Apurinic/Apyrimidinic Endonuclease Activity Is Associated with Response to Radiation and Chemotherapy in Medulloblastoma and Primitive Neuroectodermal Tumors

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Abstract

Purpose: Apurinic/apyrimidinic endonuclease (Ap endo) is a key DNA repair activity that confers resistance to radiation- and alkylator-induced cytotoxic abasic sites in human cells. We assayed apurinic/apyrimidinic endonuclease activity in medulloblastomas and primitive neuroectodermal tumors (PNET) to establish correlates with tumor and patient characteristics and with response to adjuvant radiation plus multiagent chemotherapy.

Experimental Design: Ap endo activity was assayed in 52 medulloblastomas and 10 PNETs from patients 0.4 to 21 years old. Apel/Ref-1, the predominant human Ap endo activity, was measured in 42 medulloblastomas by immunostaining. Cox proportional hazards regression models were used to analyze the association of activity with time to tumor progression (TTP).

Results: Tumor Ap endo activity varied 180-fold and was significantly associated with age and gender. Tumor Apel/Ref-1 was detected almost exclusively in nuclei. In a multivariate model, with Ap endo activity entered as a continuous variable, the hazard ratio for progression after adjuvant treatment in 46 medulloblastomas and four PNETs increased by a factor of 1.073 for every 0.01 unit increase in activity (P ≤ 0.001) and was independent of age and gender. Suppressing Ap endo activity in a human medulloblastoma cell line significantly increased sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide, suggesting that the association of tumor activity with TTP reflected, at least in part, abasic site repair.

Conclusions: Our data (a) suggest that Ap endo activity promotes resistance to radiation plus chemotherapy in medulloblastomas/PNETs, (b) provide a potential marker of treatment outcome, and (c) suggest clinical use of Ap endo inhibitors to overcome resistance.

Medulloblastomas and primitive neuroectodermal tumors (PNET) are highly malignant (i.e., WHO grade 4) cancers that account for 20% to 25% of the ~2,200 pediatric primary brain tumors diagnosed annually in the United States (1, 2). The median age at diagnosis is about 9 years, with the majority of cases (62%) occurring in males (3). Medulloblastomas and PNETs also occur infrequently in younger adults between 20 and 40 years of age (4). These malignancies, categorized as embryonal tumors in the current WHO classification (5), are histologically similar, being composed of small, morphologically undifferentiated cells of uncertain histogenesis that occasionally contain focal areas displaying morphologic and immunohistochemical features of mature neurons or glia (5, 6). Although emerging evidence suggests that medulloblastomas and PNETs harbor distinctive genetic aberrations (6), these tumors are primarily distinguished by their anatomic sites of occurrence: medulloblastomas arise in the cerebellum, whereas PNETs occur almost exclusively in the cerebral hemispheres.

Improvements in surgical technique and the development of effective postoperative treatments have dramatically increased survival rates for medulloblastomas in the last 30 years (1, 2). Better outcome has also been achieved by using patient and tumor characteristics to stratify medulloblastomas into average-risk and high-risk groups that differ in predicted response and likelihood of recurrence (6–8). The current standard of care is surgical excision to the greatest extent possible followed by adjuvant radiotherapy and chemotherapy. For patients receiving this care, overall 5-year survival rates range from 60% to 85% for average-risk medulloblastomas to about 40% for high-risk medulloblastomas (6, 7, 9). In contrast, PNETs are more aggressive and less responsive, with 5-year survival rates of <20% (10). Unfortunately, long-term survival is frequently accompanied by detrimental physical and neuropsychological sequelae produced by...
adjuvant radiotherapy and chemotherapy, especially in infants and young children (11). Moreover, there is no therapy that produces long-term remission in recurrent disease (2, 7, 10).

In light of the detrimental long-term effects of adjuvant therapy and the poor prognosis for the recurrent tumors that afflict ca. 50% of patients (2, 7, 10), development of more effective approaches to treating medulloblastomas/PNETs is an urgent priority. Little is known about the molecular mechanisms that underlie adjuvant therapy failure. Delineating resistance mechanisms will likely suggest new strategies to improve the efficacy of existing therapies and provide new markers of clinical outcome. As a group, medulloblastomas/PNETs are relatively responsive to radiation and chemotherapeutic agents that damage DNA (2), suggesting that DNA repair may be a determinant of tumor response. Ionizing radiation and the nitrosourea- and nitrogen mustard–based alkylating agents used to treat medulloblastomas/PNETs produce cytotoxic abasic sites (apurinic/apyrimidinic sites) in DNA (12–14). Abasic sites, which constitute a major lethal lesion, block progression of replicative DNA polymerases and are believed to cause replication forks to stall and/or collapse (15–17). The vast majority of abasic site repair in mammalian cells is catalyzed by Apel/Ref-1, an abundant enzyme possessing a strong apurinic/apyrimidinic endonuclease (Ap endo) activity that hydrolyzes the phosphodiester bond 5′ to baseless sites (12, 17–19).

Our recent evidence indicates that the Ap endo activity of Apel/Ref-1 mediates resistance to adjuvant radiation and alkylating agent–based chemotherapy in adult gliomas (20–22). We have observed a strong inverse correlation between glioma Ap endo activity and time to tumor progression (TTP) following either radiation or alkylator therapy (20); that is, the higher the activity, the shorter the time to recurrence. We have also found that suppressing Ap endo activity, and Apel/Ref-1 content, in a human adult glioma cell line reduces resistance to the clinical alkylators 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide (21). A concurrent increase in abasic sites supports the conclusion that abasic site repair promotes resistance and shows a functional basis for the in vivo findings. Lastly, Ap endo activity in human gliomas is positively correlated with tumor characteristics associated with greater malignancy and poor response (22). These findings in adult gliomas led us to assess the contribution of Ap endo activity to the resistance of pediatric brain tumors to adjuvant therapy. Here, we report an initial study of 52 medulloblastomas and 10 PNETs in children and young adults. Our results suggest that Ap endo activity promotes resistance to combined radiation and chemotherapy containing alkylating and/or platinating agents and is predictive of outcome following this adjuvant therapy in medulloblastomas/PNETs.

Materials and Methods

Tissue. Tumors were obtained with informed consent from patients operated at the Children's Hospital and Regional Medical Research Center and the University of Washington Medical Center. Care was taken to ensure that tumor specimens were distant from the gross interface with normal tissue. All tissues were reviewed by a neuropathologist and diagnosis was obtained from the final neuropathology report. Tissue and demographic information was obtained in accordance with protocols approved by the Institutional Review Boards at Children's Hospital and Regional Medical Research Center and the University of Washington Medical Center. Immediately upon resection, tissue was placed in ice-cold DMEM/F12 supplemented with 5% fetal bovine serum and transported to the laboratory. The precautions taken to preserve tissue viability and enzymatic activity during transport of specimens and the procedure for determining cell number are described elsewhere (23).

Apurinic/apyrimidinic endonuclease activity. Ap endo activity was quantitated in whole cell extracts as we have previously described for human brain tumors (22), brain tumor–derived cell lines (21), and normal brain (22). The highly sensitive assay measures conversion of acid-treated, supercoiled plasmid DNA to relaxed form caused by incision at an abasic site (24). Briefly, tissue was rinsed in PBS, weighed, and minced into small pieces with scalpel blades. Tissue fragments were resuspended in PBS and passed serially through 16-, 18-, and 20-gauge needles to produce a single cell suspension. Cells were pelleted by centrifugation at 2,000 × g for 5 minutes at 4°C and resuspended at 1 × 107 cells/mL in 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 100 mmol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin. Cell suspensions were sonicated on ice for four 15-second intervals, and debris was pelleted by centrifugation at 10,000 × g for 5 minutes at 4°C. In some instances, extraction was done in 1.5× Ap endo reaction buffer [75 mmol/L HEPES (pH 7.5), 225 mmol/L KCl, 75 mmol/L MgCl2, 0.75 mmol/L CoCl2, and 150 μg/mL bovine serum albumin]. Experiments with pediatric brain tumor cell lines revealed identical Ap endo activities for extracts prepared in both extraction buffers.

Assay mixtures (30 μL) contained 0.033 μg/mL depurinated pKT100 plasmid DNA (3.5 kb), containing, on average, 1.5 abasic sites per molecule, 50 mmol/L HEPES (pH 7.5), 150 mmol/L KCl, 5 mmol/L MgCl2, 0.5 mmol/L CoCl2, 100 μg/mL bovine serum albumin, and extract equivalent to 10 to 104 cells. Preparation of the DNA substrate is described in ref. 22. After incubation for 10 minutes at 37°C, reaction products were resolved on a 0.8% agarose gel in 40 mmol/L Tris-acetate, 2 mmol/L EDTA. The gel was stained with ethidium bromide to visualize supercoiled and nicked, relaxed plasmid DNA and was photographed with a Kodak DC290 digital camera. Band density was quantitated using Kodak 1D version 3.5.4 image processing software, with HindIII-linearized pKT100 as a standard. Activity (fmol abasic sites incised/cell/min, abbreviated to fmol/cell/min) is the mean of at least three separate determinations that differed, in general, by <15%, and in all cases, by <30%. Each determination comprised assay of increasing amounts of sample and yielded activity calculated by regression analysis of points on the linear portion of the curve. Normalization of activity to cell number affords a uniform basis for comparing tissues of different histology, avoiding potential problems attributable to tissue- and tumor-specific differences in the amount and composition of intracellular and extracellular proteins (e.g., ref. 25).

Immunohistochemistry. Immunoperoxidase staining was done on paraffin blocks of formalin-fixed tumors. Briefly, 7-μm tissue sections were mounted on 3-aminopropyltriethoxysilane–treated slides, de-waxed, and rinsed twice in 100% ethanol followed by treatment with 0.6% hydrogen peroxide/methanol to quench endogenous peroxidase. Slides were rehydrated by serial rinses in 95% ethanol, 80% ethanol then distilled water followed by a 5-minute incubation in TBS (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl and 12.5 mmol/L KCl). Sections were incubated for 5 minutes at room temperature in 0.1% trypsin in TBS followed by 3 × 5-minute washes with TBS. Slides were incubated with 10% goat serum and 3% bovine calf serum in TBS for 1 hour at room temperature and then incubated overnight at 4°C with a 1:5,000 dilution of a mouse monoclonal anti-Apel/Ref-1 antibody (BD Biosciences, San Jose, CA) at 1:50 dilution in TBS containing 10% goat serum. Slides were washed in TBS and incubated with a 1:100 dilution of a biotin-conjugated goat anti-mouse IgG antibody (Vector, Burlingame, CA) for 1 hour at room temperature. After washing with

Clin Cancer Res 2005;11(20) October 15, 2005  7406  www.aacrjournals.org

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TBS, the slides are incubated for 30 minutes at room temperature with streptavidin-conjugated horseradish peroxidase and washed again with TBS. Primary antibody binding is visualized by incubation with 3,3'-diaminobenzidine tetrachloride until a satisfactory signal develops. Slides were counterstained with methyl green or Gill's hematoxylin. Negative controls were treated identically except for omission of the primary antibody. Stained slides were scanned at low power to evaluate regional variation. One hundred cells from five regions were counted and the average percent of positive cells for each tumor was calculated. Both nuclear and cytoplasmic staining were considered positive. Staining in any fragments of normal brain was also assessed.

Suppression of apurinic/apyrimidinic endonuclease activity and alkylating agent sensitivity. Ap endo activity in the human medulloblastoma-derived cell line UW228-2 (26) was suppressed by transfection with either antisense oligonucleotides or siRNA targeting Ape1/Ref-1. For transfection with antisense oligonucleotides, separate 50-μL aliquots of unsupplemented Opti-MEM (Gibco, Invitrogen, Carlsbad, CA) were mixed with either 2.9 nmol of an antisense oligonucleotide complementary to the translation start site (5'-TTTCCCCAGGGTCCGATCC-3') or with 15 μl of cationic lipid (Lipofectin, Life Technologies) and held at room temperature for 30 minutes. The antisense oligonucleotide and cationic lipid mixtures were gently combined and held overnight at 4°C. Subconfluent cultures of UW228-2 incubated at 37°C in humidified 95%/5% air/CO2 overnight in 3 ml of Opti-MEM containing 5% iron-supplemented bovine serum in a 35-mm dish were washed once with unsupplemented Opti-MEM before adding the antisense oligonucleotide mixture. Cells were incubated with antisense oligonucleotides for 18 hours before adding 2 ml of Opti-MEM containing 10% iron-supplemented bovine serum and continuing incubation. Final antisense oligonucleotide concentration was 1 μmol/L. Seventy-two hours after initiating transfection, cells were harvested by trypsinization for determination of alkylator sensitivity and assay of Ap endo activity. Transfection with a random oligomer composed of the same nucleotide composition as the antisense oligonucleotide served as a control.

A protocol similar to that described by Wang et al. (27) was used for transfection with siRNA. In separate tubes, 1.6 nmol of the siRNA (5'-CUUUCGAGGUCUUCGAGCC-3', 3'-UCCAAUUGUCUCUCUCUCGU-5', corresponding to nucleotides 532-552 of the human Ape1/Ref-1 cDNA sequence) was mixed with 0.1 ml of unsupplemented Opti-MEM, and 15 μl of cationic lipid was mixed with 0.4 ml of Opti-MEM. After 10 minutes at room temperature, the two mixtures were combined and added to 35-mm dishes of UW228-2 grown as described above. Cells were incubated with siRNA for 4 to 18 hours before adding 2 ml of Opti-MEM containing 10% iron-supplemented bovine serum and continuing incubation; suppression of Ap endo activity or effect on alkylator sensitivity was assayed 18 hours after length of incubation with siRNA. Final siRNA concentration was 320 nmol/L. At 72 hours after initiating transfection, cells were harvested by trypsinization for determination of alkylator sensitivity and assay of Ap endo activity. Transfection with an inactive siRNA duplex (5'-AAUUGGUAGGGGCUUCGAGGC-3', 3'-CUUUCGAGGUCUUCGAGCC-5', corresponding to nucleotides 412-432 of the human Ape1/Ref-1 cDNA sequence) served as a control.

Alkylating agent survival. BCNU and temozolomide were obtained from the pharmacies of the University of Washington Medical Center and Children’s Hospital and Regional Medical Center, respectively. BCNU was dissolved in absolute ethanol at a concentration of 1 mol/L; temozolomide was dissolved in DMSO at a concentration of 0.15 mol/L. Both drugs were stored as single-use aliquots at −80°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 assayed as previously described in detail (e.g., ref. 28). Six-well (35 mm) trays were inoculated with 2 ml of supplemented DMEM/F12 containing 1,000 to 2,000 cells transfected with antisense oligonucleotides or siRNA. The trays were incubated for 6 to 8 hours to allow cells to attach and resume proliferation before further incubation with alkylating agent for 1 hour. In each experiment, single or duplicate wells of cells were exposed to at least 10 drug doses that serially increased by 10% to 20% increments. Alkylator sensitivity was assayed in two and three independent experiments for temozolomide and BCNU, respectively; each experiment employing different drug doses. The cells were 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/H2O (v/v) to aid visualization during counting by light microscopy. Colonies containing ≥50 cells were counted. The colony-forming efficiency of untreated UW228-2 was ~25%.

Drug sensitivity was determined by analysis of survival curves (log surviving fraction versus dose) using standard methods, as we have previously described in detail (e.g., Fig. 1 of ref. 28). The survival variables LD10 (the dose required to reduce overall survival to 10%), DT1 (the threshold dose below which cells are insensitive to alkylator cytotoxicity), and D37 (the rate of killing as indicated by the slope of the linear portion of the survival curve) were derived by regression analysis of the linear portion of a composite kill curve derived from the replicate assays. The three survival variables are presented as mean ± SE and provide a complete description of alkylator sensitivity.

Statistical analysis. Data analysis and statistical procedures were done using Microsoft Excel and the statistics program Intercooled Stata 8 (Stata Corp., College Station, TX). Comparison of means was by Student’s t test assuming unequal variances. Relationships between continuous variables were assessed by regression analysis. The association of Ap endo activity with TTP was assessed by standard methods. In these analyses, the outcome variable TTP was assessed by radiologic imaging. Tumor progression was defined as appearance of tumor growth in the case of gross total resection: increase in the largest dimension of residual tumor by at least 25% and/or tumor growth at a different site. Observations were censored at the last documented follow-up time if progression was not yet observed. Mean TTP was determined by the method of Kaplan and Meier. The hazard ratio (HR) for tumor progression as a function of Ap endo activity was calculated by using Cox regression analysis. Ap endo was entered into the regression models as a continuous variable and scaled (i.e., multiplied by 100) so that the tabulated HR represents the relative change in hazard for a 0.01 unit change in measured Ap endo activity. Possible confounding by age and gender was examined by using multivariate Cox regression analysis. Statistically significant relationships were determined at the 95% confidence level (95% CI).

Results

Patient and tumor characteristics. Fifty-two medulloblastomas and 10 cerebral PNETs were obtained from patients ranging in age from 4 days to 21 years (mean ± SD, 8.4 ± 5.0 years). The majority of tumors occurred in males (61%), in accord with previous observations of gender bias (3). Fifty-five tumors were newly diagnosed, two were recurrent after surgery, one after surgery and chemotherapy, and four after surgery, radiation, and chemotherapy.

Association of apurinic/apyrimidinic endonuclease activity with tumor and patient characteristics. Ap endo activity in the 62 tumors ranged 180-fold from 0.0028 to 0.50 fmol/cell/min (Table 1). The mean was 0.12 ± 0.12 fmol/cell/min and did not differ between medulloblastomas and PNETs (0.11 ± 0.13 vs. 0.13 ± 0.13 fmol/cell/min), indicating that anatomic location (i.e., cerebellum versus cerebrum) did not affect activity. Newly operated and previously treated tumors (see above) did not differ significantly in activity (0.11 ± 0.12
versus 0.19 ± 0.18 fmol/cell/min), suggesting that prior therapy had no lasting effect on Ap endo activity.

Regression analysis revealed a significant inverse association between activity and age (r = 0.262, P < 0.040), with activity decreasing 0.0070 fmol/cell/min per year (Fig. 1). This decline with age, illustrated by the 2-fold higher activity in infants compared with adolescents (Table 1), was observed in medulloblastomas and PNETs separately, and in males and females (data not shown). Ap endo activity also differed significantly between genders (Table 1) as evidenced by a 2-fold greater activity in females (0.16 ± 0.14 versus 0.089 ± 0.11 fmol/cell/min, P ≤ 0.040). However, the female sample population had a greater proportion of infants (33% versus 5%) than the male, suggesting that the gender difference could reflect the effect of age on activity. To address this possibility, the relationship among Ap endo activity, age, and gender was further analyzed by using a multivariate model. Controlling for variation in age, activity remained 0.060 fmol/cell/min higher in females. Controlling for gender, the age-related decrease in activity was 0.0054 fmol/cell/min per year. The consistency of the age and gender relationships with activity when controlling for one another indicates that these relationships are independent.

**Ape1/Ref-1 is localized in tumor cell nuclei.** Intracellular localization of APE1/Ref-1, the predominant Ap endo activity in human cells (18), was examined by immunohistochemistry in 42 medulloblastomas. As is characteristic of medulloblastomas, all tumors were highly cellular, composed of small, poorly differentiated, round cells that displaced rather than infiltrated surrounding normal parenchyma. Immunostaining was detected in all tumors examined, and as illustrated in Fig. 2, seemed almost exclusively nuclear in tumor cells. Between specimens, the average fraction of immunopositive cells varied from ~8% to 73% and differences in the relative intensity of staining were clearly discernible (e.g., Fig. 2A-F). However, within individual tumors, the distribution of stained cells and the intensity of staining displayed little variability when different areas of the tumor were examined; that is, there seemed no focal areas that differed in stained fraction or intensity. Moreover, the fraction of stained cells was strongly correlated with relative staining intensity, estimated visually as +1 to +3 (P ≤ 0.003). These findings suggest that the pattern of expression of Ap endo activity may be uniform within a tumor, a conclusion consistent with a near-significant association between activity and the fraction of immunopositive cells (r = 0.314, P < 0.06). Immunoreactivity was largely absent in adjacent normal cerebellum (Fig. 2G) with the exception of cytoplasmic staining of occasional Purkinje cells.

**Table 1. Ap endo activity by tumor and patient characteristics**

<table>
<thead>
<tr>
<th>Tumors</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Activity*</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>All</td>
<td>62</td>
<td>0.12 ± 0.12</td>
<td>0.0028-0.50</td>
<td></td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>52</td>
<td>0.11 ± 0.13</td>
<td>0.0028-0.50</td>
<td></td>
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<tr>
<td>PNET</td>
<td>10</td>
<td>0.13 ± 0.13</td>
<td>0.0042-0.34</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
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<td></td>
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<tr>
<td>Newly operated</td>
<td>55</td>
<td>0.11 ± 0.12</td>
<td>0.0028-0.50</td>
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<tr>
<td>Previously treated</td>
<td>7</td>
<td>0.19 ± 0.18</td>
<td>0.023-0.43</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>0.16 ± 0.14</td>
<td>0.0042-0.45</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>0.089 ± 0.11</td>
<td>0.0028-0.50</td>
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<tr>
<td>Age</td>
<td></td>
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<tr>
<td>Infants (&lt;3.0 y)</td>
<td>10</td>
<td>0.17 ± 0.14</td>
<td>0.0076-0.40</td>
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<tr>
<td>Children (≥3.0, &lt;12.0 y)</td>
<td>37</td>
<td>0.12 ± 0.13</td>
<td>0.0091-0.50</td>
<td></td>
</tr>
<tr>
<td>Adolescents (&gt;12 y)</td>
<td>15</td>
<td>0.078 ± 0.11</td>
<td>0.0028-0.34</td>
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*fmol abasic sites incised/cell/min.
and, rarely, of cells of the inner granular layer (Fig. 2H). In accord, activity in two samples of normal cerebellum was 5.9- and 29-fold lower than that in adjacent medulloblastoma (data not shown).

Apurinic/apyrimidinic endonuclease activity is inversely associated with time to tumor progression following adjuvant therapy. To examine the association of Ap endo activity with response to postoperative treatment, we analyzed the association of activity with TTP after initiation of adjuvant therapy in 46 medulloblastomas and four PNETs. All patients received craniospinal radiation (23.4-36 Gy) with additional focal irradiation to the tumor bed for a total dose of 54.4 to 58.8 Gy. Patients also received vincristine during radiation and during subsequent multiagent chemotherapy that included an alkylating agent (1-(2-chloroethyl)-3-cyclohexyl-L-nitrosourea and/or cyclophosphamide) and a platinating agent (cisplatin or carboplatin). Fourteen tumors progressed and 36 were censored: median time at risk was 47.8 months. In a univariate Cox proportional hazards regression model (Table 2) with Ap endo activity entered as a continuous variable, the HR increased by a factor of 1.067 for every increase of 0.01 in activity (95% CI, 1.029-1.106; \( P < 0.0001 \)). Thus, greater apurinic/apyrimidinic endonuclease activity was associated, on average, with an increasing risk for progression (i.e., shorter TTP). For example, in our sample population, the tumor with the highest activity is \( 25 \times \) more likely to progress than the lowest-activity tumor.

Adjusting for age and gender, variables that have been associated with treatment outcome and/or overall survival in diverse pediatric and adult (reviewed in Table 2. HR estimates for the association of Ap endo activity with TTP following combined radiotherapy and chemotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>( P )</th>
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<tr>
<td><strong>Univariate analysis</strong></td>
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<tr>
<td>Apurinic/apyrimidinic endonuclease*</td>
<td>1.067 (1.029-1.106)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age ( ^\dagger )</td>
<td>0.972 (0.863-3.318)</td>
<td>0.80</td>
</tr>
<tr>
<td>Male versus female ( ^\dagger )</td>
<td>1.218 (0.407-3.642)</td>
<td>0.72</td>
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<td><strong>Multivariate analysis</strong></td>
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<tr>
<td>Age ( ^\dagger )</td>
<td>1.048 (0.919-1.196)</td>
<td>0.49</td>
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<tr>
<td>Male versus female ( ^\dagger )</td>
<td>1.041 (0.342-3.166)</td>
<td>0.94</td>
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*Per 0.01 increase in activity.
\( ^\dagger \)Per 1-y increase.
\( ^\dagger \)Male = 0, female = 1.

These data suggest that Ap endo activity may be a useful predictor of progression following postoperative radiotherapy and/or chemotherapy in medulloblastoma/PNETs and are consistent with the proposal that Ap endo activity mediates resistance to adjuvant therapy.

Ape1/Ref-1 immunopositivity and time to tumor progression. Previous studies have suggested that the fraction of Ape1/Ref-1 immunopositive cells is associated with treatment outcome or overall survival in diverse pediatric and adult (reviewed in

Fig. 2. Nuclear staining of Ape1/Ref-1 in medulloblastoma cells. A-F, relative intensity of nuclear immunopositivity is compared at \( \times 10 \) and \( \times 40 \) power (original magnification) for weak (+1; A-B), moderate (+2; C-D), and strong (+3; E-F) staining. Tumor nuclei expressing detectable Ape1/Ref-1 are brown; negative nuclei are green (stained with 3,3'-diaminobenzidine tetrachloride and methyl green). G, Ape1/Ref-1 immunostaining in a medulloblastoma and adjacent normal cerebellum. Low-power view shows a marked contrast between a fragment of tumor (left) adjacent to cerebellum (right), with diffuse staining of tumor cells at +3 intensity compared with nearly complete absence of staining in the normal cerebellum. H, cytoplasm of large Purkinje cells has intermediate staining, whereas rare cells of the inner granular layer (arrowhead) are positive.

Table 2.

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\( ^\dagger \)Male = 0, female = 1.
ref. 18, 19, 29) tumors. TTP following adjuvant radiotherapy and chemotherapy was available for 37 of the 42 medulloblastomas (13 progressed) examined for Ape1/Ref-1 by immunohistochemistry. Univariate analysis revealed no relationship between TTP and the fraction of immunopositive cells (HR, 0.992; P < 0.61), the intensity of staining (HR, 0.998; P < 0.72), or the product of stained fraction and intensity (HR, 0.998; P < 0.68).

Apurinic/apyrimidinic endonuclease activity is associated with time to tumor progression following adjuvant therapy within both high-risk and average-risk medulloblastomas. Newly diagnosed medulloblastomas can be stratified into high risk and average risk for treatment failure and recurrence based on patient age, extent of tumor resection, and dissemination to other sites in the central nervous system (6, 7, 8). In our study, high-risk tumors were defined using the Children's Cancer Study Group criteria; that is, high-risk patients are those with >1.5 cm³ residual tumor after surgery, are younger than 3 years, and have evidence of tumor dissemination to other sites (7). In accord with the increasing hazard for progression associated with increasing Ap endo activity, the mean Ap endo activity in 20 high-risk tumors in our sample was twice that of 29 average-risk tumors (0.18 ± 0.18 versus 0.083 ± 0.14 fmol/cell/min; P ≤ 0.05). Univariate analysis revealed a significant inverse relationship between activity and TTP in both high-risk tumors (HR, 1.149; 95% CI, 1.016–1.300; P = 0.027) and average-risk tumors (HR, 1.076; 95% CI, 1.013–1.144; P = 0.018), indicating the use of Ap endo in predicting treatment outcome within each risk group.

Suppression of apurinic/apyrimidinic endonuclease activity increases alkylator killing of medulloblastoma cells. The association between Ap endo activity and TTP following postoperative therapy suggests that repair of abasic sites promotes medulloblastoma/PNET resistance to radiation and/or alkylating agents in situ. To address this hypothesis, alkylator sensitivity was determined by clonogenic colony-forming assay following suppression of Ap endo activity in the human medulloblastoma–derived cell line UW228-2. The effect of transfection with either antisense oligonucleotides or siRNA targeting Apel/Ref-1 on Ap endo activity, Apel/Ref-1 content, and alkylator sensitivity is illustrated in Fig. 3. Antisense oligonucleotide treatment reduced activity 5.4-fold (0.21 ± 0.048 to 0.039 fmol/cell/min) in three separate experiments, whereas transfection with siRNA reduced activity 3.8-fold (0.23 ± 0.044 to 0.061 ± 0.022 fmol/cell/min) in four separate experiments. In accord, Western analysis (Fig. 3B) revealed reduced abundance of Ape1/Ref-1, evident within 30 hours after transfection with antisense oligonucleotides or siRNA. Suppression of Ap endo activity was accomplished by significantly greater sensitivity to the clinical chloroethylating agent BCNU, as illustrated in Fig. 3C for siRNA. As summarized in Table 3, treatment with antisense oligonucleotides or siRNA yielded 2.4- and 1.8-fold reductions, respectively, in LD10 for BCNU, relative to cells transfected with control sequences. Greater sensitivity reflected (a) 2.3- and 1.3-fold reductions in D10, respectively, indicating that apurinic/apyrimidinic endonuclease contributes to insensitivity to low doses of BCNU and (b) increased rates of killing, as evidenced by 2.2- and 1.9-fold lower D17 values, respectively. Suppression of Ap endo activity also yielded enhanced sensitivity to the clinical methylator temozolomide. Antisense oligonucleotide–mediated reduction of Ap endo activity decreased LD10 for temozolomide by 1.9-fold (Fig. 3D; Table 2). The enhanced sensitivity to temozolomide reflected an increased rate of killing (i.e., lower D17), whereas D1 was unchanged.

Discussion

Improvements in adjuvant radiotherapy and chemotherapy have greatly increased survival for pediatric medulloblastomas/PNETs (1, 2). However, the benefit is not equally afforded to all patients, and postoperative radiotherapy is frequently accompanied by physical and neuropsychological detriments (11). Although clinical experience has identified patient and tumor features that are associated with overall survival, there are few molecular markers that predict response to the combined radiotherapy and chemotherapy used to treat medulloblastomas/PNETs. The cytotoxicity of unrepaired abasic sites induced by radiation and alkylators (12, 13), and the evidence that Ap endo activity mediates resistance of human gliomas to treatment with these agents (20–22), led us to hypothesize that Ap endo activity is a mechanistically relevant predictor of response to adjuvant therapy in medulloblastomas/PNETs. The work reported here substantiates our hypothesis, shows a functional basis for the association of Ap endo activity with recurrence, and suggests that apurinic/apyrimidinid endonuclease activity may be useful as a predictor of treatment outcome and as a target for antiresistance therapies.

Support for our hypothesis is provided by the strong, inverse association between Ap endo activity and TTP following combined radiotherapy and chemotherapy that included an alkylating and/or a platinating agent (Table 2). Based on biochemical, genetic and cell/molecular biological studies, particularly in human glioma cells (20–22), the relationship between activity and TTP likely reflects repair of abasic sites, at least in part. Both radiation and alkylating agents induce baseless sites via DNA glycosylase–mediated excision of oxidized and alkylated purines and pyrimidines (12, 13). In addition, radiation-induced oxidative free radicals can induce abasic sites by direct attack on deoxyribose (12). Unrepaired abasic sites are lethal, most likely a consequence of their ability to strongly impede the progress of DNA replication (15–17). The contribution of abasic sites to the cytotoxicity of radiation and alkylating agents is evidenced by the hypersensitivity of bacterial, yeast, and mammalian cells that are deficient in repair of this lesion (reviewed in refs. 12, 17–19). Moreover, suppression of Ape1/Ref-1, the major human Ap endo activity (18), results in hypersensitivity to oxidizing and alkylating agents in both rat (30) and human adult glioma cells (21) Similar findings have been reported for a variety of additional mammalian cell lines (e.g., refs. 27, 31, 32). The association between Ap endo activity and TTP may also reflect mechanisms in addition to, or other than, excision of abasic sites. Ape1/Ref-1 has multiple catalytic activities in vitro, including 3’-phosphodiesterase, 3’-phosphatase, and 3’-exonuclease (18, 19) that may promote resistance to radiation damage

8 J.R. Silber and M.S. Bobola, unpublished results.
by excising fragmented deoxyribose phosphate moieties at single-strand breaks (12). In addition, the reduction-oxidation activity Ref-1, located at the \textsc{NH}$_2$ terminus of Ape1/Ref-1 (18), mediates signal transduction in response to radiation and other genotoxic stress (18, 19). For example, Ref-1 has been implicated in regulating the transactivation and proapoptotic activities of p53 (33). Thus, it is possible that multiple redox-sensitive proteins that are mechanistically associated with Ref-1 contribute to the associations with outcome that we observed here. Whatever the case, involvement of the DNA repair activity of Ape1/Ref-1 is supported by the finding that a chimeric protein containing Ap endo

### Table 3. Suppression of Ape1/Ref-1-catalyzed Ap endo activity increases alkylator cytotoxicity in human medulloblastoma cells

<table>
<thead>
<tr>
<th>BCNU</th>
<th>Antisense</th>
<th>siRNA</th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (µmol/L)</td>
<td>ASO (µmol/L)</td>
<td>Con/ASO</td>
<td>( P )</td>
<td>Control (µmol/L)</td>
<td>siRNA (µmol/L)</td>
<td>Con/siRNA</td>
<td>( P )</td>
<td></td>
</tr>
<tr>
<td>LD$_{10}$</td>
<td>85 ± 2.9</td>
<td>36 ± 1.9</td>
<td>2.4</td>
<td>≤0.001</td>
<td>79 ± 2.3</td>
<td>44 ± 1.3</td>
<td>1.8</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td>( D_1 )</td>
<td>27 ± 2.0</td>
<td>12 ± 2.5</td>
<td>2.3</td>
<td>≤0.03</td>
<td>20 ± 2.4</td>
<td>15 ± 2.0</td>
<td>1.3</td>
<td>≤0.1</td>
<td></td>
</tr>
<tr>
<td>( D_{37} )</td>
<td>22 ± 2.8</td>
<td>10 ± 1.8</td>
<td>2.2</td>
<td>≤0.001</td>
<td>25 ± 1.6</td>
<td>13 ± 1.4</td>
<td>1.9</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td>Temozolomide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LD$_{10}$</td>
<td>460 ± 9.8</td>
<td>242 ± 9.0</td>
<td>3.2</td>
<td>0.001</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>( D_1 )</td>
<td>129 ± 10</td>
<td>130 ± 14</td>
<td>1</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( D_{37} )</td>
<td>143 ± 7.3</td>
<td>45 ± 10</td>
<td>3.2</td>
<td>0.001</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviation: ASO, antisense oligonucleotides.
and O\textsuperscript{6}-methylguanine-DNA methyltransferase activities, but no Ref-1, conferred greater alklylation resistance to human cells than O\textsuperscript{6}-methylguanine-DNA methyltransferase activity alone (34). These survival-promoting functions of Ref-1 may be especially important in promoting resistance to multiagent chemotherapy protocols such as those used to treat medulloblastomas/PNETs that include DNA-damaging agents that do not produce abasic sites or oxidative lesions (e.g., cisplatin) and cytotoxic drugs that do not damage DNA (e.g., vincristine).

Additional evidence associating Ap endo activity with clinical outcome following postoperative treatment in medulloblastomas is the difference in mean activity between tumors that are average risk and high risk for recurrence. One criterion of higher risk, age of $<3$ years (i.e., infants), is associated with significantly greater Ap endo activity compared with older children and adolescents (Table 1). The worse outcome observed for infants has been attributed to restriction of adjuvant treatment to chemotherapy to avoid the severe morbidity caused by radiotherapy in the very young (2). Our data suggest that greater Ap endo activity/Apel/Ref-1 content could also contribute to the poor prognosis in infants by promoting chemoresistance. In children older than $3$ years and adolescents, Ap endo activity is also greater in high-risk medulloblastoma ($0.17 \pm 0.17$ fmol/cell/min) versus average-risk medulloblastoma ($0.083 \pm 0.14$ fmol/cell/min). In these patients, high risk is defined by tumor invasion of surrounding brain that prevents gross total resection or dissemination of tumor elsewhere in the neuroaxis (7). Our data indicate that elevation of Ap endo activity accompanies and/or promotes acquisition of these aggressive characteristics.

We observed that Ap endo activity is associated with age and gender in medulloblastomas/PNETs. The inverse correlation between activity and age (Fig. 1) may reflect processes associated with physical and functional maturation of the brain that are intrinsic to the progenitor cells of medulloblastomas/PNETs. Alternatively, declining tumor Ap endo activity with age may reflect reduction in exogenous stress in the surrounding brain associated with cessation of proliferation of neural cell progenitors and neuronal maturation. Notably, reduction of Apel/Ref-1 mRNA detected by \textit{in situ} hybridization accompanies the maturation of developing brain into adulthood in rodents (35, 36). Possible mechanisms underlying the 2-fold higher activity in females are less evident. Several studies have shown hormonal and trophic factor regulation of Apel/Ref-1 expression in human and rodent cell lines (37–39), suggesting that estrogenic hormones responsible for sexual differentiation in the brain may stimulate Apel/Ref-1 expression.

It is of interest to relate our findings for medulloblastomas/PNETs to those for adult gliomas (20–22). Medulloblastomas/PNETs differ from adult gliomas in biological and clinical properties, particularly in their greater responsiveness to radiation and chemotherapy. Despite these differences, we found that mean Ap endo activity for medulloblastomas/PNETs ($0.12 \pm 0.12$ fmol/cell/min) was comparable with that for high-grade gliomas ($0.090 \pm 0.11$ fmol/cell/min; ref. 22). However, we observed no association of activity with age or gender in gliomas. In both groups of tumors, increasing aggressiveness (high grade versus low grade in gliomas and high risk versus low risk in medulloblastomas) is associated with significantly higher mean activity. Correlation with degree of malignancy may reflect elevated oxidative stress accompanying increased rates of proliferation and/or tumor cell migration. As also observed for an adult glioma line (21), suppression of Apel/Ref-1 expression in the medulloblastoma line UW228-2 increased killing by the clinical alkylators BCNU and temozolomide. Finally, in both medulloblastomas/PNETs and adult gliomas, there is a strong significant inverse association between Ap endo activity and TTP following adjuvant therapy with radiation and/or alkylating agent–based chemotherapy (both modalities combined in the present study, and each modality separately in gliomas). It is important to note that, in our sample populations, the hazard for progression differed by 25-fold within the range of Ap endo activities observed in 52 medulloblastomas/PNETs and by 13- to 14-fold within the range of activities observed in 30 and 44 adult gliomas treated with either alkylators or radiotherapy, respectively. Taken together, our results suggest that Ap endo may contribute to resistance to postoperative radio/chemotherapy and provide a predictor of outcome in a variety of primary brain tumors.

We observed extensive variation between medulloblastomas in the fraction of cells that were immunopositive for Apel/Ref-1 ($\sim 8$–73%) and in the intensity of staining scored on a scale of +1 to +3 (e.g., Fig. 2). This variability is consistent with the 180-fold range in Ap endo activity measured in our biochemical assay. The staining pattern seemed uniform within each of the 42 tumors examined, and there was a strong correlation between fraction of stained cells and staining intensity, as well as a nearly significant correlation between fraction of stained cells and activity. Apel/Ref-1 was detectable only in nuclei, as has been observed for some, but not all tumors (18, 19). Lack of cytoplasmic staining may reflect the small, round cell morphology and large nuclear volume that are histologic hallmarks of medulloblastomas. More intriguing are the mechanisms that may account for the variability in stained cell fraction and intensity. Numerous studies have implicated oxidative stress as a modulator of Ap endo activity and Apel/Ref-1 content in cell lines and tissue (19). It is possible that intertumor heterogeneity in immunopositivity reflects oxidative stress associated either with cell proliferation or episodes of transient hypoxia due to inadequate/impaired vasculature. An association with Apel/Ref-1 content, assessed by immunohistochemistry, with treatment outcome has been reported for some human tumors, including head and neck cancer, germ cell tumors, cervical cancer, ovarian carcinoma, and osteosarcomas (reviewed in refs. 18, 19, 27, 40). In contrast to these studies, a Cox univariate model did not reveal a significant relationship between either the fraction of Apel/Ref-1-immunopositive cells, the intensity of staining, or their product, and TTP following adjuvant treatment for medulloblastomas. However, this lack of association may reflect the relatively small number of tumors examined, the low proportion that had progressed (35%), and/or the relative insensitivity of a discontinuous variable (staining intensity) versus a continuous variable (activity). Nonetheless, Ap endo activity permitted detection of an association for a similar sample size and fraction of treatment failures, suggesting that activity may be a more sensitive predictor of clinical outcome. Increased nuclear Apel/Ref-1 content and localization has been observed to accompany malignant progression in
ovarian carcinomas (40), suggesting that the nuclear localization of Ape1/Ref-1 may reflect the malignant character of medulloblastomas.

Our results indicate that Ap endo activity may have utility in guiding postoperative radiation and chemotherapy in medulloblastoma/PNET. High-activity tumors may be candidates for novel, potentially more effective, treatments (e.g., immunotherapy) that spare patients the systemic toxicity caused by radiation and DNA-damaging agents. Alternatively, high activity may identify tumors that are amenable to anti-resistance therapies targeting Ape1/Ref-1. Numerous studies have shown hypersensitivity to oxidizing and alkylating agents accompanying suppression of Ap endo activity and/or Ape/Ref-1 protein level in a variety of tumor cell lines (e.g., refs. 21, 27, 30, 31). The potentiation of alkylator cytotoxicity effected by antisense- and/or siRNA-mediated suppression of Ap endo activity and Ape1/Ref-1 protein content that we observed here in the medulloblastoma line UW228-2 and earlier in an the adult glioma line (21) provide proof of principal of the efficacy and clinical potential of strategies to circumvent radiotherapy and chemoresistance in primary brain tumors. Importantly, partial elimination of Ap endo activity results in significant increases in alkylator sensitivity in vitro (Fig. 3; ref. 20), in contrast to the complete and prolonged ablation of MGMT required to produce enhanced alkylator cytotoxicity (41). A number of low molecular weight compounds have been described that inhibit the excision of abasic sites (14, 42). For example, lucanthone, a clinically used antischistosome, inhibits the Ap endo activity of human Ape1/Ref-1 in vitro and increases the abundance of abasic sites in HeLa cells (42). Lucanthone also sensitizes breast cancer cells to methylating agents (43) and adult glioma cells to both alkylating and oxidizing agents. More importantly, lucanthone has been observed to accelerate the regression of brain metastases treated with radiation, a result attributed to decreased repair of abasic sites (44). These findings suggest that antiresistance therapies targeting Ap endo activity are pharmacologically tractable and potentially efficacious.

Acknowledgments

We thank Dr. A. Blank for critical reading of the article, Dr. Kristy Seidell for guidance with statistical analysis, and Bobby Stevens and Douglas Kolsto for technical assistance.

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Apurinic/Apyrimidinic Endonuclease Activity Is Associated with Response to Radiation and Chemotherapy in Medulloblastoma and Primitive Neuroectodermal Tumors

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