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Identification of a novel ionizing radiation-induced nuclease, AEN, and its functional characterization in apoptosis

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Abstract

To investigate ionizing radiation response, we screened genes that exhibit higher expression following γ irradiation. We report here the isolation and functional characterization of a novel ionizing radiation-induced gene, AEN. Sequence analysis of AEN revealed exonuclease domain highly similar to that of exonuclease III. The AEN protein revealed DNase activity by cleaving various DNA substrates. Subcellular distribution of AEN exhibited nuclear colocalization with apoptotic nucleases such as CAD and AIF following irradiation. Moreover AEN distribution revealed perinuclear staining pattern which could be seen with other apoptotic nucleases. Irradiation of AEN-expressing cells resulted in synergistic increase of apoptosis whereas AEN deletion mutant in exonuclease domain did not. Our data, thus, suggest that radiation-induced AEN cleaves DNA in concert with other apoptotic nucleases and thereby enhances apoptosis following ionizing irradiation.
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Keywords: Apoptosis enhancing nuclease; DNase; Radiation response; Apoptosis; Ionizing radiation

Exposure of cells to ionizing radiation (IR) results in various DNA lesions including double strand breakages (DSBs). Since formation of DSBs is generally considered lethal event, irradiated cells induce various mechanisms such as cell cycle arrest, DNA damage repair, and cell death to control the damage [1]. If the damage cannot be repaired properly, cell death pathways are activated.

Radiation can produce cell death by either necrosis or apoptosis. Necrosis is a passive process where cells are characterized by a loss of membrane integrity, cell swelling, and random degradation of DNA [2]. In contrast, apoptosis is an active process characterized by programmed cell death in which a series of events is triggered in response to stress conditions including ionizing radiation [3,4]. Apoptosis is characterized by nuclear DNA fragmentation, condensed chromatin, and a fragmented nucleus.

Understanding of cellular radiation response revealed genetic factors that might be responsible for apoptotic cell death upon IR. For example, microarray analysis of IR-responsive genes showed that apoptosis-related Bax and BCL-XL are differentially expressed following IR [5]. In addition to changes in gene expression, activation of various apoptotic nucleases is implicated in the process following IR. For instance, caspase-activated DNase (CAD) and DNaseI are involved in IR-induced apoptosis [6] by generating blunt-end double-strand breaks [7] or DNA strand breaks containing 3′-OH end groups, respectively [8]. Moreover cooperation among nucleases has been observed during apoptosis. CAD is involved in apoptosis in synergy with DNase γ-like endonucleases [9] whereas EndoG is along with ExoIII, and DNaseI [10].

Considering various genetic factors and complexities of the mechanisms that are involved in irradiation-induced apoptosis, there might be still unknown genetic factors that are involved in the process. Therefore, to identify potential factors, we first employed suppressive subtractive

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hybridization method to screen differentially induced genes following IR and then examined their activity in radiation-induced apoptosis.

We report here isolation and characterization of a novel gene, called AEN (for apoptosis enhancing nuclease), and show that AEN protein acts as DNase and thereby affects cellular radiation response by enhancing apoptosis following ionizing irradiation.

Materials and methods

Subtractive hybridization, sequence analysis, and RT-PCR. Suppressive subtractive hybridization analysis was carried out using kits from Clontech (USA) following instruction manual (PCR-select cDNA subtraction (K1804-1) Manual PT1117-1). mRNA for the analysis was isolated at 2 h post-irradiation from either unirradiated or irradiated cells using oligotex bead (Qiagen; Santa Clarita, CA, USA). Isolated clones from subtractive hybridization analysis were sequenced and analyzed using Blast search program from NCBI (National Center for Biotechnology Information, USA). Standard RT-PCR analysis with β-actin as a control was performed to examine transcript level of AEN. The following AEN primers produced 335 bp RT-PCR product: left primer, CGCAAGGCTG TCCCCTTCCA; right primer, CTGCGGGCCTCCTGCTGTTC. β-Actin RT-PCR primers are as follows: left primer, GGGAAATCGTGCG TGACATTAAG; right primer, TGTGT TGG CGTACAG GTCTTTG.

Cell culture and treatment. Lung cancer cell lines, NCI-H460, were obtained from American Type Culture Collection (Manassas, VA). The cell line was grown in RPMI 1640 (Life Technologies, Rockville, MD) containing 5 mM glutamate and 10% fetal bovine serum (Invitrogen), and maintained at 37 °C in an atmosphere of 5% CO_2 and 95% room air. Cell cultures were irradiated using a ^{137}Cs gamma-ray source (Atomic Energy of Canada, Canada) at a dose rate of 3.81 Gy/min Cells were transfected by using Lipofectamine Reagent (Invitrogen, USA) following instruction manual from the company.

Preparation of cell extracts, immunoblotting, and immunoprecipitation. Cells were collected and lysed in lysis buffer (40 mM Tris–Cl, pH 8.0, 0.1% Nonidet P-40, 100 mM NaCl, 2.5 mM Na_3VO_4, and 10 μM leupeptin, aprotinin, and AEBS). After centrifugation (for 15 min at 14,000 rpm), supernatants were collected and used as whole cell extracts for Western blot analysis and immunoprecipitation.

For cell fractionation, harvested cells were incubated for 15 min on ice in buffer A (10 mM Hepes–KOH (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitors). Cells were lysed by adding 0.6% NP-40, centrifuged (1400 rpm for 15 min at 4 °C), and supernatant was collected as cytoplasmic fraction. Nuclei pellets were washed once by buffer A. Nuclear protein was extracted for 30 min at 4 °C in cold extraction buffer (10 mM Hepes–KOH (pH 7.2), 422 mM NaCl, 0.1 mM EGTA, 0.5 mM PMSF, and 5.3% glycerol). The protein extracts were obtained by centrifugation at 1400 rpm for 15 min at 4 °C and used as nuclear extracts. Bradford assay was used for protein quantification.

For Western blot analysis of proteins, samples were electrophoresed at 120 V in 10% SDS–polyacrylamide gels. After transfer to nitrocellulose membranes, membranes were blocked overnight in PBS/Tween solution containing 5% non-fat dried milk, probed for 3 h with primary antibody (1:1000 dilution) and for 1 h with secondary antibody (1:3000 dilution) as specified in the figures. Visualization was achieved using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

For immunoprecipitation, cell extracts were incubated with 2 μg of Flag antibody (Sigma) overnight at 4 °C. Protein A was added and incubated further for 3 h at 4 °C and centrifuged. Precipitated beads were washed three times in PBS and used in nuclease assay.

DNA constructs. The following primers were used to make plasmid constructs. Sequence is from 5′ to 3′. AEN Eco 5′: CGGAATTCATG GTACCCCGGGAGGCCCCT AEN Eco 3′: CGGAATTCTCAATTC CTTCTGTCCTGTGCCT Myc AEN 5′: CCG GAA TTC ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG GTA CCC CGG GAG AEN Bam 3′: CGC GGATCC ATT CCT TCT GTC CTG TGC CTC Flag AEN 5′: CGGAATTC ATG GAC TAC AAG GAC GAC GAT GAC AAG GGA GTA CCC CGG GAG GCC CCT GA AEN flag left primer: CGCGGATCCCACCATGGACTACAAGGACGACGA TGACAAGGGAGTACCCCGGGAGGCCCCTGAGT AEN N-terminal down primer: CCAGAGGCTGCG GGCCTCCTGGTCGACA TAGGTGGTATCCCG AEN C-terminus up primer: CCAGAGGCT GCGGGCCTCCTGGTCGACATAGGTGGTATCCCG AEN C-terminus up primer: CAGACCCGGGATACCACCTATGTCGACCAGGAG GCCCGCAGC AEN right: CGGAATTCTCAATTCCTTCTGTCCT GTGCC AEN flag left primer: CGCGGATCCCACCATGGACTACAA GGACGACGATGACAAGGGAGTACCCCGGGAGGCCCCTGAGT AEN right: CGGAATTCTCAATTCCTTCTGTCCTGTGCC. AEN cDNA was cloned by RT-PCR using AEN Eco 5′and AEN Eco 3′ primer set (refer to the primer sequences). Amplified cDNA was inserted into the *Eco*RI site of pCDNA3 vector to make pcDNA3-AEN.

To make amino-terminus Myc tagged AEN construct, pcDNA3-myc AEN, myc AEN 5′ primer and AEN Eco 3′ primer were used with pcDNA3-AEN as a template in PCR.

To add both myc and GFP tags at N- and C-termini of AEN, respectively, PCR primer myc AEN 5′ primer and AEN Bam 3′ primer were used. PCR products were inserted into the *Eco*RI and *Bam*HI sites of pGFP3 vector to make pMyc-AEN-GFP. pMyc-AEN-GFP plasmid was used to examine the efficiency of transfection or to overexpress and immunoprecipitate AEN protein.

Flag tagged full-length AEN was amplified with Flag AEN 5′/AEN Eco 3′ primers and pcDNA3-AEN as a template in PCR. The resulting fragments were inserted into the *Eco*RI site of pCDNA3 to make pFlag AEN FL.

To make a construct which deleted amino acid 217–275 of AEN (ΔEX AEN), we used a two-step PCR method [11]. Briefly, the first two separate reactions of PCR were carried out to amplify N- and C-terminal portion of the clone using the following primer sets.

First PCR1: AEN flag left primer and AEN N-terminal down primer with pcDNA3-AEN as a template were used to amplify sequences encoding amino acids 1–217.

First PCR2: AEN C-terminus up primer and AEN right with pcDNA3-AEN as a template were used to amplify the C-terminal part. Since the resulting two PCR products possess an overlapping sequence, a mixture of the two can serve as a template for the next round of PCR which would produce a longer PCR product with deletion.

To do that, gel isolated PCR products from the first reactions (reactions 1 and 2) were mixed and run for second PCR with AEN flag left primer and AEN right primer.

The resulting PCR product was digested with *Bam*HI and *Eco*RI, and inserted into the corresponding sites in pCDNA3 to make ΔEX AEN.

PCR cycles for most plasmid construction are as follows: 1 cycle of 94 °C, 30 cycles of {94 °C, 30 s–56 °C, 1 min–72 °C, 1 min}.

Nuclease assay. A nuclease activity of AEN was assayed by following the protocol [12]. Briefly, overexpressed AEN in transfected cells was immunoprecipitated using antibodies to the tag attached to the amino terminus of AEN. The immunoprecipitates dissolved in a reaction buffer (50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 g/ml bovine serum albumin, and 0.2 mM DTT) were added to the reaction mixture, which contained ^{32}P 5′-end labeled DNA substrates and incubated for 20 min at 37 °C. The reactions were terminated by the addition of stop solution (89 mM Tris borate, 2 mM EDTA, pH 8.0, 12% Ficoll, 0.01% bromphenol blue, 0.02% xylene cyanol FF, and 7 M urea) and immediately heated at 98 °C for 3 min Reaction products were analyzed by running on 20% polyacrylamide 1× TBE/7 M urea gel and visualized by autoradiography. DNA substrates were prepared by annealing various single stranded oligonucleotides (see below). The sequence of the single stranded oligonucleotides is as follows.

Oligonucleotide	Sequence (5′ to 3′)
1	TGGTACAGAACATGTCTAAGCATGCTGGG GACTG
2	CAGTCCCCAGCATGCTTAGACATGTTCTGT ACCA
3	TGGTACAGAACATGTCTAAGCATGCGAAG TTTCA
4	TGGTACAGAACATGTCT
5	AACATGTCTAAGCATGC
6	A AGC ATG CTG GGG ACT G

These single stranded oligonucleotides were either used as a single stranded DNA substrate or combined with each other to make double stranded substrates. The combinations are as follows:

Oligonucleotide 1 alone as single stranded substrate (SS), 1 + 2 as blunt ended double stranded (DS), 2 + 4 as 3′ recessed (3′), 2 + 5 as 5′ and 3′ recessed (Mid), 1 + 3 as branched, 2 + 4 + 6 as nicked, and 2 + 6 as 5′ recessed (5′). Abbreviated names are in parentheses. DNA substrates were ^{32}P labeled at the 5′ end by using polynucleotide kinase using standard protocol.

Immunofluorescence microscopy. AEN in the cell was detected by immunofluorescence microscopy. Briefly, NCI-H460 cells grown on glass coverslips in 35 mm dishes were either unirradiated or γ-irradiated with 10 Gy. After 1 or 2 h, cells were fixed with ethanol. Flag M2 (Sigma) primary antibody and Alexa fluor 488 labeled secondary antibody (Molecular probes) were used to detect flag tagged AEN whereas nuclear DNA was stained with propidium iodide (PI). Slides were viewed using confocal laser scanning microscopy (CLSM) (Leica). Images obtained in double or triple excitation mode were processed using SlideBook and Adobe Photoshop.

Apoptosis assay. In order to evaluate the effect of AEN on apoptosis, we measured apoptosis by counting subG1 fraction, AnnexinV/propidium iodide stained cells, and DNA fragmentation as described previously [13,14]. Specific conditions for cell treatment are specified in figures.

Results and discussion

We have conducted subtractive hybridization assay to identify genes that are induced upon exposure of cells to γ-ionizing radiation. From a screen of candidate genes, we identified a clone with high homology to exonucleases. Here we report the isolation and functional characterization of the clone and its implication in ionizing radiation response.

A novel ionizing radiation-induced nuclease

The complementary DNA of the clone encodes a polypeptide of 325 amino acids with unknown function (Fig. 1A) and is identical to the gene in GenBank (Accession No. NM_022767). We named the clone as AEN (apoptosis enhancing nuclease).

We confirmed induction of the gene by ionizing radiation by RT-PCR analysis (Fig. 1B). The level of AEN transcript increased upon gamma-irradiation whereas that of control β-actin gene remained constant (Fig. 1B). We found increased gene expression from samples irradiated with as low as 1 cGy and up to 10 Gy. Unirradiated (0 Gy) sample appeared to have very low or undetectable level of AEN transcript (Fig. 1B). This result indicates that AEN is induced by a wide range of ionizing radiation doses.

We hypothesized that highly expressed genes in irradiated cells might affect cellular radiation response. In order to investigate how AEN affects cellular radiation response, we first analyzed its nucleotide sequence. BLAST analysis and alignment of AEN nucleotide sequence with homologues revealed significant homology to various exonucleases (Fig. 1C). In particular, we noticed the highest homology to exonuclease domain of EXO III, Exonuclease X-T, and DnaQ.

DNase activity of AEN

In order to confirm whether AEN protein has a nuclease activity, we have tried purification of AEN recombinant proteins from bacterial overexpression system. It was not successful, however, due to very low viability of the bacteria harboring the AEN cDNA (data not shown). Therefore, we adopted cell transfection system which allowed production and isolation of enough amount of AEN protein for enzyme assay.

For cellular transfection, we used plasmid constructs that contain either full-length AEN (FL AEN) or deletion in exonuclease domain of AEN (ΔEX AEN) (Fig. 2A). Cells were transfected with the constructs and the expressed proteins were isolated by immunoprecipitation. Addition of the immunoprecipitate from the FL AEN-transfected cells cleaved a 5′-labeled blunt end double-stranded DNA substrate (Fig. 2B) whereas that from the ΔEX AEN or from vector alone transfected-control did not (Fig. 2C). These results indicate that AEN encodes a DNase and that the conserved exonuclease domain is critical for the enzyme activity.

We also tested whether this protein could cleave circular plasmid DNA, which would allow us to determine whether it has endonuclease activity. We were unable to detect the cleavage of circular plasmid DNA substrate indicating that AEN is likely to act as an exonuclease (data not shown).

AEN might be involved in cellular radiation response by digesting DNA substrates generated by cell's exposure to ionizing radiation. For instance, ionizing radiation (IR) generates ~35 double-strand breaks (DSBs) and ~1000 single-strand breaks (SSBs) per diploid G1 cell per Gy as well as numerous base damages [15,16]. Or DNA fragmentation by IR-induced apoptosis might provide DNA substrates for AEN. In order to test the hypothesis that AEN participates in radiation response by cleaving DNA intermediates with DSB or SSB generated as a result of irradiation, we first determined the substrate requirement of AEN.

For an enzyme assay, we used various DNA substrates which have structural features similar to those of DNA intermediates generated following irradiation (Fig. 2B). The FL AEN immunoprecipitate cleaved various substrates with varying efficiency. AEN cleaved efficiently substrates such as single stranded, blunt end double stranded, 3′ recessed, branched DNA whereas it performed less efficiently with nicked, 5′ recessed, and mid-DNA substrates (Fig. 2D).

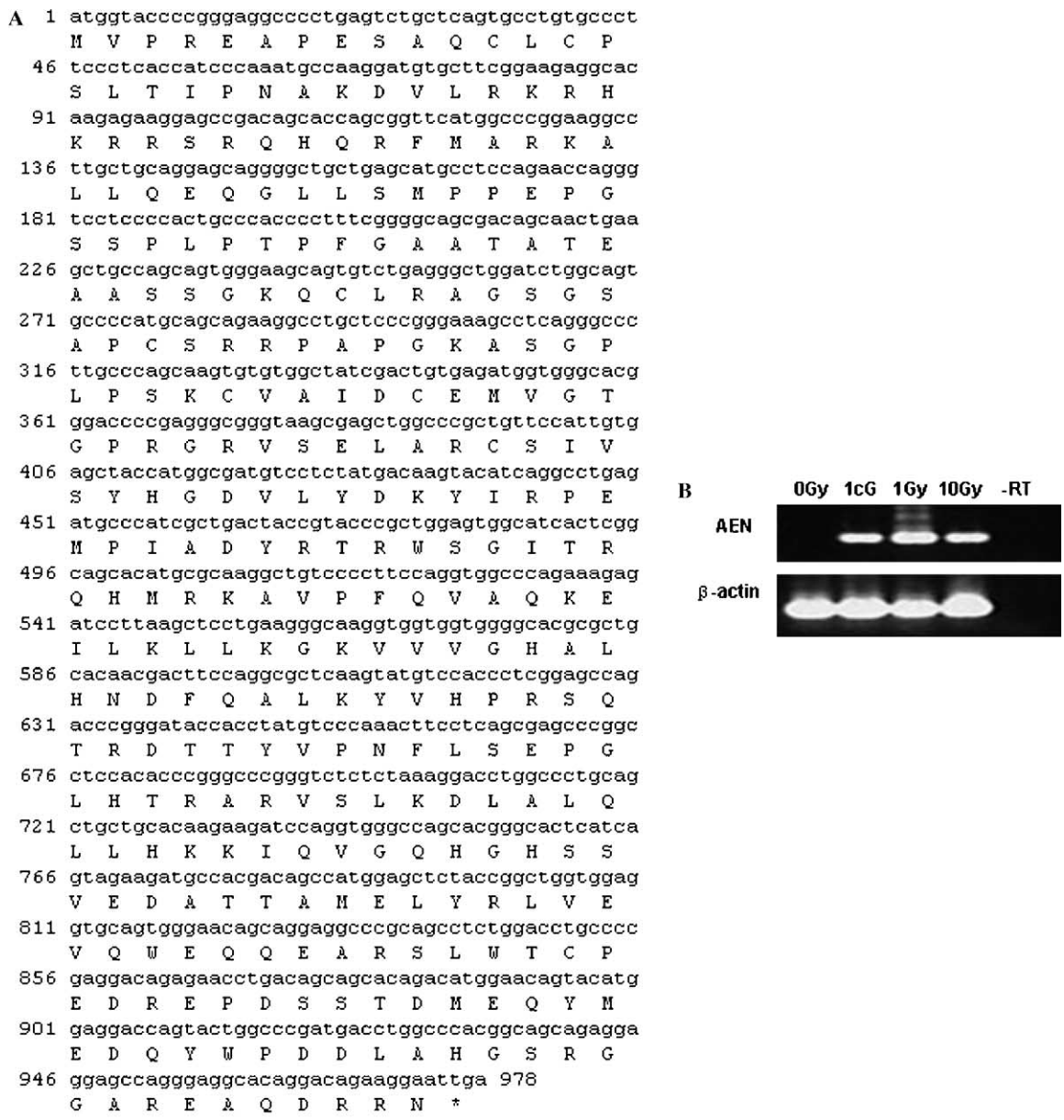

Fig. 1. Ionizing radiation-induced AEN encodes a putative nuclease. (A) Open reading frame (ORF) of AEN. Nucleotide sequence of AEN is identical to the gene in GenBank (Accession No. NM_022767). ORF was generated by using ORF finder program. Number of nucleotide sequence is indicated on the left. (B) RT-PCR analysis of AEN transcript to show its induction following IR. NCI-H460 cells were irradiated with 0, 0.01, 1, and 10 Gy. At 2 h following irradiation, total RNA was isolated and used for RT-PCR. −RT refers to reverse transcription reaction without reverse transcriptase and serves as a negative control. β-Actin is a positive control. (C) Comparison of exonuclease domains with that of AEN. Sequences with homology to that of AEN were searched and aligned by running BLAST program. Number of amino acid of the proteins is indicated.

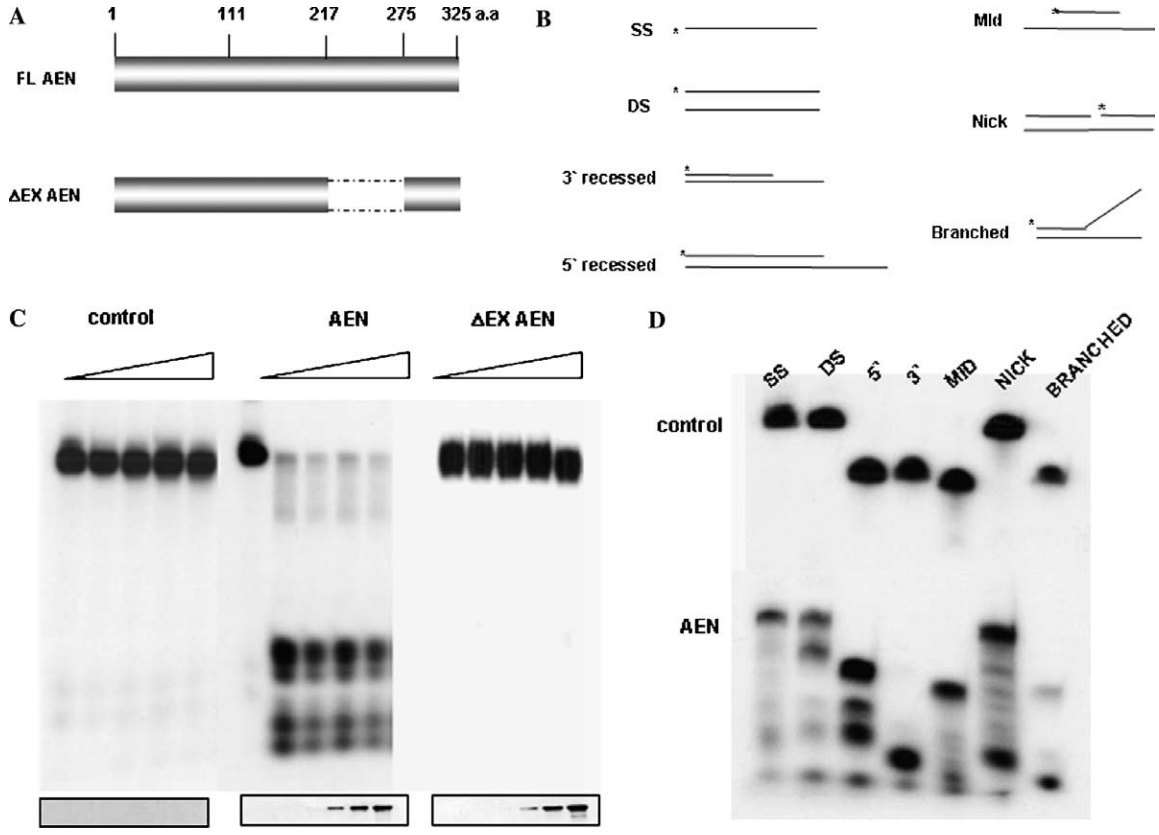

Fig. 2. AEN protein contains nuclease activity. (A) Schematic representation of AEN recombinant proteins used in enzyme assay. Construction of each plasmid is described under Materials and methods. ΔEX AEN deleted part of the conserved exonuclease domain amino acid 217–275. (B) Various DNA substrates used in the enzyme assay. All the substrates were labeled at 5′ ends with p^{32}. Full names and nucleotide sequence of the substrates are found under Materials and methods. (C) Cleavage of DNA substrates by FL AEN but not by ΔEX AEN. The result is a typical nuclease assay out of triplicate experiments. Expressed AEN and ΔEX AEN proteins were isolated by immunoprecipitation using antibody to GFP tag or FLAG tag, respectively. Increasing amount of the proteins was used in enzyme assay. The amount of the proteins used in the enzyme assay was equal as assessed by Western blot analysis and can be seen under the figure of nuclease assay. (D) Substrate requirement of AEN. Various DNA substrates as in (B) were used in nuclease enzyme assay. Control and AEN immunoprecipitaes were isolated and used. Control refers to addition of immunoprecipitates from non-AEN expressing cells, that is, control vector-transfected cells. Upper gel shows control reactions and lower one is for AEN reactions.

Since the substrates that we used in the enzyme assay mimic DNA intermediates generated by IR or by apoptotic DNA cleavage, AEN is likely to further cleave damaged DNA thereby affecting cellular radiation response.

Co-localization of AEN protein with AIF and CAD following IR

DNA degradation is a critical step in apoptosis and requires the involvement of several endo- and exonucleases [17]. For example, caspase-activated DNase (CAD) is activated by γ-irradiation and is involved in the nuclear DNA fragmentation [6]. CAD cleaves double-stranded DNA substrates and generates blunt-end double-strand breaks whereas EndonucleasesG cleaves single strand and double-strand DNA or RNA to produce single strand nicks [7]. Moreover DNaseI has been implicated in radiation induced lymphoid cell death [18] by producing DNA strand breaks containing 3′-OH end groups—the same end groups found in DNA fragmentation products during apoptosis [8].

Since the cleaved DNA products by apoptosis have structural features on which AEN can act, AEN protein might facilitate further cleavage of DNA with SSB or DSB generated by the apoptotic nucleases [17,19,20]. These apoptotic nucleases move into the nucleus or are activated in the nucleus from inactive state and attack chromatin to yield 50–300 kb cleavage products as well as internucleosomal DNA fragmentation [7,21].

In order to test the possibility whether AEN is involved in apoptotic DNA fragmentation with the aforementioned nucleases, we first investigated localization of AEN in the cell (Fig. 3).

Both FL AEN and ΔEX AEN were located mainly in the nucleus as revealed by immunofluorescence microscopy (Fig. 3A) and by cell fractionation (Fig. 3B) suggesting AEN functions in the nucleus. Nuclear localization of AEN was maintained even after γ-irradiation with subtle difference in staining pattern (Fig. 3A). FL AEN displayed pronounced perinuclear staining pattern at 1 h post-irradiation. By 2 h after irradiation, we noticed scattered FL AEN. By contrast, ΔEX AEN did not exhibit such patterns

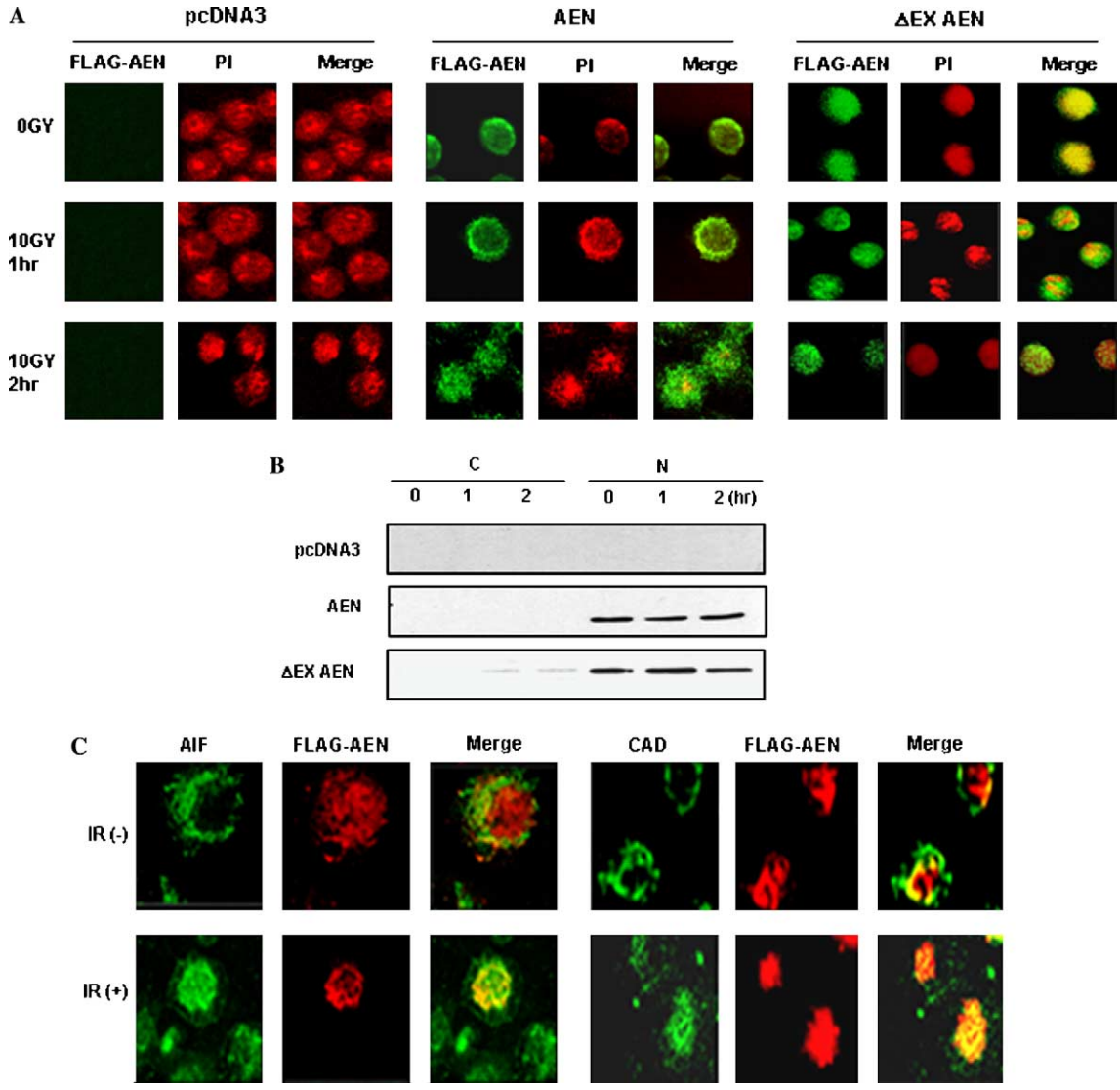

Fig. 3. Subcellular distribution of AEN. (A) Nuclear localization of FLAG-tagged FL AEN and ΔEX AEN as assessed by immunofluorescence microscopy. Cells were transfected with the plasmid constructs as indicated. Cells were either unirradiated or irradiated with 10 Gy and fixed at 1 or 2 h post-irradiation. The proteins were detected by using FLAG tag antibody. (B) Nuclear localization shown by cell fractionation and Western blot analysis. FLAG tag antibody was used in Western blot detection of the proteins. C is for cytoplasmic extracts and N for nuclear extracts. (C) Colocalization of AEN with AIF and CAD following ionizing irradiation. Antibodies to AIF and CAD for immunofluorescence microscopy are from Santa Cruz (USA). Colocalization is revealed by yellow color in merged picture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

after irradiation as observed in FL AEN. ΔEX AEN displayed a rather uniform nucleoplasmic localization (Fig. 3A). The unique perinuclear staining pattern of FL AEN following irradiation might be related to its nuclease function as non-functional ΔEX AEN did not display such pattern. Moreover colocalization of various apoptotic nucleases in apoptotic ring-like perinuclear condensed chromatin supports the notion that the AEN's perinuclear localization is related to its DNase activity [21,22]. Similar localization and a unique perinuclear pattern may suggest the involvement of AEN in apoptotic DNA cleavage along with other apoptotic nucleases.

Therefore, we examined whether AEN colocalizes with apoptotic proteins such as AIF and CAD.

DFF40/CAD is present in the nucleus in complex with its inhibitor DFF45/ICAD as an inactive form [7]. Once activated by cleavage of the inhibitor by caspases, it cleaves the internucleosomal linker regions in chromatin.

AIF is a mitochondrial flavoprotein. Following apoptotic stress it translocates to the nucleus together with Endonuclease G and induces DNA fragmentation resulting in perinuclear consensed chromatin [7,23].

We found that AIF localized in cytoplasm in unirradiated cells (Fig. 3C) as expected. Upon γ-irradiation, AIF moved into the nucleus and colocalized with most of AEN (Fig. 3C). CAD overlapped with AEN in the nucleus in unstressed cells (Fig. 3C) consistent with previous result that CAD is present in the nucleus as an inactive form and

activated following stress. Following γ irradiation, we found more extensive overlapping between AEN and CAD. This result suggests that FL AEN might be involved in apoptotic process upon ionizing irradiation in concert with other apoptotic nucleases such as CAD and AIF/EndoG.

Enhancement of apoptosis by AEN

To confirm whether AEN is involved in apoptosis, we expressed either FL AEN or ΔEX AEN by transfection and measured apoptosis in the presence or absence of γ-irradiation (Figs. 4A–C).

Expression of FL AEN itself without irradiation increased apoptosis more than 2-fold compared to control whereas concurrent irradiation resulted in more apoptosis as measured by subG1 fraction and Annexin V/PI staining (Figs. 4A and B). Expression of ΔAEN, which removed nuclease domain and consequently does not have enzyme activity as a nuclease, had no significant increase in apoptosis although a little increase was observed upon irradiation (Fig. 4A).

Increased subG1 fraction by FL AEN expression alone might be caused by cleavage of limited number of endogenous SSB and DSB present in unstressed cells.

Upon γ irradiation, subG1 fraction increased in control sample as a result of IR-induced DNA lesions. Irradiation of FL AEN-expressing cells resulted in a significantly higher subG1 fraction than the simple sum of radiation and AEN expression alone (Fig. 4A, compare lane 5 with 2 and 4). It suggests cooperation between IR and AEN in apoptotic DNA degradation.

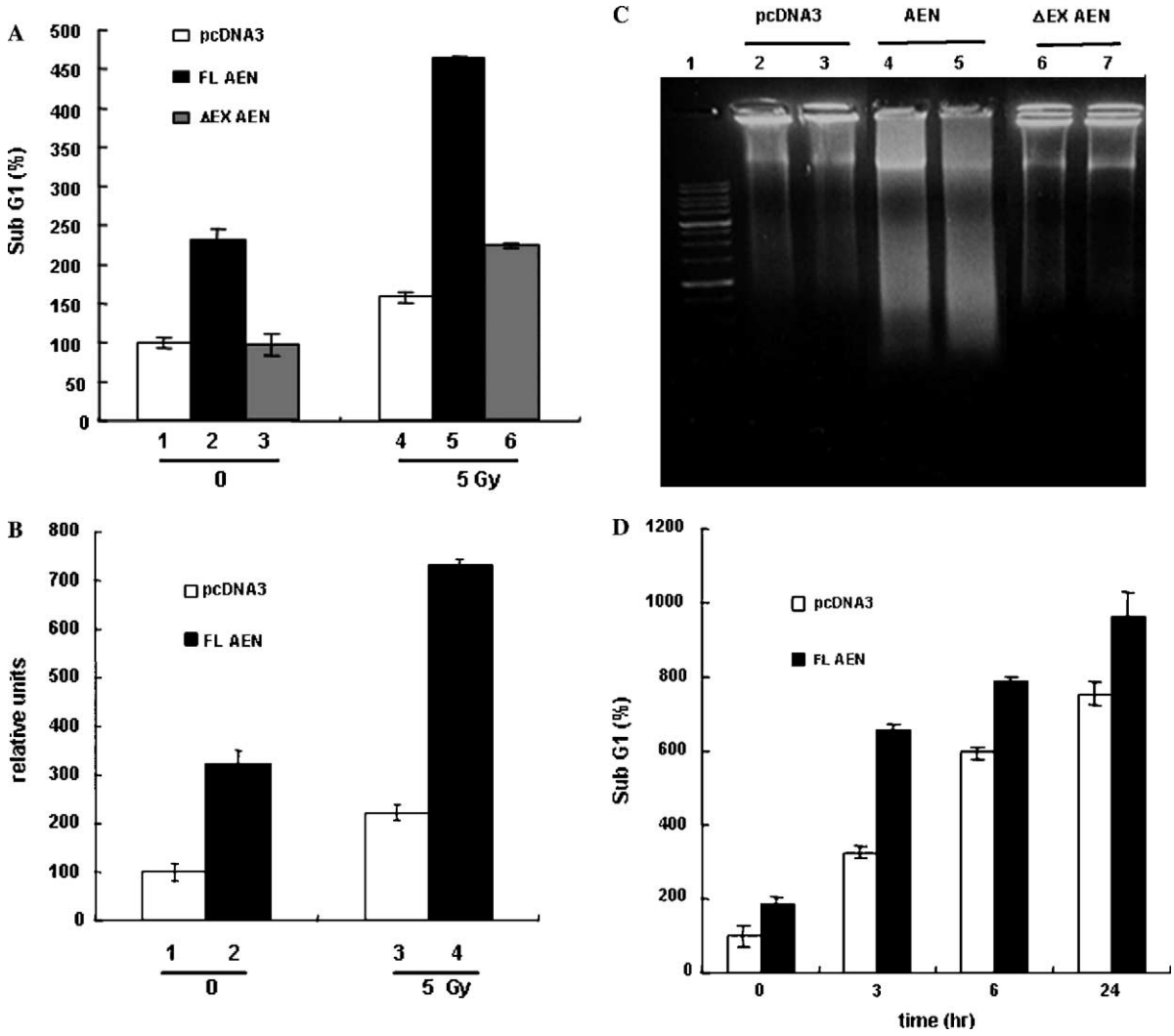

Fig. 4. AEN enhances apoptotic cell death following IR. (A) Increased formation of subG1 fraction by AEN expression. SubG1 fraction was measured by FACS as described under Materials and methods. 1, 2, and 3 are unirradiated whereas 4, 5, and 6 are 5 Gy irradiated. (B) Increased apoptosis by AEN as assessed by Annexin V/PI staining. 1 and 2 were unirradiated samples whereas 3 and 4 were irradiated with 5 Gy. (C) DNA fragmentation assay. Lane 1 is a molecular marker, 1 kb ladder. Lanes 2, 4, and 6 are unirradiated samples. Lanes 3, 5, and 7 are 10 Gy irradiated and harvested at 24 h post-irradiation. Plasmids used for cellular transfection are indicated on top of the lanes. This is a typical result out of four independent experiments. (D) Increased subG1 fraction by AEN following straurosporine treatment. One micromolar of staurosporine was used to treat either vector alone or FL AEN transfected cells. Samples were harvested at specified time points and subG1 fraction was measured. Bars on the graphs represent standard deviation from three separate measurements (A,B,D). Unirradiated control samples were set as 100% to compare with other samples (A,B,D).

Further cleavage of IR-induced single (SSB) and double-strand breakages (DSB) might account for the cooperation. We observed a similar increase in apoptosis with FL AEN when measured cells were stained with AnnexinV and PI (Fig. 4B). Enhanced apoptosis by FL AEN was also observed with DNA fragmentation assay (Fig. 4C). While pCDNA vector alone or ΔEX AEN transfection did not result in significant DNA degradation, FL AEN alone or FL AEN together with irradiation caused significant DNA degradation consistent with results from other assays (Fig. 4C).

In order to determine whether the apoptosis enhancing effect of FL AEN could be induced by different stimulus, we induced apoptosis with staurosporine and found a similar apoptosis-enhancing activity of AEN (Fig. 4D). This result suggests that AEN is likely to enhance apoptosis not only by ionizing radiation but also by other stimuli.

Results of increased subG1 fraction and apoptosis suggest cooperative nature of AEN and apoptotic nucleases. Such cooperation between nucleases has been observed during apoptosis. For example, CAD is involved in apoptosis in association with AIF [9], cyclophilin B [24] or in synergy with DNase γ-like endonucleases [25]. Similarly, EndoG is involved in apoptotic DNA processing along with ExoIII, and DNaseI [10]. The presence in AEN of exonuclease domain homologous to that of ExoIII supports our notion that apoptotic nucleases might cooperate with AEN as EndoD does with ExoIII. Unlike other apoptotic nucleases, which are generally activated or move into the nucleus for activity following stimuli, AEN is unique in that it is transcriptionally induced by ionizing radiation and cleaves DNA together with other apoptotic nucleases to enhance apoptosis.

Our results do not provide explanation for regulatory mechanism of how AEN expression is tightly regulated by the presence or absence of IR. Moreover the nature of AEN cooperation with other apoptotic nucleases is still open for further investigation. Our findings, however, reveal a role of AEN as a DNase in IR-induced apoptosis and suggest requirement of tight regulation in cellular radiation response.

Taken together with these results, we conclude that AEN might contribute to radiation response during ionizing radiation induced-apoptosis by cooperatively cleaving DNA substrates along with other apoptotic nucleases and enhance apoptosis. Further analysis as to how AEN gene expression is induced by irradiation and how molecular and functional interaction is achieved for the cooperative nuclease activity will be necessary.

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