Elevated and Altered Expression of the Multifunctional DNA Base Excision Repair and Redox Enzyme Ape1/ref-1 in Prostate Cancer

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ABSTRACT

The DNA base excision repair pathway is responsible for the repair of cellular alkylation and oxidative DNA damage. A crucial step in the BER pathway involves the cleavage of baseless sites in DNA by an apurinic/apyrimidinic or baseless (AP) endonuclease (Ape1/ref-1), which is a multifunctional enzyme that acts not only as an AP endonuclease but also as a redox-modifying factor for a variety of transcription factors including Fos, Jun, paired box containing genes (PAX), nuclear factor-κ, hypoxia-inducible factor α (HIF-1α), HIF-like factor (HLF), p53, and others. The expression of Ape1/ref-1 in prostate has not been characterized previously. Ape1/ref-1 nuclear immunohistochemistry levels, scored for intensity as 1+, 2+, or 3+, were 91, 3, and 6% in benign hypertrophy (BPH), 0, 42, and 58% in prostatic intraepithelial neoplasia (PIN) and 3, 30, and 67% in prostate cancer, respectively, clearly showing an increase in Ape1/ref-1 nuclear staining in the PIN and cancer compared with BPH. Furthermore, the level of cytoplasmic staining of Ape1/ref-1 in cancer and PIN were elevated (42 and 36%, respectively) compared with BPH (5%). There was no correlation with prostate-specific antigen values or doubling time of prostate cancer, respectively. In conclusion, we have demonstrated that Ape1/ref-1 is dramatically elevated in prostate cancer, the level of staining of Ape1/ref-1 increases from low in BPH to intense in PIN and cancer, and there is an increase in the amount of Ape1/ref-1 in the cytoplasm of PIN and cancer compared with BPH. Given these results, we conclude that Ape1/ref-1 may be a diagnostic marker for early prostate cancer and play a role, through its repair, redox, or both functions, in the physiology of the early development of prostate cancer.

INTRODUCTION

The DNA BER pathway is responsible for the repair of IR, oxidative, or alkylation DNA damage resulting in protection against the deleterious effects of endogenous and exogenous agents encountered on a daily basis. Removal of the incorrect or damaged base by a DNA glycosylase (e.g., methylpurine DNA glycosylase) comprises the first step of the BER pathway, followed by Ape1/ref-1, the second enzyme in the BER pathway, and the focus of these studies. Ape1/ref-1 hydrolizes the phosphodiester backbone immediately 5’ to an AP site. This incision generates a normal 3’-hydroxyl group and an abasic deoxyribose-5-phosphate, which is processed by enzymes in the subsequent steps of the BER pathway. AP sites are the most common form of DNA damage with some 50–200,000 sites produced in every cell each day under normal physiological conditions (1, 2). AP sites are generated from spontaneous and chemically initiated hydrolysis, IR, UV irradiation, oxidative stress, oxidizing agents, and removal of altered (such as alkylated) bases by DNA glycosylases (3, 4). The persistence of AP sites in DNA results in a block to DNA replication, cytotoxic mutations, and genetic instability (4). Ape1/ref-1 appears to be the rate-limiting step in this branch of the BER pathway (5).

Ape1/ref-1 also contains repair activity for 3’-terminal oxidative lesions, i.e., 3’-P or 3’-PG damages, which are formed by IR or free radical attack of DNA (3, 6–10). By hydrolyzing 3’-blocking fragments from oxidized DNA, Ape1/ref-1 produces normal 3’-hydroxyl nucleotide termini that are necessary for DNA repair synthesis and ligation at single- or double-strand breaks (5, 11).

Interestingly, Ape1/ref-1 is additionally a multifunctional protein that not only is responsible for repair of AP sites, but also functions as a redox factor maintaining transcription factors in an active reduced state (12). In these initial studies, Ape1/ref-1 was given the name redox effector factor 1 (ref-1) and appears in the literature under this name as well as APE, hAPE, HAP1, and others (13). Ape1/ref-1 has been shown to stimulate the DNA binding activity of numerous transcription factors that
are involved in cancer promotion and progression such as Fos, Jun, nuclear factor-κB, PAX, HIF-1α, HLF, and p53 (13). Ape1/ref-1 has also been implicated in the activation of bioreductive drugs requiring reduction to be active (14) and has been shown to interact with a subunit of the Ku antigen to act as a negative regulator of the parathyroid hormone promoter (15, 16), as well as part of the hemin response element binding protein transcription factor complex (17).

Ape1/ref-1 levels have also been found to be elevated in a number of other cancers such as ovarian, cervical, rhabdomyosarcoma, and germ-cell tumors (18–21) correlated with the radiosensitivity of cervical cancers (22), but have not been found to be elevated in breast cancer (23).

Given the importance of Ape1/ref-1 in both DNA repair and redox regulation of oncoproteins, including p53, and its relationship to HIF-1α and HLF posttranslational regulation, we have undertaken investigations into the expression level and relationship of Ape1/ref-1 to prostate cancer. Our results demonstrate that Ape1/ref-1 is elevated in very early stages of prostate cancer and may implicate Ape1/ref-1 as an early diagnostic marker for prostate cancer.

MATERIALS AND METHODS

Cell Lines

The DU145, LnCAP, and PC-3 cell lines were maintained as adherent cells in 100 mm × 20-mm Falcon tissue culture dishes (Fisher, Becton Dickinson, NJ) in DMEM media (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) plus 1% Penstrep (Life Technologies, Inc.). Cells were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air.

Tissue

Prostate tissues used in this study were obtained (under Institutional Review Board study no. EX9812–05) by urology surgeons in the Department of Urology at Indiana University School of Medicine. We chose to examine the paraffin-embedded blocks of prostate tissue at Indiana University from patients who had undergone prostatectomy for prostate cancer and in whom we had sufficient follow-up to determine treatment outcome. We specifically chose blocks from 34 patients who had experienced disease recurrence during follow-up as determined by a detectable serum PSA. Serum PSA is used as a biomarker of residual prostate tissue, and the level of PSA in the serum correlates with disease volume. Rate of disease progression after prostatectomy is determined by PSADT. Linear regression analysis is then performed after logarithmically transforming PSA data. The PSADT is then calculated by the formula (ln2)/slope.

Archival tissue blocks were also obtained from patients who underwent prostatectomy for BPH and/or underwent prostate biopsy and were determined to have PIN to determine the relationship of APE to benign (BPH) and premalignant (PIN) conditions. In patients with cancer, the pathological tumor stage, Gleason score, PSA levels, and calculated doubling time were not available to the investigators performing tissue analysis.

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 and Western Blot Analysis

The monoclonal antibody used in these studies (clone 13B8E5C2) has been extensively characterized and was from Novus Biologicals (Littleton, CO; Refs. 19, 20, 24, 25). Antibody purity was confirmed with Western blot analysis before each use, and each batch of slides was simultaneously processed using the same antibody concentration. Western blot analysis was performed as described previously (19, 20, 24, 25).

IHC and Scoring

Tissue sections were processed on a Ventana automated machine with anti-Ape1/ref-1 monoclonal antibody at a 1:500 dilution and were detected using a DAKO detection system. Hand-processed tissues were initially examined to determine the appropriate dilution and to confirm the specificity of the antibody reaction as described previously (19, 20). Tissues that had been previously used and characterized for Ape1/ref-1 staining were included as positive controls (19). As a negative control, preimmune IgG (50 μg/ml) was used as the primary antibody in place of anti-Ape1.

The specimens were stained with antibody against Ape1/ref-1 protein. Both nuclear and cytoplasmic staining were noted. Any appreciable brown staining was considered positive and graded as 1+, if barely detectable, 2+ if easily seen fine granules were present diffusely throughout the nucleus or cytoplasm, and 3+ when dark coarse granules were observed. Also, the percentage of cells exhibiting positive staining was estimated. Less than 10% of the cells showing presence of stain were considered negative, 10–30% of the cells positive were graded as 1+, 30–60% as 2+, and >60% were graded as 3+. The nuclear and cytoplasmic staining were recorded separately. The H&E slides were then reviewed to determine diagnosis and to map the location of the various histological patterns, such as carcinoma, glandular hyperplasia, atrophy, inflammation, and normal, to correlate with the staining patterns observed in the immunohistochemical preparations.

Statistical Analysis of Ape1/ref-1 IHC Staining and PSADTs and Gleason Score

Overall, cytoplasmic, and nuclear APE staining by IHC were then correlated with PSADT, PSA prior to resection, and tumor grade (Gleason score) for those patients with cancer, by linear regression analysis.

RESULTS

Although the Ape1/ref-1 antibody has been previously characterized in other cell lines and tissue IHC studies, we wanted to confirm the specificity of the Ape1/ref-1 monoclonal antibody before analyzing the prostate tissues. Total cell extracts from the prostate cell lines DU145, LnCAP, and PC-3 were analyzed using Western blot analysis (Fig. 1). As can be seen in the figure, the antibody is very specific for the Mr 37,000 Ape1/ref-1 protein. Further subcellular fractionation into nuclear and cytoplasmic components illustrates that Ape1/ref-1 is primarily localized in the nucleus in these cell lines (Fig. 1).
We have determined that Ape1/ref-1 levels of 66 scored regions for PIN and cancer were 2 staining levels, whereas in the PIN and cancer regions, only 1 case stained at a 3. The intensity and location of staining was determined for each of the cases and tabulated (Table 1). Thirty of the 33 BPH stained regions demonstrated 1 staining for Ape1/ref-1, whereas benign epithelial cells showed little, if any, Ape1/ref-1 staining (Fig. 2A). Cribiform pattern high-grade PIN also demonstrated high Ape1/ref-1 staining (Fig. 2B), whereas the adjacent benign glands showed no immunostaining (Fig. 2B). Also, the expression of Ape1/ref-1 immunoreactivity was contained only within the malignant glands (Fig. 2C). The intensity and location of staining was determined for each of the cases and tabulated (Table 1). Thirty of the 33 BPH stained regions demonstrated 1+ intensity, whereas in the PIN and cancer regions, only 1 case stained at a 1+ intensity level. However, when comparing the 2+ and 3+ staining levels, only 3 fell into this category for BPH, whereas 65 of 66 scored regions for PIN and cancer were 2+ or 3+. From this analysis, we have determined that Ape1/ref-1 levels are low (1+) in BPH, yet change dramatically to predominantly 2+ or 3+ in PIN and cancer (Table 1). Furthermore, the location of Ape1/ref-1 becomes altered in the PIN and cancer cases compared with BPH, with an increase in the amount of Ape1/ref-1 in the cytoplasm (Table 1). We also observed a strong correlation between the intensity of staining of PIN and cancer. Of the samples analyzed, in 24 cases, the level of staining of PIN was equal to that of prostate cancer; in 3 cases, PIN staining was less than that of cancer and in 3 cases greater than that of cancer (Table 2). This pattern of Ape1/ref-1 altering its location in the more advanced or aggressive cancer has been seen in two other studies: cervical (20) and ovarian (19). However, the cytoplasmic versus nuclear location appears to be a more distinct and dramatic alteration in PIN and prostate cancer compared with the other cancers analyzed previously (19, 20).

We also determined whether there was any correlation in the doubling-time time of PSA values or PSA levels and Ape1/ref-1 staining. We did not see any relationship between PSADTs and the overall level of Ape1/ref-1 staining (Fig. 3A), nor of nuclear (Fig. 3B) or cytoplasmic Ape1/ref-1 staining (Fig. 3C), nor did we find any relationship between the Gleason score and Ape1/ref-1 expression (Fig. 3D).

**DISCUSSION**

The repair of AP sites, which are the most common form of DNA damage (1, 2) and are both cytotoxic and mutagenic (4), requires the major human AP endonuclease, Ape1/ref-1, both for AP site recognition and cleavage 5’ to the AP site via a hydrolytic mechanism. It has also been shown to contain repair activity for 3’-terminal oxidative lesions, such as those formed by IR (3, 5–7). However, the predominant human AP endonuclease, Ape1/ref-1, (12, 30, 31) is a multifunctional protein that not only is responsible for the repair of AP sites but also functions as a redox factor maintaining transcription factors in an active reduced state (26–29).

Ape1/ref-1 has been shown to stimulate the DNA binding activity of Fos-Jun dimers, as well as nuclear factor-κB, Myb, AP-1 proteins, and members of the ATF/CREB family, HIF-1α, HLF, and PAX5 (12, 32–37). The various transcription factors that require reduction to bind to DNA contain a cysteine residue within the DNA binding motif that, if oxidized, prevents DNA binding and, if reduced, augments DNA binding. Therefore, the DNA binding activity of these proteins is sensitive to redox (38). Ape1/ref-1, which is the major AP-1 redox activity in cells, represents a novel redox component of the signal transduction processes that regulate eukaryotic gene expression (32) and links Ape1/ref-1 as a critical player in DNA repair and redox regulation (13). Recent developments also have intimately linked Ape1/ref-1 as a major controlling factor for p53 activity through a redox-dependent and -independent mechanism (39, 40) and have established its interaction with p53 in vivo (41). These findings, along with the fact that hypoxia stresses induce the accumulation of both Ape1/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 (25, 42–44). Additionally, Ape1/ref-1 has been shown to posttranslationally regulate HIF-1α and HLF through a redox mechanism. This finding is of interest because HIF-1α and HLF, when induced by hypoxia in tumor cells, regulate the expression of a number of downstream targets involved in angiogenesis (36, 45, 46). Furthermore, HIF-1α levels have been shown to be elevated in rat and human prostate cancer (47).

The relationship of Ape1/ref-1 levels and chemoresistance of cells or correlation with tumorigenicity has begun to be addressed in a variety of systems. For example, bacteria or human cells lacking AP endonuclease repair activity are hypersensitive to alkylating or oxidizing agents that induce the formation of AP sites (3). Moreover, targeted reduction of Ape1/ref-1 protein by specific antisense RNA expression renders mammalian cells hypersensitive to methyl methanesulfonate, H2O2, and bleomycin. More recent studies have shown that radiosensitivity of cervical cancers is directly correlated with the levels of Ape1/ref-1 activity (22, 48, 49). Furthermore, Ape1/ref-1 levels have been found to be elevated in a number of other...
cancers, such as ovarian, cervical, rhabdomyosarcoma, and germ cell tumors (18–21).

In this study, we determined that: (a) Ape1/ref-1 is dramatically elevated in prostate cancer; (b) the level of staining of Ape1/ref-1 increases from low in BPH, to intense in PIN and cancer; (c) there is no correlation between Ape1/ref-1 and PSA values or PSA doubling times; and (d) there is no correlation between Ape1/ref-1 and Gleason score. Furthermore, there is an increase in the amount of Ape1/ref-1 in the cytoplasm of PIN and cancer compared with BPH. Thus, if the increased expression of Ape1/ref-1 observed in PIN and prostate 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 the tumor cells’ accumulating mutations because of decreased BER, leading to a more aggressive oncogenic phenotype. Alternatively, the cytoplasmic location of Ape1/ref-1 could have an impact on the redox function of Ape1/ref-1. However, given that prostate cancer cells are slow growing, the implication that Ape1/ref-1 is elevated because of rapid growth or turnover is unfounded. Furthermore, we have previously demonstrated an inverse relationship between Ape1/ref-1 protein levels and apoptosis (25), which has recently been observed in other cell types (50), such that prostate cells with elevated Ape1/ref-1 levels may also have a decreased apoptotic index.

Movement of Ape1/ref-1 between the nucleus and cytoplasm has been seen in some other studies. For example, Ape1/ref-1 translocates from the cytoplasm to the nucleus after reac-

**Table 1** Level of intensity and cellular location of Ape1/ref-1 expression

<table>
<thead>
<tr>
<th>Cell types</th>
<th>1+ intensity %&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2+ intensity %</th>
<th>3+ intensity %</th>
<th>Nuclear&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cytoplasm&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH</td>
<td>91</td>
<td>6</td>
<td>3</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>PIN</td>
<td>0</td>
<td>42</td>
<td>58</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>Cancer</td>
<td>3</td>
<td>30</td>
<td>67</td>
<td>58</td>
<td>42</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were scored for staining intensity and numbers as described in the “Materials and Methods” section.

<sup>b</sup> The percentage of cells exhibiting positive staining were determined for either nuclear or cytoplasmic location (see “Materials and Methods”).

**Table 2** Correlation between consistency of intensity and BPH, PIN, and prostate cancer

<table>
<thead>
<tr>
<th>Comparison of staining intensity of PIN vs. prostate cancer</th>
<th>No. of samples</th>
</tr>
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<tbody>
<tr>
<td>PIN = cancer</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIN &gt; cancer</td>
<td>3</td>
</tr>
<tr>
<td>PIN &lt; cancer</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> In only three cases, BPH = PIN = cancer.
tive oxygen species production in B lymphocytes and thyroid cells, in which the translocation is fairly rapid and occurs within 1 h (24, 43, 51, 52). However, in HeLa cells and in the rat dentate gyrus, the process is in hours (43, 51). Additionally, in rat thyroid cells, the translocation of Ape1/ref-1 to the nucleus is hormone dependent and under the control of thyrotropin (52). However, in no experimental system to date, has it been observed that Ape1/ref-1 moves from the nucleus to the cytoplasm, although steady-state elevated levels have been observed in cervical cancer cell lines (19), but apparently not in prostate cell lines, or at least not in those analyzed by us (Fig. 1).

The implications of cytoplasmic staining in prostate tumors are harder to understand. In these particular studies, we have not determined the actual status in which Ape1/ref-1 exists in the tumor, i.e., is it in a reduced or oxidized state and is there a difference in the redox status of the cytoplasmic versus nuclear Ape1/ref-1 protein? Furthermore, we haven’t determined whether the Ape1/ref-1 found in the various prostate samples is phosphorylated or not. Recent data have emerged showing that Ape1/ref-1 is modified posttranslationally by phosphorylation (53, 54); however, there is a disagreement on whether the phosphorylation of Ape1/ref-1 affects the repair (53) or redox function (54) of the protein. Nevertheless, this will be an important issue to clarify, not only for mechanistic studies but also to develop Ape1/ref-1 antibodies that will be able to determine the phosphorylation status of Ape1/ref-1 in prostate cancer and its impact on Ape1/ref-1 function in these cells. These types of analyses will go further in determining the role that Ape1/ref-1 plays in prostate cancer initiation and progression and, possibly, in clarifying the cytoplasmic location result presented here.

Finally, there could be a relationship between Ape1/ref-1 levels and the type of BER pathway that is invoked. It has recently been shown that oxidative DNA base damage is repaired by the short-patch DNA repair pathway (11). The steps invoked in this pathway, after the removal of the damaged base by a glycosylase/lyase require the processing of the 3'-terminus, a function carried out by Ape1/ref-1. It has also been shown that the removal of the oxidatively damaged base is not the rate-limiting step, but rather it is Ape1/ref-1 through its phosphodiesterase activity that is the rate-limiting step in BER repair of oxidative DNA damage (5, 11). Given that it has been shown that a number of cancers display an oxidative DNA damage phenotype (55), it can be hypothesized that the overexpression of Ape1/ref-1 in prostate cells is in response to increased oxidative DNA damage, for either its DNA repair role or its role as a redox modification factor in maintaining the expressed oncoproteins in a redox state, or both.

A greater understanding of alterations in BER and redox functions of Ape1/ref-1 in human cancers has epidemiological and therapeutic significance. The dramatic increase in Ape1/
ref-1 protein in PIN and prostate cancer, compared with normal prostate tissue and BPH, clearly supports a role of Ape1/ref-1 in early prostate cancer initiation and progression from normal and hyperplasia to a cancerous state. How this occurs, mechanistically, and the exact function Ape1/ref-1 is playing in prostate cancer awaits further investigation.

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