Alterations in the Expression of the DNA Repair/Redox Enzyme APE/ref-1 in Epithelial Ovarian Cancers

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ABSTRACT

The DNA base excision repair pathway is responsible for the repair of alkylation and oxidative DNA damage. A crucial step in the base excision repair pathway involves the cleavage of an apurinic/apyrimidinic (AP) site in DNA by an AP endonuclease (APE). The major AP endonuclease in mammalian cells is APE/ref-1, a multifunctional enzyme that acts not only as an AP endonuclease but as a redox-modifying factor for a variety of transcription factors. The purpose of this study was to determine the expression of APE/redox factor-1 (ref-1) in ovarian tissues, particularly ovarian cancers. Formalin-fixed, paraffin-embedded specimens of ovarian tissues (normal, various benign conditions, and epithelial cancers) were studied using both polyclonal and monoclonal antibodies to APE/ref-1. The relationship between APE/ref-1 protein levels and DNA repair activity was studied in ovarian Hey and Hey-C2 cell lines using Western blot and a specific AP-site oligonucleotide cleavage assay. Hey and Hey-C2 cells were fractionated, and the nuclear and cytoplasmic extracts were quantitated for protein levels and assessed for APE/ref-1 with Western blot. Normal ovarian tissues consistently demonstrated strong nuclear staining of the surface epithelium, epithelial inclusions, corpora lutea and albicantia, and stroma. Cytoplasmic staining was absent. A similar pattern was seen for genomic damage: direct reversal, mismatch repair, nucleotide excision repair, and base excision repair.

DNA base excision repair involves two major classes of repair enzymes, the DNA glycosylases and APEs (3–5). Glycosylases remove damaged bases, creating AP sites that in turn are incised by APE (APE/ref-1) 5' to the AP site, followed by removal of the abasic residue and insertion of a new base by DNA β-polymerase and ligation. The rate-limiting step in the base excision repair pathway has not been defined in vivo (6–8). However, in vitro evidence suggests that APE/ref-1 activity is the rate-limiting step in the repair of DNA oxidative damage (9).

INTRODUCTION

DNA damage occurs as a result of ionizing radiation, UV irradiation, oxidizing agents, hydrolysis, or alkylating agent chemotherapy. AP sites are the most common form of DNA damage and an estimated 10–20,000 apurinic and 500 apyrimidinic sites are produced per cell/day under normal physiological conditions (1). AP sites may block DNA replication, leading to cytotoxic mutations or genetic instability (2). Several repair pathways have evolved to repair genomic damage: direct reversal, mismatch repair, nucleotide excision repair, and base excision repair.

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There is recent evidence that APE/ref-1 expression and base excision repair capabilities may not be equivalent in all cell types and tissues. APE/ref-1 is differentially expressed during fetal development and in the adult rat brain and testis, as well as in various human tissues (10–12). Little is known about base excision repair functions in malignant tumors. A significant elevation in APE/ref-1 expression has been demonstrated in cervical cancer tissues and cell lines, as well as in germ cell tumors (13, 14). The expression of APE/ref-1 in ovarian tissues has not been characterized and is the focus of this investigation.

MATERIALS AND METHODS

Tissue. Ovarian tissues used in this study were obtained by gynecological surgeons in the Department of Obstetrics and Gynecology at the Indiana University School of Medicine. Surgical specimens were fixed in 4% buffered formaldehyde and embedded in paraffin. Archival tissue blocks from patients

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The abbreviations used are: AP, apurinic/apyrimidinic; APE, AP endonuclease; ref-1, redox factor-1; LMP, low malignant potential.

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determined previously to have normal ovaries, various nonneoplastic and benign conditions, or epithelial ovarian cancer were obtained. Tissues were cut in 6-μm sections and fixed on glass slides. Slides were immunostained and analyzed using a blinded coding system such that staining procedures and microscopic assessments were performed without knowledge of the histopathological diagnosis.

**Antibody Preparation.** The initial antibody used in these studies was produced using overexpression of the human APE/ref-1 protein in a pGEX-glutathione-S-transferase *Escherichia coli* system (Pharmacia, Uppsala, Sweden), as described previously (12, 13, 15), and was obtained from Novus Biologicals (Littleton, CO). However, we have recently produced a monoclonal antibody that was subsequently used in the studies presented here. Both polyclonal and monoclonal antibodies yielded identical results. Antibody purity was confirmed with Western blot analysis before each use (12, 13), and each batch of slides was simultaneously processed using the same antibody concentration. Tissues determined previously to express APE/ref-1 were used as positive controls.

**Immunohistochemistry.** Tissue sections were coated with anti-APE antibody (mouse anti-human APE/ref-1 monoclonal) and incubated overnight at 4°C in a 1:200 dilution in 10% goat serum in PBS. The following day, sections were washed three times for 5 min in PBS and incubated with biotinylated goat antimouse IgG (Vector Labs, Burlingame, CA) at 15 μg/ml in 10% goat serum for 1 h. After two PBS washes for 5 min each, sections were incubated with avidin and biotinylated horseradish peroxidase complex (ABC elite kit; Vector Labs) for 45 min. Slides were then incubated with diaminobenzidine (Vector Labs). After the development of color signal, the products (5 m) were separated on a 20% polyacrylamide gel containing 7 M urea. Gels were wrapped in Saran wrap and subjected to autoradiography. Protein levels in the extracts were quantitated using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), and equal amounts (20 μg) of protein were loaded/lane.

**Cell Lines.** Two ovarian cancer cell lines, Hey and Hey-C2, were supplied to us by Dr. Gordon Mills (M. D. Anderson Cancer Center, Houston, TX). The Hey cells were originally grown from an ovarian adenocarcinoma. By serially passing Hey-C2 cells, 2.5 pmol of 5’ 32P end-labeled double-stranded tetrahydrofuran-containing oligonucleotide, 50 mM HEPEs, 50 mM KCl, 10 mM MgCl2, 2 mM DTT, 1 μg/ml BSA, and 0.05% Triton X-100 (pH 7.5) were allowed to proceed for 15 min in a 37°C water bath. Reactions were halted by adding 10 μl of 96% formamide, 10 mM EDTA, xylene cyanol, and bromphenol blue. AP assay products (5 μl) were separated on a 20% polyacrylamide gel containing 7 M urea. Gels were wrapped in Saran wrap and exposed to film for visualization. The amount of 14-mer to 26-mer was determined after scanning the exposed film into SigmaScan.

**Cellular Fractionation.** Fractionation of the Hey and Hey-C2 cells into nuclear and cytoplasmic components was performed according to the method of Tell et al. (20) Briefly, cells (106) were washed in PBS and resuspended in 5 ml of hypotonic lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM MgCl2, 0.1 mM EDTA, 2 mg/ml leupeptin, 2 mg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The cells were allowed to sit for 10 min and were then homogenized in a Dounce homogenizer for 10 strokes. The nuclei were collected by centrifugation for 5 min at 500 × g at 4°C in a microcentrifuge. The supernatant (cytoplasmic fraction) was decanted from the nuclei, and nuclear proteins were extracted with 10 ml of buffer B (10 mM HEPES, 400 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 2 mg/ml leupeptin, 2 mg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). After incubating for 20 min at 4°C, samples were centrifuged at 12,000 × g at 4°C for 15 min. Nuclear and cytoplasmic extracts were quantitated for protein levels (Bio-Rad assay) and used immediately for Western blot analysis.

**RESULTS**

A total of 38 separate ovarian tissue blocks were processed and analyzed for APE/ref-1 immunostaining (Table 1). Normal ovarian tissues consistently demonstrated strong nuclear stain-

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* LMP, adenocarcinoma of low malignant potential.
ing of the surface epithelium, epithelial inclusions, corpora lutea and albicantia, and stroma (Fig. 1). In one specimen obtained from a postmenopausal woman, there were luteinized stromal cells with strong nuclear staining. Cytoplasmic staining was absent in normal ovarian tissues.

A similar pattern was seen for benign, nonneoplastic conditions. Strong nuclear staining was seen in the stroma and in the epithelial cells of both serous and mucinous cystadenomas (Fig. 2). Endometriosis demonstrated moderate to strong nuclear staining in both glands and stroma elements (Fig. 3).

LMP ovarian cancers stained in a pattern similar to the normal ovarian and nonneoplastic tissues. Moderate to strong nuclear staining was seen in the epithelia of all seven LMP tumors; however, two specimens also demonstrated areas of cytoplasmic staining (Fig. 4). Ovarian cancers were remarkably different from all other tissues studied in the intensity and subcellular localization of APE/ref-1 immunoreactivity. Nuclear staining of the malignant epithelium ranged from strong to weak, with considerable staining heterogeneity noted within the same tumor (Fig. 5). Cytoplasmic staining of the malignant epithelium was frequently encountered and often predominant as the subcellular location of immunoreactivity (Fig. 6). In general, considerable variation was seen within and between malignant ovarian tumors with respect to intensity and predominance of nuclear versus cytoplasmic staining. There appeared to be no relationship between the presence, intensity, or subcellular location of APE/ref-1 immunostaining and either tumor grade or histological subtype.

Using the Hey and Hey-C2 ovarian cancer cell lines, we compared APE/ref-1 protein levels (Western blots) to DNA repair APE activity levels. This analysis was performed to begin to understand the relationship between altered levels of APE/ref-1 and what this altered expression indicates in the human samples. Is the altered expression of APE/ref-1 affecting its
DNA repair capacity, redox function, or both? Western blot analysis was performed on the Hey and Hey-C2 cells, and the data are presented in Fig. 7. We detected a 4-fold increase in the level of APE/ref-1 protein. Western blot analysis of protein extracts from nuclear versus cytoplasmic components indicated that the overall increased level of APE/ref-1 in the Hey-C2 cells, relative to the Hey cells, was mainly attributable to an increase in cytoplasmic levels of the enzyme (Fig. 7). These data complemented the clinical data, showing an elevated and altered expression of APE/ref-1 in the epithelial ovarian tumors and in repair activity shown in the following experiments.

To determine whether the changes in APE/ref-1 expression observed at the protein level translated into changes in APE/ref-1 repair function, we used a specific AP-site oligonucleotide cleavage assay (Fig. 8). As can be seen by the activity assay, the Hey cell line had no detectable levels (<150 ng of total cell extract, whereas the Hey-C2 cells (same cell extract as shown in Fig. 7, Lane 2) still had detectable activity at 40 ng of cellular extract. Therefore, the Hey-C2 cells demonstrated a 4-fold increase in APE activity in the AP oligonucleotide assay compared with the Hey cells. The AP-site oligonucleotide cleavage DNA repair assay results indicated that APE/ref-1 protein levels (Fig. 7) correlated well with appropriate APE/ref-1 repair activity changes (Fig. 8). Assuming this in vitro relationship of increased APE/ref-1 protein signifying increased APE/ref-1 repair activity, then the increase of APE/ref-1 observed in the immunohistochemical analysis of human samples could correlate with increased in vivo DNA repair activity. However, these observations do not preclude additional alterations in redox functions of the APE/ref-1 protein, given that DNA repair and redox functions reside in separate domains of the protein. Thus, if the APE/ref-1 seen in ovarian cancer cells is primarily functioning as a DNA repair enzyme, its movement out of the nucleus and into the cytoplasm would have an impact on the ability of this protein to carry out nuclear DNA repair activities. This could lead to increased sensitivity of these tumor cells to DNA-damaging agents, or the tumor cells may accumulate mutations because of decreased base excision repair, leading to a more aggressive oncogenic phenotype.

**DISCUSSION**

AP sites are possibly the most common DNA lesions and may occur spontaneously or during the repair of modified bases. Base excision repair of AP sites requires multiple steps, beginning with the cleavage of the DNA strand adjacent to AP sites. DNA strand cleavage is catalyzed by an APE (APE/ref-1) and occurs 5' to the AP site (3, 4). APE/ref-1 is believed to be the rate-limiting step in the base excision repair pathway. The reduction of APE/ref-1 levels by antisense RNA sensitizes cells to oxidative damage and to various DNA-damaging agents (21–23), and APE/ref-1 knockout mice are lethal very early in embryonic development (24).

There is accumulating evidence that APE has additional cellular functions beyond DNA repair. Xanthoudakis et al. (25) identified a human gene encoding a product involved in the redox-activated DNA binding of several transcription factors including FOS, JUN, and nuclear factor-κB. The gene product (ref-1) proved to be identical to APE. A number of other transcriptional activators are also regulated by redox modulation including: c-myc, Ets, early growth response-1, the glucocorti-
Fig. 7 Immunohistochemical and Western blot analysis of Hey and Hey-C2 cell lines. Panel A, immunohistochemical staining of Hey (A and B) and Hey-C2 (C and D) cells with monoclonal APE/ref-1 antibody. A and C, ×10; B and D, ×40. Panel B, Western blot analysis using monoclonal APE/ref-1 antibody on Hey and Hey-C2 cells. Total protein extract from each cell line was run on the gel. Panel C, same blot as in Panel B, but the blot was reprobed with antibody to actin to normalize for loading. Panel D, Hey-C2 (Lanes 1 and 2) and Hey (Lanes 3 and 4) cells were fractionated into nuclear and cytoplasmic components, and equal amounts of protein were run, followed by Western blot analysis using APE/ref-1 monoclonal antibody as described in the text.
coid receptor, members of the activating transcription factor/cAMP-responsive element binding family, and HIF-1α (26–31). Redox regulation of transcription factors may therefore exert an enormous influence on cellular physiology, the end result being a net effect of positive and negative interactions between the various transcription factors and their downstream targets or effector genes.

Recently, it has been shown that the redox state of p53 affects its ability to bind to specific DNA sequences and subsequently regulate the transcription of adjacent genes (32). The p53 redox state can be altered in vivo with increased oxidation, correlating with an impaired ability to effect downstream functions (33). It is not surprising that APE/ref-1 has been shown to be an important regulator of p53 function through both redox-independent and -dependent means (34, 35). These findings, along with the fact that hypoxic stresses induce the accumulation of both APE/ref-1 and p53, suggest plausible links between oxidative damage, the activation of DNA repair and redox transcriptional functions, and consequent influences on cellular proliferation and apoptotic processes.

In this study, we found marked differences in the pattern and intensity of APE/ref-1 immunostaining in ovarian cancer specimens versus all other ovarian conditions: normal ovaries, benign lesions, and LMP ovarian cancers. Strong nuclear staining was found in cells of the normal ovary surface epithelium, the epithelium of benign tumors and endometriosis, and the epithelium of LMP tumors. In contrast, nuclear staining of cells in the epithelium of ovarian malignancies ranged from strong to weak, with considerable staining heterogeneity frequently found within the same tumor. Cytoplasmic staining was frequently encountered in ovarian malignancies and often the predominant site of APE/ref-1 immunoreactivity. Two LMP tumors also had infrequent areas of cytoplasmic staining. These immunohistochemical differences parallel the clinical differences in the respective ovarian states. Although LMP tumors may metastasize, they are generally associated with a good prognosis and may be treated with conservative surgery and without need for adjuvant chemotherapy (36, 37). Clinical data for the patients from whom specimens were obtained are unknown. It would be interesting to know whether the two LMP tumors with (infrequent) APE/ref-1 cytoplasmic immunoreactivity came from patients with metastatic disease.

On the basis of our observations, wide variations in nuclear staining intensity and the presence of strong cytoplasmic staining in ovarian tissues are correlates of malignant behavior. In previous studies, APE/ref-1 expression during fetal development and in a variety of adult tissues was predominantly nuclear, although cytoplasmic staining was seen in certain parts of the brain and in the liver (10, 12). Using animal models, others have reported both nuclear and cytoplasmic APE/ref-1 expression in certain brain tissues (pyramidal neurons of the hippocampus and granular cells of the dentate gyrus) in response to ischemia (38). APE/ref-1 expression is markedly elevated in preinvasive and invasive cervical neoplasia (13) and in germ cell tumors (14) but is exclusively nuclear. Our observations in ovarian tumors parallel those by Kakolyris et al. (39), who

Fig. 8  Functional AP-site oligonucleotide cleavage assay. A, schematic representation of the APE DNA repair activity assay. The amount of APE activity is calculated using the relative amount of the 14-mer oligo fragment compared with the unreacted 26-mer band. B, Hey and Hey-C2 cell extracts were incubated with a 32P-labeled 26-mer containing an artificial AP site and then assayed for cleavage by denaturing SDS-PAGE as described in “Materials and Methods.” The 14-mer band indicates APE activity. Recombinant APE is included as a positive control. Dilutions of extracts are shown to demonstrate differences in activity between the Hey (top) and Hey-C2 (bottom) extract, which correlates with the Western blot analysis. Lane APE, a reaction using recombinant APE/ref-1 protein that is included as a positive control for the assay. In the ovarian cell extract lanes, an excess of 26-mer is always used to not inhibit the reaction because of substrate limitation.
reported a marked disruption in the APE/ref-1 staining pattern in colonic adenomas/carcinomas. In the normal colonic mucosa, APE/ref-1 staining was predominantly nuclear in the lower part of the crypts (less differentiated cells) and cytoplasmic in the superficial colonic epithelium (more differentiated cells). The expression and localization of APE/ref-1 in hyperplastic polyps were similar to the normal colonic epithelium. For adenomas and carcinomas, however, immunostaining was exclusively cytoplasmic in 37 and 50%, respectively, and both nuclear and cytoplasmic staining were seen in 53% of adenomas and 39% of carcinomas (39).

The implications of cytoplasmic staining in ovarian tumors are unknown. We cannot determine by these studies the in vivo functional status of APE/ref-1 but can only speculate whether the observed differences in ovarian malignancies represent alterations in DNA repair functions, redox functions, or both. Increased expression does not signify that both DNA repair and redox functions are changed. The DNA repair and redox activities of APE/ref-1 reside in two separate domains, with the NH2 terminus primarily involved in redox regulation and the COOH terminus primarily involved in DNA repair (30). The DNA repair activity of APE/Ref-1 may be inactivated by phosphorylation (19). Furthermore, AP sites may occur in mitochondrial DNA as a result of oxidative damage, and base excision repair is important for the repair of AP sites in mitochondrial DNA (40, 41). Thus, APE/ref-1 may have both cytoplasmic and nuclear functions in base excision repair.

Given the abundance of APE/ref-1 and its pleiotropic cellular functions, further work will likely identify a number of posttranslational events for regulating specific APE/ref-1 activities. A greater understanding of alterations in base excision repair and redox functions of APE/ref-1 in human cancers has epidemiological and therapeutic significance.

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