Altered Expression of Ape1/ref-1 in Germ Cell Tumors and Overexpression in NT2 Cells Confers Resistance to Bleomycin and Radiation

Kent A. Robertson, Heather A. Bullock, Yi Xu, Renee Tritt, Erika Zimmerman, Thomas M. Ulbright, Richard S. Foster, Lawrence H. Einhorn, and Mark R. Kelley

ABSTRACT

The human AP endonuclease (Ape1 or ref-1) DNA base excision repair (BER) enzyme is a multifunctional protein that has an impact on a wide variety of important cellular functions including oxidative signaling, transcription factor regulation, and cell cycle control. It acts on mutagenic AP (baseless) sites in DNA as a critical member of the DNA BER repair pathway. Moreover, Ape1/ref-1 stimulates the DNA-binding activity of transcription factors (Fos-Jun, nuclear factor-xB, Myb, ATF/cyclic AMP-responsive element binding protein family, HIF-1α, HLF, PAX, and p53) through a redox mechanism and thus represents a novel component of signal transduction processes that regulate eukaryotic gene expression. Ape1/ref-1 has also been shown to be closely linked to apoptosis associated with thiorerodoxin, and altered levels of Ape1/ref-1 have been found in some cancers. In a pilot study, we have examined Ape1/ref-1 expression by immunohistochemistry in sections of germ cell tumors (GCTs) from 10 patients with testicular cancer of various histologies including seminomas, yolk sac tumors, and malignant teratomas. Ape1/ref-1 was expressed at relatively high levels in the tumor cells of nearly all sections. We hypothesized that elevated expression of Ape1/ref-1 is responsible in part for the resistance to therapeutic agents. To answer this hypothesis, we overexpressed the Ape1/ref-1 cDNA in the GCT cell line NT2/D1 using retroviral gene transduction with the vector LAPS. An overexpression of nucleotide cleavage assay and immunohistochemistry to assess Ape1/ref-1 repair activity and expression, respectively, we found that the repair activity and relative Ape1/ref-1 expression in GCT cell lines are directly related. NT2/D1 cells transduced with Ape1/ref-1 exhibited 2-fold higher AP endonuclease activity in the oligonucleotide cleavage assay, and this was reflected in a 2.3-fold increase in protection against bleomycin. Lesser protection was observed with γ-irradiation. We conclude that: (a) Ape1/ref-1 is expressed at relatively high levels in some GCTs; (b) elevated expression of Ape1/ref-1 in testicular cancer cell lines results in resistance to certain therapeutic agents; and (c) Ape1/ref-1 expression in GCT cell lines determined by immunohistochemistry and repair activity assays parallels the level of protection from bleomycin. We further hypothesize that elevated Ape1/ref-1 levels observed in human testicular cancer may be related to their relative resistance to therapy and may serve as a diagnostic marker for refractory disease. To our knowledge, this is the first example of overexpressing Ape1/ref-1 in a mammalian system resulting in enhanced protection to DNA-damaging agents.

INTRODUCTION

Therapy for disseminated testicular cancer/GCT has been successful, with 70–80% of patients being cured with front-line chemotherapy (1). However, for those 20–30% of patients with extragonadal primaries or relapsed/refractory disease, the response to therapy is poor, with only 3–30% surviving disease free after second-line agents (1). Active chemotherapeutic agents against GCTs include vinblastine, etoposide, bleomycin, cisplatin, cyclophosphamide, and ifosfamide (2). Except for vinblastine and etoposide, each of these agents exerts their toxic effect on tumor cells by directly reacting with DNA, inducing damage including baseless mutagenic AP sites, and ultimately, cell death. Although little is known about the expression and role of DNA repair systems in GCTs and their response to therapeutic agents, it is of interest that biopsies of GCTs reveal that patients with tumors responding (complete remission) to cisplatin have a higher number of platinum-DNA adducts/tumor cell than patients who respond poorly (partial remission; REF. 3). This observation suggests that resistant cells are able to repair cisplatin-induced DNA damage more effectively than sensitive GCT cells.

Preliminary investigations have been performed examining GCTs that are refractory to cisplatin and by establishing cell lines from these cells (4). These initial studies have demonstrated that cisplatin-resistant GCT cell lines are able to repair DNA-platinum adducts and cross-links better than cisplatin-responsive cell lines. These two studies suggested a hypothesis that resistance to cisplatin may be related to the relative efficacy of DNA repair (5). More recently, cisplatin adducts have been found to inhibit DNA glycosylases (6), which may alter the BER pathway.

The human AP endonuclease (Ape1 or ref-1) DNA BER enzyme is a multifunctional protein that has an impact on a wide variety of important cellular functions, including oxidative signaling, transcription factor regulation, cell cycle control, and cancer (7). It acts on mutagenic AP (baseless) sites in DNA as a major member of the DNA BER repair pathway. Simple glycosylases, such as methylpurine DNA glycosylase, excise damaged/alkylated bases, resulting in AP sites that are subsequently incised 5’ to the AP site by Ape1/ref-1, allowing repair to be completed by deoxyribosyl phosphate activity, provided by DNA β-polymersase, to remove the deoxyribose phosphate termini, followed by insertion of the correct base and ligation. Additionally, Ape1/ref-1 is able to repair the 3’ phosphate and phosphoglycolate lesions generated in single-strand breaks by ionizing radiation and bleomycin (8). Moreover, Ape1/ref-1 stimulates the DNA-binding activity of transcription factors (Fos-Jun, NFκB, Myb, ATF/cyclic AMP-responsive element binding protein family, HIF-1α, HLF, PAX, and p53) through a redox mechanism and thus represents a novel component of signal transduction processes that regulate eukaryotic gene expression (9–15). Ape1/ref-1 has also been shown to be closely linked to apoptosis (16), associated with thiorerodoxin (17, 18), and
altered levels of Ape1/ref-1 have been found in some cancers (19–21). More recently, in a B-lymphocyte system, Ape1/ref-1 has demonstrated a response to reactive oxygen species by rapid translocation from the cytoplasm to the nucleus (22).

In a pilot study, we found Ape1/ref-1 to be expressed at a relatively high level in a small group of GSTs (23). Because of the multifaceted role of Ape1/ref-1 in cells, we have begun to try and determine whether the elevated level of Ape1/ref-1 in GCTs is responsible for an increase in repair, redox, or both activities. We hypothesized that elevated expression of Ape1/ref-1 is responsible in part for the resistance to therapeutic agents. To answer this hypothesis, we overexpressed the Ape1/ref-1 cDNA in the GCT cell line NT2/D1 using retroviral gene transduction with LAPESN. Using an oligonucleotide cleavage assay and IHC to assess Ape1/ref-1 repair activity and expression, respectively, we found that the repair activity and relative Ape1/ref-1 expression in GCT cell lines to be directly related. NT2/D1 cells transduced with Ape1/ref-1 exhibited a 2-fold higher AP endonuclease activity in the oligonucleotide cleavage assay, and this was reflected in a 2–3-fold increase in protection against bleomycin. Lesser protection was observed with γ-irradiation. We conclude that elevated expression of Ape1/ref-1 in testicular cancer cell lines results in resistance to certain therapeutic agents. We further hypothesize that elevated Ape1/ref-1 levels observed in human testicular cancer may be related to their relative resistance to therapy and may serve as a diagnostic marker for refractory disease. This is the first example of overexpressing Ape1/ref-1 in a mammalian model system that leads to cellular protection from chemotherapeutic agents.

MATERIALS AND METHODS

Patients and Tumors. Tissue sections of biopsy material from treated and untreated patients with disseminated GCTs were obtained from the Indiana University Medical Center, University Hospital, under an Indiana University Institutional Review Board approved protocol (IU Study No. 9908–47) as 4% formaldehyde-fixed tissues embedded in paraffin blocks, which were sectioned at 3 μm and fixed onto slides. Diagnosis was made from morphological examination of H&E-stained sections of biopsy material.

IHC. Tissue sections were stained for Ape1/ref-1 expression using an anti-Ape1 monoclonal antibody that has been characterized extensively (19, 20) and is available commercially (Novus Biologicals, Littleton, CO). The staining process used was identical to that described previously (19, 20), with the exception that a Dako Universal Staining System was used to automate the process (Dako Corp., Carpinteria, CA). Sections were treated with a 10-min incubation in 3% H2O2 to block endogenous peroxidases, incubated with the biotinylated goat antimouse IgG for 10 min, streptavidin-horseradish peroxidase for 10 min, and diaminobenzidine for 5 min, per Dako recommendations and empiric secondary antibody for 10 min, streptavidin-horseradish peroxidase for 10 min, and diaminobenzidine for 5 min, per Dako recommendations and empiric determination. Preimmune IgG was used as a control for antibody specificity. AP assay products (5 μg/ml) were separated on a 20% polyacrylamide gel containing 7 M urea. Gels were scanned for analysis.

RESULTS

Expression of Human Ape1/ref-1 in GCTs. We examined the expression of Ape1/ref-1 in 10 GCT cases from a variety of histological subtypes. Four tumors were yolk sac tumors, four were metastatic teratomas, including one with primitive neuroectodermal tumor elements and one with cystic trophoblastic elements, and two were seminomas. Normal cells, including fibroblasts and vascular endothelium, had a predominant nuclear staining pattern with occasional cells demonstrating a mixed nuclear-cytoplasmic staining pattern (Fig. 1). Infiltrating lymphocytes had little Ape1/ref-1 expression. Tumor cells identified in each section displayed an increased level of expression of nuclear Ape1/ref-1 compared with stromal elements on the same slides (Fig. 1 and Table 1). The distribution of Ape1/ref-1 staining in the GCTs was predominantly nuclear in 50% of the cases and mixed nuclear-cytoplasmic in the other 50% (Table 1). There appeared to be a trend for the more differentiated subtypes (teratomas) to have less cytoplasmic expression of Ape1/ref-1 compared with other subtypes (Table 1). Given the small number of samples examined in this pilot study, no statistical analysis was performed; however, a larger trial has been initiated that should permit more conclusive analysis of Ape1/ref-1 expression in GCTs and its relationship to clinical parameters, such as relapse and chemotherapy resistance.

Retroviral Gene Transfer of Ape1/ref-1 into NT2 Cells. As detailed in “Materials and Methods,” we inserted a cDNA fragment
harboring the complete coding sequence of human Ape1/ref-1 into the LXSN retroviral vector (Ref. 28; Fig. 2A). This vector includes a convenient cloning site downstream from the Moloney murine leukemia virus long terminal repeat as well as the neomycin phosphotransferase gene (neo) for use as a selectable marker. NT2 clones transduced with LAPESN were selected in 10-cm dishes and isolated with cloning rings. Screening by Southern blot (Ape1/ref-1 probe) identified two clones (data not shown) with single inserts, which were used for further study. It was difficult to distinguish on Northern blot analysis the mRNA from NT2, NT2-LXSN, and NT2-LAPESN clones 1 and 2 of the endogenous Ape1/ref-1 (1.6 kb) from the retroviral-derived transcript (1.7 kb); however, probing for neo revealed the full-length, 2.9-kb LXSN transcript in the LXSN vector-only transduced cells and the truncated 1.6-kb Neo transcript from the

Table 1 Ten clinical GCT samples scored for Ape1/ref-1 expression by IHC including both tumor cells and surrounding normal tissues*

<table>
<thead>
<tr>
<th>GCT no.</th>
<th>Histology</th>
<th>% APE-tumor cells</th>
<th>% APE-Tumor Nuclei</th>
<th>% APE-Tumor Cell Cytoplasm</th>
<th>Tumor nuclear intensity (1–3)</th>
<th>Tumor cytoplasmic intensity (1–3)</th>
<th>% APE-Stromal Cells</th>
<th>Stromal cell intensity (1–3)</th>
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<tr>
<td>011</td>
<td>Solid and glandular yolk sac tumor</td>
<td>90%</td>
<td>90%</td>
<td>10%</td>
<td>2–3</td>
<td>1</td>
<td>50%</td>
<td>2</td>
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<td>Seminoma</td>
<td>95%</td>
<td>80%</td>
<td>80%</td>
<td>1–2</td>
<td>2</td>
<td>40%</td>
<td>1–2</td>
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<td>Glandular yolk sac tumor</td>
<td>90%</td>
<td>&gt;95%</td>
<td>30%</td>
<td>3</td>
<td>1</td>
<td>60%</td>
<td>2–3</td>
</tr>
<tr>
<td>014</td>
<td>Solid yolk sac tumor</td>
<td>90%</td>
<td>90%</td>
<td>50%</td>
<td>2–3</td>
<td>1</td>
<td>70%</td>
<td>3</td>
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<tr>
<td>015</td>
<td>Teratoma with PNET elements</td>
<td>75%</td>
<td>80%</td>
<td>0%</td>
<td>3</td>
<td>0</td>
<td>70%</td>
<td>3</td>
</tr>
<tr>
<td>016</td>
<td>Seminoma</td>
<td>80%</td>
<td>60%</td>
<td>50%</td>
<td>2–3</td>
<td>2</td>
<td>50%</td>
<td>2</td>
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<td>017</td>
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<td>90%</td>
<td>90%</td>
<td>1%</td>
<td>3</td>
<td>2</td>
<td>40%</td>
<td>2–3</td>
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<tr>
<td>018</td>
<td>Cystic trophoblast</td>
<td>90%</td>
<td>90%</td>
<td>40%</td>
<td>3</td>
<td>2</td>
<td>20%</td>
<td>2–3</td>
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<tr>
<td>019</td>
<td>Teratoma</td>
<td>70%</td>
<td>70%</td>
<td>0%</td>
<td>3</td>
<td>0</td>
<td>50%</td>
<td>2–3</td>
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<tr>
<td>020</td>
<td>Teratoma</td>
<td>60%</td>
<td>60%</td>
<td>0%</td>
<td>3</td>
<td>0</td>
<td>70%</td>
<td>3</td>
</tr>
</tbody>
</table>

* Any appreciable staining is considered positive and graded for intensity: 1, barely detectable; 2, fine granules diffusely present throughout the nucleus or cytoplasm; 3, dark coarse granules are observed.
The absence of a full-length LAPESN transcript likely results from an occult polyadenylation signal within the 3' sequence of the Ape1/ref-1 cDNA, similar to the transcript pattern observed with other LXSN constructs (24).

Expression of Human Ape1/ref-1 in GCT Cell Lines. Immuno-histochemical staining experiments using the antihuman Ape1 antibody were performed on the human GCT cell lines NTD2 and 833K to begin to characterize the role that Ape1/ref-1 might play in the response of GCTs to therapeutic agents. The NTD2 cell line is derived from a human testicular embryonal carcinoma/teratoma lung metastasis (29), and the 833K cell line is derived from an abdominal metastasis of a human testicular GCT with teratoma, embryonal carcinoma, choriocarcinoma, and seminoma elements (30). In both cell lines, there was a predominance of nuclear staining with little cytoplasmic staining (Fig. 3A), similar to the pattern described above in sections of primary teratomas (Table 1). The nuclei displayed a distinctive punctate staining pattern that we observed previously in cervical carcinoma cells (19). The NT2 cells overexpressing Ape1/ref-1 demonstrated an elevated level of nuclear staining for Ape1/ref-1 again with little cytoplasmic staining, similar to the wild-type NT2 cells (Fig. 3A). Vector-only transduced cells were identical to the wild-type NT2 cells in Ape1/ref-1 staining, and morphologically, the transduced cells (LXSN and LAPESN) were indistinguishable from the wild-type cells (Fig. 3A).

Ape1/ref-1 Repair Function in GCT Cell Lines. To determine whether the increased expression of Ape1/ref-1 detected by IHC correlated with increased AP site repair activity, we used an oligonucleotide cleavage assay (20, 27). The assay uses a radiolabeled oligonucleotide (26-mer; Fig. 3B) containing an artificial THF AP site, which when cleaved by AP endonuclease produces a labeled 14-mer (Fig. 3B). Sequential dilutions of cell extracts from each of the cell types were assayed for endonuclease activity and are shown in Fig. 3B. The increasing expression of Ape1/ref-1 noted in parental NT2 cells, 833K cells, and NT2-LAPESN cells (Fig. 3A) was reflected in increasing AP endonuclease activity, as measured with the oligonucleotide cleavage assay. Thus, Ape1/ref-1 repair activity appears to correlate with the degree of Ape1/ref-1 expression by IHC.

Ape1/ref-1 Overexpression Protects GCT Cells from Bleomycin and Radiation. To determine whether Ape1/ref-1 expression correlated with sensitivity to therapeutic agents, we quantitated the AP-site cleavage activity of parental NT2 cells and NT2-LAPESN clones 1 and 2 (Fig. 4, A–C). Both clones as well as the bulk-infected NT2 cells from which the clones were selected displayed a 2-fold higher endonuclease activity compared with NT2 cells (Fig. 4D). We then examined the response of NT2 and NT2-LAPESN cells to bleomycin, an antitumor antibiotic known for its effectiveness in treating GCTs. Cells transduced with the empty vector, LXSN, and the parental NT2 cells responded similarly to treatment (Fig. 5). The NT2 cells over-expressing Ape1/ref-1 demonstrated an elevated level of nuclear staining for Ape1/ref-1 again with little cytoplasmic staining, similar to the wild-type NT2 cells (Fig. 3A). Vector-only transduced cells were identical to the wild-type NT2 cells in Ape1/ref-1 staining, and morphologically, the transduced cells (LXSN and LAPESN) were indistinguishable from the wild-type cells (Fig. 3A).

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expressing Ape1/ref-1 demonstrated a 2-fold increase in protection from bleomycin in a dose-dependent fashion (Fig. 5A), paralleling the 2-fold increase in endonuclease activity observed in the oligonucleotide cleavage assay (Fig. 4). A similar trend for protection from γ-irradiation was observed but with not quite the same degree of protection that was afforded to bleomycin (Fig. 5B). Thus, the AP endonuclease activity of Ape1/ref-1 as measured by the oligonucleotide cleavage assay appears to reflect the degree of protection from bleomycin and radiation.

DISCUSSION

We have made the observation that a key enzyme in the DNA BER pathway, Ape1/ref-1, is expressed at a high level in GCTs of varying histological subtypes. The implications of this observation are that cells expressing a higher level of Ape1/ref-1 may be more efficient at repair of chemotherapy-induced damage and thereby become resistant to the therapeutic agents. To approach this question, we used the human embryonal carcinoma cell line, NT2, and overexpressed Ape1/ref-1 using retroviral gene transduction. Although alteration in Ape1/ref-1 levels in various cell types has resulted in altered sensitivity to cytotoxic agents, few studies have been performed in mammalian cells, and almost none have been performed in human-derived cells (26, 31–33). IHC has been an effective technique to identify and characterize expression of molecular cellular components in clinical samples, particularly for Ape1/ref-1 (20, 34–36). IHC for Ape1/ref-1 expression in GCT clinical samples in this study clearly demonstrated an elevated expression of Ape1/ref-1. To determine whether this elevated expression was meaningful in terms of repair, we evaluated the expression of Ape1/ref-1 in transduced NT2 cells using IHC and correlated the expression with BER activity in the same cells using an oligonucleotide cleavage assay. Overexpression of Ape1/ref-1 resulted in a 2-fold increase in repair activity, which correlated with a 2–3-fold higher protection against bleomycin. Although we frequently see results in molecular biology on the level of orders of magnitude, a 2-fold increase in the resistance of a tumor to therapy is significant. There was less of an effect of protection with radiation likely because damage induced by radiation is complex, with many different types of DNA lesions being produced, many of which are not amenable to repair via the BER pathway. Bleomycin produces single-strand breaks and 4'-oxidized AP sites, which are recognized and repaired by Ape1/ref-1 primarily through its phosphodiesterase function. Furthermore, some of the damage created by bleomycin results in oxidized AP sites in the form of C-4-keto-C-1-aldehydes in the intact DNA strands, as well as 3'-phosphoglycolate esters that terminate strand breaks (8). In previous studies, Ape1/ref-1 catalyzed incision at the C-4-keto-C-1-aldehyde sites at a rate similar to its hydrolytic incision of AP sites. Ape1/ref-1 also incised DNA at hydrolyzed 3'-phosphoglycolates, albeit more slowly than incisions at C-4-keto-C-1-aldehydes (8). Other studies have documented similar types of damage caused by bleomycin or ionizing radiation (8, 37–39). γ-Irradiation produces a variety of damage to DNA, including oxidative damage to bases resulting in base excision and the formation of mutagenic/cytotoxic AP sites, which are substrates for Ape1/ref-1 (33). Thus, we conclude that elevated expression of Ape1/ref-1 in GCTs likely contributes to resistance to therapeutic agents that induce damage responsive to BER.

However, we must recognize that Ape1/ref-1 is a complex enzyme
with multiple functions including a well-documented redox function (7). Although Ape1/ref-1 appears to protect from bleomycin and γ-irradiation presumably by means of repair activity, another aspect, and somewhat contrary to these results, is its potential role in augmentation of the response to certain therapeutic agents. Ape1/ref-1 involvement in activation of p53 and induction of cyclin G may be integral to the apoptotic response to cisplatin in GCs (9, 40). The high level expression of Ape1/ref-1 in GCs in conjunction with its role in activation of apoptosis through p53/cyclin G may account in part for the sensitivity of GCs to therapy. Additionally, elevation of Ape1/ref-1 may be important in maintaining the malignant phenotype through redox activation of transcription factors such as Fox, Jun, and HIF-1α (7). One way to sort out these issues is the use of site-specific Ape1/ref-1 mutants to characterize the role of the repair/redox domains in response of GCs to specific therapeutic agents. Mechanistic studies should be complemented with the examination of Ape1/ref-1 expression in a large sample of GCT patients to determine the correlation, if any, of expression with response to therapy. We have an ongoing protocol to collect such data retrospectively and prospectively, with the objective to analyze the relationship of Ape1/ref-1 expression to histological subtypes of GCs, risk of relapse, response to therapy, and role in tumor progression. Other questions also exist, such as: Does the cytoplasmic distribution of Ape1/ref-1 observed in some GCs result from an alteration in the nuclear localization signal harbored in the 5‘-end of Ape1/ref-1? How does this change in distribution affect repair and redox function? In one primary teratoma of the testis, we examined only the intratubular GCT cells marked with antihuman Ape1/ref-1 antibody, sparing the rest of the normal cellular elements, suggesting that in certain settings, staining with anti-Ape1 may be useful in detecting tumor cells. Similar observations have been made in staining sections of cervical carcinoma in situ for Ape1/ref-1 (19).

ACKNOWLEDGMENTS

We thank Steve Parsons for technical assistance.

REFERENCES

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Cancer Res 2001;61:2220-2225.

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