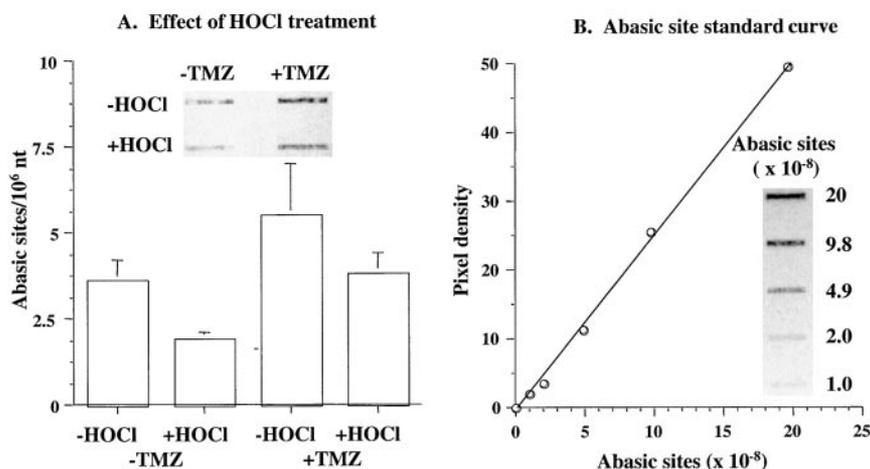
have shown to be a major cytotoxic lesion in human glioma cell lines (5, 7). Our preliminary experiments show that ASO directed against Ape1/Ref-1 also potentiate TMZ and BCNU cytotoxicity in SF763 and SF767, two MGMT-proficient human glioma lines, indicating that Ape1/Ref-1 contributes to alkylating agent resistance in Mer<sup>+</sup> as well as in Mer<sup>-</sup> cells. This finding is significant in light of our earlier observation that ~80% of human gliomas possess detectable MGMT activity (12).

Ape1/Ref-1 reduces the cytotoxicity of BCNU, as well as methylators, in SNB19 glioma cells. Importantly, the potentiation of BCNU-induced killing produced by ~50% depletion of Ap endo activity is comparable with the mean 1.8-fold potentiation produced by total ablation of MGMT in seven Mer<sup>+</sup> human glioma lines (6). This finding suggests that abasic sites may contribute as much to chloroethylator cytotoxicity as O<sup>6</sup>-

chloroethylguanine and its derivative lesions and that Ape1/Ref-1 may be as important as MGMT in promoting glioma resistance to BCNU. It is noteworthy in the context of both BCNU and TMZ resistance that the substrate analogue inhibitor O<sup>6</sup>-benzylguanine, used to ablate MGMT, is in clinical trial (47). Our results suggest that Ape1/Ref-1 may promote glioma cell survival *in vivo*, even in the presence of O<sup>6</sup>-benzylguanine.

We have recently found that elevation of Ape1/Ref-1-mediated Ap endo activity is characteristic of human gliomas (30). Elevated activity was observed in 93% (54 of 58) of gliomas, relative to adjacent normal brain, and the median elevation was large, *i.e.*, 13-fold. These results, taken together with the data presented here, suggest that the elevation of Ap endo activity that accompanies glial tumorigenesis may contribute to intrinsic resistance to alkylators. It is relevant in this regard that 7 of 9 human glioma cell lines that we have examined (6, 7) either depended little, or not at all, on MGMT for resistance to BCNU and/or TMZ or harbored subpopulations with this resistance phenotype. Clearly, other repair mechanisms, possibly including Ape1/Ref-1, mediated resistance in these cells. In the present work, we found that even subtotal depletion of Ape1/Ref-1 greatly reduced or eliminated tolerance of low doses of BCNU and TMZ. The finding that Ape1/Ref-1 was responsible for maintaining survival at doses that are likely within clinically achievable ranges (48) suggests that Ape1/Ref-1 is an attractive candidate for antiresistance therapy. Crystal structures of Ape1/Ref-1 are now available (49, 50), enabling the design of low molecular weight inhibitors. Concurrent ablation of MGMT and Ape1/Ref-1 might be an effective strategy to increase the levels of different types of major lethal lesions.

We observed that low-level oxidative stress significantly elevated Ape1/Ref-1 levels, Ap endo activity, and resistance to MMS, TMZ, and BCNU in SNB19 cells. The abasic site incision activity of Ape1/Ref-1 was at least partially responsible for increased resistance as evidenced by a reduction in the level of apurinic/aprimidinic sites. In addition to its DNA repair activity, the redox function of Ape1/Ref-1 may contribute to the ROS-induced elevation of resistance. Ape1/Ref-1 promotes stress-induced signal transduction cascades by activating tran-



**Fig. 6** Reduced abasic site levels implicate the DNA repair activity of Ape-1/Ref-1 in oxidative stress-induced enhancement of alkylating agent resistance. **A**, effect of HOCl on abasic site levels. SNB19 cells were treated with 18  $\mu$ M HOCl for 1 h, returned to complete medium for 24 h, and then exposed to 45  $\mu$ M TMZ or its carrier for 1 h. DNA was extracted immediately for determination of abasic site levels by a slot-blot procedure using an aldehyde-reactive reagent (36). **B**, standard curve for abasic site measurement. A standard curve is shown, constructed by digital image analysis of a blot and loaded with 0.3  $\mu$ g/slot of calf thymus DNA containing increasing amounts of abasic sites, prepared as described in "Materials and Methods." The DNA standards were run on the same blot as the samples in Fig. 2, E and F.

scriptional regulatory elements that have been implicated in stress responses (reviewed in Ref. 21). The role of Ape1/Ref-1 in regulating the transactivation and proapoptotic activities of p53 (27) is not relevant, however, in the case of SNB19 cells, which do not express p53.<sup>4</sup> Oxidative stress-inducible gene products in addition to Ape1/Ref-1 likely contribute to SNB19 alkylator resistance. Increased expression of numerous genes occurs in mammalian cells after exposure to ROS as part of an adaptive response (51). These include Hsp72, an inducible member of the Hsp70 family (Fig. 3). Notably, Hsp70 has been shown to stimulate the Ap endo activity of purified Ape1/Ref-1 up to 100-fold (52). Of particular relevance for alkylator resistance are reports of elevated expression of genes that promote growth arrest (53) or participate in base excision repair, including DNA polymerase  $\beta$  (54), 7,8-dihydro-8-oxoguanine-DNA glycosylase (55) and N<sup>3</sup>-methyladenine-DNA glycosylase (55).

Oxidative stress appears to be characteristic of gliomas (31) and may contribute to intrinsic alkylating agent resistance. Endogenous generation of ROS in gliomas may result from greater metabolic rates associated with proliferation and from seizures (56), hypoxia (57), and increased synthesis of nitric oxide associated with angiogenesis (58). Therapeutic intervention may exacerbate these processes. For example, surgery produces inflammation, and radiotherapy may deliver low, inducing doses of ROS to residual glioma cells that have infiltrated beyond the tumor margin. Notably, 2 Gy of ionizing radiation, the standard daily fractionated dose, have been shown to elevate Ape1/Ref-1 in HeLa cells (24). Heightened oxidative stress may be prolonged well into the postsurgical period, due, for example, to the elevation of nitric oxide that promotes revascularization. Thus, endogenous and/or exogenous sources

of ROS may compromise the efficacy of alkylating agent-based chemotherapy for malignant gliomas by inducing an adaptive response that includes elevation of Ape1/Ref-1. This presumptive elevation could be greater than that observed here because the cell line we used is adapted to greater than physiological oxygen tension ( $\sim$ 20% versus 5%; Ref. 51).

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<sup>4</sup> R. Morrison, personal communication.

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