Down-Regulation of Apurinic/Apyrimidinic Endonuclease Expression Is Associated with the Induction of Apoptosis in Differentiating Myeloid Leukemia Cells

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Abstract

The human DNA repair enzyme apurinic/apyrimidinic endonuclease (APE/ref-1) is a multifunctional protein in the DNA base excision repair (BER) pathway that is responsible for repair of apurinic/apyrimidinic (AP) sites in DNA. DNA repair and programmed cell death both function using different mechanisms to protect the organism from the consequences of extensive cellular damage; however, little is known about the relationship of the DNA BER repair pathway to apoptosis. We have determined the relationship of a BER DNA repair enzyme, APE, to apoptosis using the myeloid leukemia cell line HL-60, which can be induced to differentiate down the granulocytic or monocytic/macrophage pathway. Treatment of HL-60 cells with retinoic acid/DMSO (granulocytic) or phorbol 12-myristate 13-acetate (monocytic) results in apoptosis and in down-regulation of APE expression at both the RNA and protein levels. Moreover, double-labeling experiments using APE immunohistochemistry and the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling assay for apoptosis demonstrate that individual cells undergoing apoptosis lose expression of APE regardless of their state of differentiation. Blocking apoptosis by overexpression of the bcl-2 proto-oncogene in HL-60 cells or by a bcr-abl-related mechanism in K562 cells and subsequent differentiation results in morphological differentiation but no loss of APE expression. These studies establish that down-regulation of APE expression is associated with programmed cell death in cells of the myeloid lineage.

Introduction

AP sites are the most common form of DNA damage, with approximately 10,000–20,000 AP sites produced in every cell each day under normal physiological conditions (1). AP sites are generated from spontaneous and chemically initiated hydrolysis, ionizing radiation, oxidizing agents, and removal of altered (such as alkylated) bases by DNA glycosylases (2–4). Mutations and genetic instability can result from AP sites that are not repaired (5).

DNA BER primarily requires two types of repair enzymes: DNA glycosylases and APEs (2, 5–7). DNA glycosylases remove a damaged base, creating an AP site in the DNA that is then acted on by an APE (2, 5, 8). AP sites produced either by N-glycosylase action on damaged bases, directly by agents such as bleomycin, or by spontaneous hydrolysis are incised by APEs either 3' to the AP site (class I APE/lyase) or 5' to the AP site (class II APE, Refs. 2 and 5). Repair is completed by loss of the abasic residue followed by synthesis of a new base by DNA polymerase and ligation. The predominant human class II APE (9–12) is 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 (e.g., Jun/Fos) to facilitate DNA binding (13).

The process of programmed cell death or apoptosis involves activation of a specific cellular pathway that results in death of the cell both as a response to cell injury and as part of normal development. Details of the factors involved in apoptosis and their regulation have been the focus of intense interest in recent years (14, 15). Both DNA repair and apoptosis function to protect the organism from the sequelae of serious cellular damage. DNA repair systems serve to prevent deleterious genetic damage from being passed on to the next cellular division, and apoptosis eliminates more
extensively damaged cells that are beyond the normal repair mechanisms. Little is known about the interaction of these two processes, although recent data have linked apoptosis to PARP (16). The mammalian ICE is a proapoptotic member of the family of cysteine proteases that is critical for the process of programmed cell death (16, 17). One substrate for ICE is the DNA repair enzyme PARP. The actual role of PARP in DNA BER has been studied extensively, but no clear role has been determined. However, it may be possible for chromatin rearrangement to enhance DNA repair (18). The proteolytic cleavage of PARP results in a dysfunctional PARP unable to contribute to repair or genomic maintenance (16, 17). Moreover, the Ca²⁺/Mg²⁺-dependent endonuclease that generates internucleosomal DNA cleavage characteristic of apoptosis is regulated negatively by poly(ADP-ribosylation). Thus, inactivation of PARP may clear the way for activation of DNA cleavage to complete the process of programmed cell death.

In this report, we describe the relationship of major DNA BER enzyme, APE, to programmed cell death. The HL-60 myeloid leukemia cell line is a well-characterized system that we have used previously to study the relationship of apoptosis to cell differentiation (19). Although RA is capable of inducing terminal granulocytic differentiation with apoptosis in parental HL-60 cells, when the proto-oncogene bcl-2 is overexpressed in HL-60 cells, retinoic acid will still induce granulocytic differentiation, but the process of programmed cell death is blocked. These experiments indicated that the processes of apoptosis and myeloid differentiation are distinct and separable. We have used these same HL-60 and HL-60-bcl-2 cells to characterize the correlative inverse relationship of APE expression to apoptosis.

Results
APE Expression in Differentiating Myeloid Leukemia Cells. To investigate possible changes in expression of the DNA repair enzyme APE in differentiating myeloid cells, we used Northern and Western blot analyses with the HL-60 myeloid leukemia cell line, which can be induced to terminally differentiate into mature granulocytes or monocyte/macrophage (20). Western blot analysis revealed a progressive decrease in APE protein expression over 6 days in HL-60 cells induced to differentiate along the granulocytic pathway with 10⁻⁵ M RA compared with ethanol-treated controls (Fig. 1A). A more rapid decrease in APE expression was observed with granulocytic induction using DMSO (Fig. 1A) with nearly undetectable APE after 6 days. Our observations were confirmed by Northern blot analysis (Fig. 1A) in which a similar progressive decrease in APE transcript expression was observed with induction of granulocytic differentiation. Again, DMSO induces a more rapid decrease to a lower level compared with RA. To determine whether the decrease in APE expression was confined to granulocytic differentiation, we induced HL-60 cells down the monocyte/macrophage pathway with PMA. Expression of the APE protein decreased to a very low level by Western blot (Fig. 1B) after 2 days of exposure to PMA. Decreased expression of the APE transcript on Northern blot was also evident after exposure to PMA (Fig. 1B). Thus, induction of granulocytic
and monocytic differentiation in HL-60 cells results in decreased expression of APE at the RNA and protein level.

APE Expression and bcl-2 Expression. To ascertain whether APE expression was associated with differentiation or programmed cell death, we blocked apoptosis by overexpression of the proto-oncogene bcl-2 using HL-60 cells transduced with the retroviral construct Lbc12SN (19). Western blot analysis of the HL-60-bcl-2 cells induced down the granulocytic pathway with RA or DMSO revealed continuous expression of APE compared with the rapid decrease in expression observed with similar treatment of the parental HL-60 cells (Figs. 1A and 2A). Expression of APE mRNA in the HL-60-bcl-2 cells after exposure to RA or DMSO is similarly maintained, paralleling the protein expression (Fig. 2A). The apparent decrease in APE transcript expression after 6 days of exposure to RA or DMSO is a result of loading differences as evidenced by the GAPDH Northern control blot (Fig. 2). Monocyte/macrophage differentiation induction of HL-60-bcl-2 cells with PMA also revealed continuous expression of APE both at the protein and RNA levels (Fig. 2B). Thus, constitutive expression of bcl-2 inhibits the decrease in APE expression, as well as in programmed cell death normally observed with granulocytic or monocytic/macrophage-induced differentiation of HL-60 cells.

APE Expression and Programmed Cell Death. We examined how the decreased expression of APE in induced HL-60 cells correlated with programmed cell death by quantitating the number of cells undergoing apoptosis in induced HL-60 and HL-60-bcl-2 cells using the in situ TUNEL assay. There is a baseline low incidence (1–2%) of apoptosis in HL-60 cells that does not change after ethanol (Fig. 3) and

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**Fig. 2.** Expression of APE in HL-60-bcl-2 cells (19). A, HL-60-bcl-2 cells at 0, 2, 4, and 6 days after treatment with 10^{-5} μM RA or 1.25% DMSO. Western blot (top) probed with an affinity-purified polyclonal rabbit anti-APE antibody. Northern blots (middle and bottom) probed for the 1.6-kb APE and GAPDH transcripts. B, HL-60-bcl-2 cells treated with 100 nM PMA.

**Fig. 3.** Apoptosis in HL-60 (open boxes) and HL-60-bcl-2 (black boxes) cells after differentiation induction. The percentage of apoptotic cells was determined by counting cells staining positive for the TUNEL reaction (see "Materials and Methods"). Cells were treated with ethanol as control (EtOH), retinoic acid (Retinoic Acid), DMSO, or PMA (see "Materials and Methods"). Bars, SD. * P value < 0.05.
probably reflects the few HL-60 cells that can be observed to spontaneously differentiate in untreated growing cultures. Overexpression of bcl-2 depresses the baseline apoptosis to <1% (Fig. 3). Granulocytic differentiation induction (RA and DMSO), as well as monocyte/macrophage differentiation induction (PMA), resulted in a statistically significant inhibition of apoptosis by day 6 in the HL-60-bcl-2 cells compared with the wild-type HL-60 cells (Fig. 3). The high level expression of bcl-2 in HL-60-bcl-2 cells appears to be responsible for not only the block in apoptosis (19), but also the failure to down-regulate expression of APE with induction of differentiation. Thus, the decrease in expression of APE observed on Northern and Western blots (Fig. 1) in induced HL-60 cells appears to be associated with programmed cell death.

To determine whether the cells undergoing apoptosis were also the cells down-regulating APE, we performed double-labeling experiments on a 50:50 mix of untreated and RA-treated (6 days, 10^{-9} M) HL-60 cells. Cytocentrifuge preparations were stained for (a) fragmented DNA using fluorescein-dUTP in the TUNEL assay and (b) APE using polyclonal rabbit anti-APE with a rhodamine-labeled goat anti-rabbit secondary antibody. Cells undergoing apoptosis fluoresce “red” (Fig. 4B), and cells expressing APE fluoresce “green” (Fig. 4C), and cells expressing APE fluorescence “red” (Fig. 4B). Examination of the cells revealed that cells staining positive with the TUNEL assay, thus undergoing apoptosis, had little or no APE as evidenced by the absence of rhodamine fluorescence (Fig. 4). Conversely, TUNEL-negative cells not undergoing apoptosis stained strongly positive for APE (Fig. 4). Thus, HL-60 cells undergoing apoptosis appear to lose expression of APE.

We further characterized the temporal decline in APE expression to see whether it was an early event in programmed cell death or whether the decline coincided with fragmentation of DNA (TUNEL assay). We assayed APE expression and DNA fragmentation on cytospun preparations of cells after exposure to DMSO. The expression of APE started to fall with fewer rhodamine-positive cells after 2 days of DMSO, whereas statistically significant evidence of fragmented DNA was not apparent until after 4–6 days (Table 1, Fig. 3). A similar pattern was observed, but not as rapid a loss of APE expression, after exposure to RA or PMA (data not shown). Thus, the decrease in APE expression associated with programmed cell death appears to be an early event occurring before the final pathological consequences of DNA fragmentation in apoptosis.

Apoptosis is inhibited in K562 cells by a different mechanism related to bcr-abl expression (21, 22). As a means to rule out the possibility that the lack of down-regulation of APE in bcl-2-transduced cells after the induction of differentiation was not simply due to the overexpression of the bcl-2 gene, but was linked to the process of apoptosis, we induced K562 cells to differentiate with PMA. Expression of the APE protein and transcript remains essentially unchanged over time (data not shown) with exposure to PMA by Western and Northern blot analyses. This result confirms our hypothesis that APE expression is related to programmed cell death via a general apoptosis process and is not due to a specific bcl-2 effect.

Discussion
DNA repair and apoptosis are important processes that protect the organism from the effects of cellular damage. Little is known about the relationship between these two basic cellular processes, but evidence is emerging that they may be linked biologically. There are many examples in which impairment of DNA repair results in an excessive incidence of malignancies and cellular damage (xeroderma pigmentosa,
Fanconi anemia). Likewise, inhibition of apoptosis by overexpression of the proto-oncogene bcl-2 in transgenic mice allows retention of mutated cells, normally destined for apoptosis, and progression to malignancy with a high incidence of cancer in the cells overexpressing bcl-2 (22). Recently, the DNA repair-associated enzyme PARP has been observed to be proteolytically cleaved by the apoptotic cysteine protease ICE (16, 17), which may contribute not only to inactivation of some DNA repair pathways, but also to activation of the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease responsible for nucleosomal cleavage of DNA. The experiments described in this report characterize the relationship of a major DNA repair enzyme, APE, to programmed cell death.

The human myeloid leukemia cell line HL-60 can be induced to terminally differentiate down the granulocytic pathway with retinoic acid, resulting in the development of morphologically and functionally mature granulocytes (20). APE is expressed in the uninduced HL-60 cells but decreases dramatically upon RA induction of granulocytic differentiation. We have noted previously that retinoic acid nuclear receptors are essential to retinoid-induced granulocytic differentiation of HL-60 cells (24, 25). The decreased expression of APE in differentiating HL-60 cells, however, does not appear to be a retinoid-specific effect, because treatment of HL-60 cells with another inducer of granulocytic differentiation, DMSO, also results in decreased APE expression. Additionally, although expression of the APE transcript declines in a similar fashion after RA and DMSO (Fig. 1), expression of the APE protein persists at a lower level after 6 days of RA but becomes undetectable after 6 days of DMSO, suggesting that there may be some differences in APE protein stability or processing in HL-60 cells after RA or DMSO. This may reflect different mechanisms for reduction of APE by RA and DMSO, and perhaps has some significance in that DMSO may prove to be a more effective modulator of APE expression than RA. Moreover, the reduced APE expression does not appear to be confined to granulocytic differentiation, because treatment of HL-60 cells with PMA to induce monocytic/macrophage differentiation also results in a decrease in APE expression.

HL-60 cells induced to differentiate with retinoic acid also undergo programmed cell death or apoptosis similar to normal granulocytes (26). Previously, we transduced HL-60 cells with the proto-oncogene bcl-2 to characterize the relationship of RA-induced HL-60 cell differentiation and apoptosis and found that RA-induced granulocytic differentiation and programmed cell death were distinct pathways that could be separated by suppressing apoptosis with overexpression of bcl-2 (19). Using a similar strategy, we examined APE expression in HL-60-bcl-2 cells induced to differentiate with RA, PMA, and DMSO to determine whether APE expression was associated with differentiation or apoptosis. The bcl-2-transduced HL-60 cells differentiate (RA, DMSO, or PMA) morphologically but do not undergo apoptosis and do not exhibit decreased expression in APE, thus establishing the inverse relationship between programmed cell death and APE expression. On a certain level, this observation makes sense in that if there has been so much cellular damage that apoptosis is initiated, it is probably not worth trying to save the genetic integrity of the cell by attempting to repair damaged DNA so the cell shuts down the DNA repair systems.

BER enzymes have been presumed previously to be general housekeeping genes with ubiquitous expression; however, recent data have shown differential APE expression within tissues and even between neighboring cells of the same tissue (27–29). Although APE levels have been reported to be induced in HT29 colon carcinoma cells during hypoxia (30) and in baby hamster kidney or HeLa cells under low Ca\(^{2+}\) conditions in the culture medium (31), there has only been one previous report of APE levels declining, and that was in porcine epidermal tissue during wound healing (32). In contrast, APE protein levels have been shown to be significantly and dramatically elevated in cervical cancer tissues.\(^4\) This and other data\(^5\) have demonstrated a much higher degree of regulation of the APE gene than previously presumed and warrant additional detailed studies. Furthermore, the results presented here add another level of complexity regarding the numerous roles the APE gene appears to play in mammalian cells. Discrete domains of APE are responsible for specific functions, including a 3′ COOH terminus repair domain essential for BER and a 5′ NH\(_2\) terminus redox domain that maintains critical sulfhydryl groups in AP-1 binding transcription factors in an active reduced state (30, 33). The reduced form of AP-1 binding transcription factors (e.g., Fos and Jun) is essential for activity and initiation of apoptosis (13, 34). More recently, APE has been identified as part of the transcriptional complex involving the Ku antigen to negatively regulate the parathyroid promoter (35), although the exact APE domain important to this interaction is presently unknown. RNase H activity of APE has recently been characterized and appears to be embedded within the 3′ repair domain (36). In the present study, we have identified another potential function for APE by making the association of apoptosis with down-regulation of APE. Although these findings are correlative, the identification of a functional relationship and which APE domains might be involved in programmed cell death awaits additional experiments with retroviral transduction of various APE constructs into cells.

### Materials and Methods

**Cells and Chemicals.** K562 cells were derived from a patient with chronic myelogenous leukemia in blast crisis (37). HL-60 myeloid leuka-


\(^5\) D. A. Williams, K. A. Robertson, and M. R. Kelley, unpublished data.

\(^6\) S. Mitra, personal communication.
nia cells (20) were transduced with the 1.9-kb bcl-2 proto-oncogene as described previously (19). Briefly, the 1.9-kb cDNA of bcl-2 was subcloned into the retroviral vector LXS to produce Lbc2/SN. The ecotropic packaging cell line P3F01 was transduced and used to transduce the amphotropic packaging cell line PA317. High titer clones of PA317 + Lbc2/SN were selected for transduction of HL-60 cells to produce HL-60 + Lbc2/SN. HL-60 cells (HL-60-bcl-2) were grown in suspension culture at 37°C in 5% CO2 humidified incubators in RPMI 1640 with 5% heat-inactivated FCS plus antibiotics. RA (Sigma) stock solutions were prepared fresh under amber light the day of use as a 4-nmol solution in ethanol and diluted into culture to the desired concentration. DMSO (Sigma), a hybridoma grade, was added to cultures to give a concentration of 1.25%. PMA (Sigma) was solubilized in DMSO to give a 1-μmol solution kept at -20°C. PMA was added to cultures to give a final concentration of 100 nM with the concentration of DMSO at 0.1%. HL-60 cells were incubated to differentiate down the granulocytic pathway with retinoic acid (10-7 M) or DMSO (1.25%) and down the monocyte/macrophage pathway with PMA (100 nM) and analyzed after 0.2, 4, or 6 days of exposure. Morphological evaluation of cells was performed by sedimenting 5 x 10^4 cells onto glass slides with a Shandon Cytospin 3 cytocentrifuge (Pittsburgh, PA), followed by Wright's staining. Cells were enumerated using a hemocytometer after trypan blue staining.

**Northern Blotting.** Total RNA was extracted from the cells using RNA STAT (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's directions. Northern blotting and probe labeling were performed as described previously (38), except we used Hyb-9 (PureGene, Research Triangle Park, NC) prehybridization and hybridization solution at 65°C. The blots were hybridized using 32P-labeled APE and GAPDH cDNA clones. GAPDH expression was used to normalize for RNA loading differences. Blots were scanned and normalized using the Sigma Scan software (Jandel Scientific, San Rafael, CA).

**Western Blotting.** Western blots were performed using whole-cell extracts electrophoresed on 12% SDS-PAGE and electroblotted onto 0.2-μm nitrocellulose filters (27). Protein levels in the extracts were quantitated using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), and equal amounts (20 μg) of protein were loaded per lane. The gels were transferred for 1 h onto nitrocellulose and stained with Ponceau red to confirm equal loading between lanes. The filter was incubated in blocking buffer, which contains 5% nonfat milk for 1 h. Rabbit polyclonal antihuman APE (27) diluted to the appropriate concentration was incubated with the filter overnight at 4°C. The filter was washed in TBST and incubated in TBST containing 5% milk and then detected using the Boehringer Mannheim chemiluminescent system (Boehringer Mannheim, Indianapolis, IN). The bands were visualized using autoradiographic film.

**Immunohistochemistry.** Cells (5 x 10^5) were cytospun on glass slides as described above and fixed in 10% formalin for 30 min. Slides were rinsed with TBS, then preblocked with 10% goat IgG (Sigma) in TBS for 30 min. The blocking solution was aspirated, and rabbit polyclonal antihuman APE (1:200; Ref. 27) in 10% goat IgG-TBS was placed on the cells and incubated for 3 h at room temperature in a humidified chamber. Slides were rinsed 3 times with TBS followed by incubation with rhodamine-labeled goat anti-rabbit antibody (Sigma) for 1 h in a humidified chamber. Cells were visualized using a Zeiss fluorescence microscope. The percentage of cells expressing APE was determined by counting fields of cells under phase-contrast microscopy, followed by scoring rhodamine-positive cells under fluorescent microscopy and expressing the value as the percentage of APE-positive cells.

**Apoptosis Assays.** The percentage of cells undergoing apoptosis was determined using DUTP-fluorescein-labeled TUNEL reaction following the manufacturer's instructions (Boehringer Mannheim). Random fields of cells were photographed under phase and fluorescent microscopy (Zeiss). Photomicrographs of phase-contrast fields were collected on a marker board and outlined, followed by scoring fluorescein-positive cells with projection of the same field photographed by fluorescent microscopy. For each sample, 400-1000 cells were counted, and the number of cells undergoing apoptosis was expressed as a percentage.

**Acknowledgments**

We thank David A. Williams for his reading of the manuscript and many helpful suggestions.

**References**


