Regulation of Apoptotic Endonucleases by EndoG

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Cells contain several apoptotic endonucleases, which appear to act simultaneously before and after cell death by destroying the host cell DNA. It is largely unknown how the endonucleases are being induced and whether they can regulate each other. This study was performed to determine whether apoptotic mitochondrial endonuclease G (EndoG) can regulate expression of other apoptotic endonucleases. The study showed that overexpression of mature EndoG in kidney tubular epithelial NRK-52E cells can increase expression of caspase-activated DNase (CAD) and four endonucleases that belong to DNase I group including DNase I, DNase X, DNase IL2, and DNase γ , but not endonucleases of the DNase 2 group. The induction of DNase I-type endonucleases was associated with DNA degradation in promoter/exon 1 regions of the endonuclease genes. These results together with findings on colocalization of immunostained endonucleases and TUNEL suggest that DNA fragmentation after EndoG overexpression was caused by DNase I endonucleases and CAD in addition to EndoG itself. Overall, these data provide first evidence for the existence of the integral network of apoptotic endonucleases regulated by EndoG.

Introduction

DNA ORIGINATING FROM DEAD CELLS can be cytotoxic to other living cells. Consumption of DNA fragments from dead cells can activate DNA damage pathways in new host cells, and it also can lead to recombinations and mutations (Ermakov *et al.*, 2008). The body reacts to circulating DNA by producing anti-DNA antibodies and expressing a lupus-like syndrome (Napirei *et al.*, 2000). In addition, cells contain several DNA endonucleases, which localize tissue injury and limit the spread of DNA after cell death. Although endonucleases have been accepted as apoptotic enzymes, they are also actively involved in other types of cell death. Apoptotic endonucleases are inhibited and compartmentalized outside of the nucleus, thereby avoiding contact with DNA in healthy cells. However, in response to stress, endonucleases become activated and are released from cellular compartments to reach nuclear DNA, thus completely destroying it within a few hours after cell injury or cell death. Therefore, inactivation of endonucleases before cell/tissue injury is usually cytoprotective (Basnakian *et al.*, 2005; Napirei *et al.*, 2006; Apostolov *et al.*, 2007, 2009, 2011).

There are nine apoptotic endonucleases. Four of them belong to the deoxyribonuclease 1 (DNase I) group, which, in addition to DNase I itself, includes its three homologs:

DNase I-like 1 (DNase X), DNase I-like 2, and DNase I-like 3 (DNase γ) (Evans and Aguilera, 2003; Shiokawa and Tanuma, 2004; Roszer *et al.*, 2006). Three other endonucleases of the DNase 2 group include DNase 2 α , DNase 2 β , and L-DNase 2 (Evans and Aguilera, 2003; Chahory *et al.*, 2004). Another endonuclease known as caspase-activated DNase (CAD) has been tightly linked to apoptosis (Nagata, 2000). The last member of the group, endonuclease G (EndoG), is a nuclease capable of destroying both DNA and RNA, which makes it unique among the endonucleases.

EndoG is the key modulator of caspase-independent apoptosis (Li *et al.*, 2001; van Loo *et al.*, 2001). This enzyme was first described by Ruiz-Carrillo and Renaud (1987) as an endonuclease with a unique site-selectivity and the ability to perform initial attacking of poly(dG) sequences in double- or single-stranded DNA. The EndoG gene in rodents and humans is a single copy gene, which consists of three exons (Prats *et al.*, 1997). The level of EndoG expression widely varies between tissues. The kidney is one of the primary organs of EndoG expression (Ruiz-Carrillo and Renaud, 1987; Prats *et al.*, 1997). EndoG appears to be the most active endonuclease in cancer cells (Basnakian *et al.*, 2006; Hamada *et al.*, 2006; Wang *et al.*, 2008; Schneiders *et al.*, 2009; Mercer *et al.*, 2010).

Mammalian EndoG is synthesized in the cytoplasm as an inactive 32-kDa propeptide (Ikeda and Kawasaki, 2001) and

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imported into mitochondria due to its amino-terminal mitochondrion-targeting sequence (Ruiz-Carrillo and Renaud, 1987; Cote and Ruiz-Carrillo, 1993). In mitochondria, the enzyme was localized in the intermembrane space (Ohsato *et al.*, 2002). The signal peptide is cleaved off upon entering mitochondria. The mature active 27-kDa EndoG can be released from mitochondria, translocated to the nuclei and cleave DNA without sequence specificity (Li *et al.*, 2001). The EndoG protein appears to be very abundant in cells. Unlike other apoptotic endonucleases, EndoG can often be seen in the nucleus during DNA fragmentation visualized by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay (Lee *et al.*, 2005). This is quite surprising because EndoG is cytotoxic (Schafer *et al.*, 2004), and its presence in the nucleus should kill the host cell instantly.

Another unique feature of EndoG is that it is regulated by an enzyme of the same family, namely DNase I (Yin *et al.*, 2007). The presence of DNase I is necessary for EndoG induction during kidney injury by cisplatin. DNase I was also shown to stimulate the ability of EndoG to generate double-strand DNA breaks at physiological ionic strengths, suggesting that these enzymes work in concert in apoptotic cells to ensure efficient DNA breakdown (Widlak *et al.*, 2001). Consequently, the crosstalk between DNase I and EndoG appears to represent a new regulatory pathway of apoptosis. In this regard, the role of EndoG in regulating DNase I and/or other apoptotic endonucleases is very important, and has not been previously studied. Therefore, in this study, we examined the consequences of EndoG overexpression in kidney tubular epithelial cells, which are notorious for their expression of several endonucleases (Yin *et al.*, 2007). Our results revealed the regulatory role EndoG is playing toward other apoptotic endonucleases.

Materials and Methods

EndoG cloning and recombinant EndoG purification

Rat *EndoG* gene was inserted in pECFP-N1 vector (Clontech, Mountain View, CA) upstream of the ECFP gene. The recombinant EndoG (recEndoG) was cloned as a histidine-tagged mature mouse EndoG gene sequence (A45 through K294 positions) in pET29b plasmid. It was expressed in *Escherichia coli* BL21 (DE3) and purified using a HisTrap FF crude column (Amersham Bioscience, Piscataway, NJ) followed by PD-10 column desalting.

Cell culture

Normal rat tubular epithelial NRK-52E cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (ATCC) supplemented with 5% fetal bovine sera at 5% CO₂/95% air in a humidified atmosphere at 37°C and used within a day after confluence. The cells were transfected with plasmids expressing rat EndoG-cyan fluorescent protein (CFP), fusion protein or CFP alone (as control) using Lipofectamine-2000 (Invitrogen, Grand Island, NY) for up to 24 h. The expression efficiency was usually 90–100% cells as determined by fluorescent microscopy.

Flow cytometry

Cells were trypsinized, resuspended in phosphate-buffered saline (PBS), and treated with fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI) from the FITC Annexin V/Dead Cell Apoptosis kit (Invitrogen) according to the manufacturer's recommendations. Counting 30,000 cells at each point was performed in a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) using Annexin V/PI dot plot with quadrant statistics.

TABLE 1. PRIMERS USED FOR REAL-TIME RT-PCR AND PCR (ALL GENES ORIGINATE FROM *RATTUS NORVEGICUS* UNLESS OTHERWISE INDICATED)

Target	Sense (5'-3')	Antisense (5'-3')	Product size, bp	Annealing temp., °C
Real-time RT-PCR primers				
EndoG	GCATGCCTGGAACAACCTTGAGAA	TGCCTCCAGGATCAACACCTTGAA	193	64
DNase X	GCATACATAGCAATGCGTGCCCTT	TCAGTGTCACTCTGTGGGCATTGA	109	64
DNase I-like 2	AGACCTAAGTGCAGTGTCTTGTCT	ATACTGGTCACGTCCAAGTGGCTT	100	65
DNase γ	TCGGCCAGCTTTATAAGTCAGCGA	TGGGTACAGGGACATCTTTGCACT	85	65
DNase 2 α	AGTTTCCAGGGATTGGCAAGCAAC	ATTTGGCAAAGCTCTGGAAGGTGG	195	64
DNase 2 β	AAGTGGCCTCTGCATCACTCTCAA	TGTGGATGAGTTCACGCGAAAGA	125	65
L-DNase 2	TGCCCCAAGACATTGAGGATGAGT	CACGTTTGGTCCATTCACGCAGTT	90	64
CAD	AGGACCCATCCTGGTTTGAAGGTT	TGTACTGCACGGACTTGAGCTTGT	198	64
Mouse EndoG	GAATGCCTGGAACAACCTTGAGAG	TTCAAGGTGCTGATCCTGGAGGCA	193	65
Mouse β -actin	TGAGAGGGAAATCGTGCGTGACAT	AACGCTCATTGCCGATAGTGATGA	152	65
18s	GGATCCATTGGAGGGCAAGT	ACGAGCTTTTAACTGCAGCAA	91	64
PCR primers				
EndoG	TACGGGCCGGTTTGACCCTGG	TGGGAACATATGTATGGTGGGGACC	3039	56
DNase X	TTGGCTGAGGCAAGCGTCGG	GCCAGGTCCTCATCAGGCCG	2949	59
DNase I-like 2	GCTTTTGCCCTGATCCTCAGTTAATGG	TGAACATTAAGATCAAGCTCTGCAGCC	2473	59
DNase γ	AAAGTGGCCAGAGGGATCCAGTAATT	CCTGGGACTATGCCCTTTTGAGAATCCA	2903	60
DNase 2 α	AGACCCAGGTCCCTGAAGTCTTTAG	TTCCCTTGAGACAGTGTCTGTGTTG	2586	56.5
DNase 2 β	GCATCAGATGACATGAGACTCAGCCTC	TCTTCAGCAACACCCCTCAGCTATTTTC	2997	58
L-DNase 2	CAGCTAAGCAAGAGGTAAGGTCCT	TTCTGGGATCCAAACTCAGCTTCTC	3064	57.5
CAD	TGGGCTTGAATGGGCGCTGG	TAGACGTGTACCGCCCGCCA	2510	59
GAPDH	CTCTCTGCTCCTCCCTGTTCTAGAGACA	ATGGCATGGACTGTGGTCTAGAAAGCAT	3008	61

CAD, caspase-activated DNase; EndoG, endonuclease G; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction.

Isolation of cell nuclei and *in vitro* transcription

NRK-52E cells, rat kidney or brain nuclei were isolated as previously described (Pravdenkova *et al.*, 1996). The nuclei were resuspended in 50 mM Tris-HCl, pH 7.9, 0.25 M sucrose, 1 mM CaCl₂, and 5 mM 2-mercaptoethanol and exposed with varying concentrations of recEndoG or bleomycin (Sigma-Aldrich, St. Louis, MO) for 1 h. Total RNA was extracted and real-time RT-PCR was performed as described below. For *in vitro* transcription, recEndoG or bleomycin were exposed to mouse mature *EndoG* gene in pET29b vector (Novagen, Madison, WI) or linearized pTRI- β -actin-mouse plasmid (Ambion, Austin, TX), and Maxiscript T7 kit (Ambion) was used according to the manufacturer's protocol.

RNA and DNA extraction

Total RNA from nuclei was extracted using RNeasy Mini kit from Qiagen (Valencia, CA). DNA was extracted from cultured NRK-52E cells using DNeasy blood and tissue kit

(Qiagen). DNA or RNA electrophoresis in agarose gel was performed as described previously (Basnakanian *et al.*, 2005).

PCR and real-time RT-PCR

Our protocol described previously was followed (Basnakanian *et al.*, 2006). Total RNA (5 μ g) was reverse transcribed in a 50- μ L reaction followed by real-time RT-PCR in a 25- μ L reaction using SmartCycler (Cepheid, Sunnyvale, CA). The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) according to manufacturer's recommendations. Primers are shown in Table 1. Two-temperature annealing/extension cycles were used. The fluorescence was measured at the end of the annealing step. The melting curve analyses were performed at the end of the reaction (after the 45th cycle) between 60°C and 95°C to assess the quality of the final PCR products. The threshold cycle values, C(t) were calculated by fixing the basal fluorescence at 15 units. The standard curve of the reaction effectiveness was performed using the serially diluted

mixture (1:40, 1:80, 1:160, and 1:320) of all experimental cDNA samples in duplicate for each endonuclease and 18S RNA separately. Calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (Version 2.0d). Data were presented as ratios of endonuclease/18S mRNA. PCR products were separated in 1% agarose gel, stained with ethidium bromide, and photographed under UV light in a Bio-Rad imaging system (Hercules, CA).

Western blotting

Protein content was measured using the Bradford protein assay (Pierce Biotechnology, Rockford, IL). Bovine serum albumin (BSA) was used as the standard. Proteins were separated in 11.5% gel according to Laemmli's procedure (Laemmli, 1970). The total protein extract from cells (10–100 µg) was dissolved in 50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 2 mM EDTA, 1% 2-mercaptoethanol, 7.5% glycerol, and denatured by heating at 100°C for 10 min. Electrophoresis was performed at 100 V for 2 h. Proteins were transferred onto the nitrocellulose membrane in NuPAGE® transferring buffer (Invitrogen) at 40 V for 3 h. The membranes were stained with Ponceau S (Sigma-Aldrich) to control equal protein load as described elsewhere

(Hofnagel *et al.*, 2004). After soaking in the blocking solution overnight at 4°C, the membrane was incubated with polyclonal anti-DNase I antibody (Rockland, Gilbertsville, PA) diluted 1:1000, polyclonal anti-CAD antibody (Abbotec, San Diego, CA) diluted 1:500, polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (Abcam, Cambridge, MA) antibody diluted 1:1000, or polyclonal anti-DNase X, anti-DNase I-like 2, or anti-DNase γ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, then washed in Tris-buffered saline, pH 7.6. Primary antibodies, anti-DNase I, anti-CAD, and anti-GAPDH were detected with anti-rabbit IgG-horseradish peroxidase (HRP) while anti-DNase X, anti-DNase I-like 2, and anti-DNase γ were detected with donkey anti-goat IgG-HRP using SuperSignal chemiluminescent kit (Pierce Biotechnology).

Immunocytochemistry and TUNEL assay

Cells were fixed with 5% formalin and the immunostaining was performed as described previously (Apostolov *et al.*, 2011). The cells were immunostained with rabbit anti-EndoG at 1:200 dilution (Millipore, Billerica, MA), goat anti-DNase γ at 1:200 dilution (Santa Cruz Biotechnology), or anti-CAD at 1:100 dilution (Abbotec) in dilution buffer (0.5% BSA, 0.05% Tween-20, PBS). The primary antibodies were detected with 1:400

FIG. 2. EndoG over-expression causes induction of DNase I-like endonucleases. NRK-52E cells were transfected with pEndoG-ECFP or pECFP. mRNA expressions of endonucleases were measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR) ($n=4$, $*p \leq 0.05$ vs. pECFP-transfected cells). (Labels A–I are explained in the text.)

diluted anti-rabbit IgG-AlexaFluor 647 conjugates (Invitrogen) or anti-goat IgG-AlexaFluor 594. Control sections were performed by substituting the primary antibody with the dilution buffer. Cells were then subjected to TUNEL staining using the *in situ* Cell Death Detection kit from Roche Diagnostics (Indianapolis, IN). After washing and DNA counterstaining with 4',6-diamidino-2-phenylindole (DAPI), cells were mounted under cover slips with Prolong[®] Antifade kit (Invitrogen) and acquired using the Olympus IX-51 inverted microscope (Olympus America, Center Valley, PA) equipped with a Hamamatsu ORCA-ER monochrome camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan).

Image analysis

Image analysis was performed using the SlideBook v4.2 software. For quantification, 10 independent fields of view were collected per each experimental subset, and the mean optical density was recorded for the channels used. Red color fluorescence in images was used to mask DNase γ and CAD for the entire cell population. Purple was used to mask EndoG. Blue and green were used to mask nuclei stained with DAPI and DNA fragmentation detected by TUNEL, respectively. Colocalization and pixel-to-pixel correlation analysis was performed using Pearson and Manders algo-

rithm available within SlideBook software (Manders *et al.*, 1993). R coefficients for both correlations were calculated with 10 different images per experimental subset. In the analyses, brightnesses of all stained endonucleases prevailed over TUNEL brightness.

Statistics

Statistical analysis was performed with Student's *t*-test. Results were expressed as mean $\pm$ standard error of mean. $p < 0.05$ was considered significant. Bonferroni modification of Student's *t*-test was used for calculating *p* in experiments with recEndoG and bleomycin treatment of nuclei.

Results

EndoG overexpression causes cell death

We have previously shown that *in vitro* cisplatin exposure of kidney tubular epithelial cells results in a mild induction of EndoG ($\sim 10\%$ of total) associated with a significant redistribution of EndoG to the cytoplasm and nucleus. This suggests that cisplatin toxicity increases the maturation of EndoG (removal of the signal peptide) (Yin *et al.*, 2007). To model the elevation of mature EndoG, in the first series of experiments, we have tested transfection of NRK-52E

FIG. 3. Induction of DNase I-like endonucleases in EndoG-overexpressing cells. **(A)** Western blotting for DNase I, DNase X (both at 8 h), DNase 1-like 2, DNase γ (both at 12 h), caspase-activated DNase (CAD) (at 8 h post-transfection), and GAPDH (as a loading control) in NRK-52E cells overexpressing EndoG. **(B–F)** Quantification of DNase I, DNase X, DNase 1-like 2, DNase γ , and CAD by densitometric analysis of band mean optical densities ($n = 4$, $*p \leq 0.05$ vs. pECFP transfected NRK-52E cells). CAD, caspase-activated DNase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells with pEndoG-ECFP or “empty” pECFP, to determine whether mature EndoG cytotoxicity was induced within the time frame of the experiment. The results show that while some cell detachment was observed starting 16 h after transfection with pEndoG-ECFP due to EndoG cytotoxicity, most of the cells remained alive and attached up to 24 h after transfection (Fig. 1A). The transfection reached a plateau in 8 h. It was close to 100% of cells as assessed by cyan fluorescence under the microscope for both plasmids (Fig. 1B, C). Annexin V/PI staining followed by FACS analysis showed that the cell death was more due to apoptosis rather than to necrosis (Fig. 1D–G).

EndoG overexpression causes induction of DNase I-like endonucleases

To determine whether EndoG overexpression can affect expression of other endonucleases, total RNA was isolated from NRK-52E cells transfected with pEndoG-ECFP or pECFP and real-time RT-PCR was performed for all known apoptotic endonucleases including EndoG itself every 4 h up to 24 h after transfection. No difference in 18S expression between pEndoG-ECFP and pECFP transfected samples was observed. Elevation of EndoG expression occurred within first 12 h after transfection, followed by a sharp decrease (Fig. 2A). The latter was associated with the cytotoxicity (detachment of cells) described above. Yet, EndoG mRNA expression remained higher than in pECFP-transfected control cells for up to 24 h. Interestingly, the entire DNase I group of endonucleases was found to be induced during the same time period, including DNase I, DNase X, DNase I-like 2, and DNase γ (Fig. 2B–E). None of the DNase 2 group of endonucleases was significantly induced by EndoG overexpression (Fig. 2F–H). Similar to DNase I group, CAD was significantly induced at 20–24 h above the control pECFP (Fig. 2I). Western blotting followed by quantitative densitometric analysis confirmed that DNase I and DNase I-like endonucleases were induced by 8–12 h after transfection thus coinciding with the overexpression of EndoG (Fig. 3). CAD was induced within 24 h.

EndoG overexpression induces DNA fragmentation

We then tested whether EndoG induces DNA fragmentation, and if DNA is indeed degraded by EndoG or another EndoG-induced endonuclease. Quantitative TUNEL assay was used to measure DNA fragmentation. Quantitative immunocytochemistry was used to measure endonucleases expression. In addition to EndoG, two representative endonucleases were chosen, namely DNase γ , as an example of DNase I-like endonuclease, and CAD. Results show that EndoG and DNase γ were simultaneously induced at 12 h post-transfection, while CAD and TUNEL were maximal at 24 h (Figs. 4 and 5). After localizing of endonucleases by immunohistochemistry and DNA breaks by TUNEL, a potential relation between these events was assessed by Person's and Manders' colocalization algorithms. The results shown in Table 2 suggested that at 12 h, DNase γ and CAD had a higher degree of association with TUNEL than EndoG ($p=0.45$ – 0.50 vs. $p=0.25$). However, at 24 h, the association between EndoG and TUNEL became stronger while correlation

FIG. 4. EndoG overexpression induces DNase γ protein and DNA fragmentation. (A) EndoG, DNase γ immunostaining, TUNEL and DAPI staining of pEndoG-ECFP and pECFP transfected NRK-52E cells with 12-h intervals (purple, EndoG, red, DNase gamma or CAD, green, TUNEL and blue, DAPI). (B, C) Quantification of EndoG and DNase γ ; mean optical density in transfected NRK-52E cells ($n=4$, $*p \leq 0.05$ vs. pECFP-transfected cells). (D) Quantification of TUNEL-positive transfected NRK-52E cells.

between DNase γ and TUNEL plunged down ($p=0.56$ – 0.68 vs. $p=0.29$). The association between TUNEL and CAD was not different from that of controls and remained unchanged at both time points. These data suggest that in the conditions leading to overexpression of EndoG, the majority of early DNA damage events is in fact not actually related to EndoG, while delayed DNA damage is likely to occur with the involvement of EndoG. To measure the degree of localization overlap between endonucleases and TUNEL-positive DNA damage, Manders' correlation was carried out. Our data confirmed that at 12 h, the majority of TUNEL (>95%) was overlapping with DNase γ and CAD. At a later point in time, the association between CAD and TUNEL diminished, while the association between EndoG and TUNEL appeared (Fig. 6). *Vice versa* analysis demonstrated that only about half of the expressed endonuclease pool was topologically associated with TUNEL, while EndoG appears to be the only

FIG. 5. EndoG overexpression induces CAD protein and DNA fragmentation. (A) EndoG, CAD immunostaining, TUNEL and DAPI assay for pEndoG-ECFP and pECFP transfected NRK-52E cells with 12-h intervals (purple, EndoG, red, DNase gamma or CAD, green, TUNEL and blue, DAPI). (B, C) Quantification of EndoG and CAD; mean optical density in transfected NRK-52E ($n=4$, $*p \leq 0.05$ vs. pECFP transfected NRK-52E cells). (D) Quantification of TUNEL-positive transfected NRK-52E cells.

endonuclease, 80% of which was associated with TUNEL at the 24-h time point. These data confirm that in the EndoG-overexpression cellular microenvironment, DNA damage is likely associated, in addition to EndoG, with other nucleases as well.

TABLE 2. PEARSON'S PIXEL-TO-PIXEL CORRELATION BETWEEN EXPRESSION OF ENDONUCLEASE AND DNA BREAKS MEASURED BY TUNEL ASSAY

Transfection	EndoG	DNase γ	CAD
Control	0.47	0.34	0.55
12 h			
pECFP	0.43	0.29	0.31
pEndoG-ECFP	0.25	0.45	0.50
24 h			
pECFP	0.44	0.28	0.38
pEndoG-ECFP	0.68	0.29	0.56

FIG. 6. Colocalization of endonucleases and TUNEL. (A) Manders' colocalization index for TUNEL versus endonucleases. (B) Manders' colocalization index for total cellular endonucleases versus TUNEL.

EndoG overexpression induces DNA breaks in promoter/exon 1 regions of DNase I-like endonucleases genes

In the next set of experiments, we determined whether EndoG overexpression would cause degradation of endonuclease genes, and whether DNA degradation would coincide with the induction of other endonuclease mRNAs. NRK-52E cells were transfected with pEndoG-ECFP. DNA was isolated every 4 h for up to 24 h and PCR was performed at every time point. PCR for endonuclease genes was performed in the promoter/exon 1 areas, which are likely to be involved in regulating of genes. To allow for comparison, PCR was designed to generate products of the same length of ~ 3 kb. In good correlation with the previous observations, the results showed some DNA degradation (one or more double-strand DNA breaks per 3 kb) in the promoter/exon 1 areas in the majority of endonuclease genes (Fig. 7). It occurred at 16–24 h after transfection in DNase X gene, and at 20–24 h in DNase I, DNase I-like 2, and DNase γ genes. Much weaker degradation occurred in DNase 2 β and CAD genes, while other genes showed no degradation. These results suggest that DNA degradation correlates with induction of endonucleases. Some RNA degradation was also observed 16–24 h after EndoG was overexpressed (data not shown).

Recombinant EndoG and bleomycin induce DNase I and DNase X transcription in isolated rat brain cell nuclei

To determine whether EndoG can directly, without protein synthesis, induce expression of other endonucleases by acting on chromatin, we used rat brain nuclei, which are known to have very low endogenous endonuclease activity (Pravdenkova *et al.*, 1996). The cell nuclei were isolated and exposed with varying concentrations of recEndoG for 60 min. Total RNA was extracted and real-time RT-PCR was performed for DNase I and DNase X (the two endonucleases prominently induced by overexpression of EndoG in NRK-52E cells). Both mRNAs were significantly induced by EndoG in the range 1–100 ng/mL DNase I and 0.1–100 ng/mL DNase X (Fig. 8A, B). In another approach, to

FIG. 7. EndoG overexpression induces breaks in promoter/exon 1 regions of DNase I-like endonuclease genes and degradation of RNA. PCRs of genomic DNA in promoter/exon 1 regions of endonucleases and GAPDH were performed using primers shown in Table 1.

determine whether DNA breaks of nonenzymatic origin would induce endonucleases in the same manner, we tested DNase I and DNase X expression in isolated rat brain nuclei treated with varying concentrations of bleomycin. Real-time RT-PCR showed a significant increase in the expression of these endonucleases at bleomycin concentrations ranging

between 3 and 100 $\mu\text{g}/\text{mL}$. These data suggest that nonenzymatic DNA breaks induce endonucleases similar to the endonuclease-mediated breaks (Fig. 8C, D).

Recombinant EndoG induces gene transcription in supercoiled DNA

To explain how endonuclease expression could be induced by DNA breaks, we then tested whether transcription of DNA can be increased by DNA breaks. This may occur if the substrate DNA is supercoiled and thus less accessible to RNA polymerase, while DNA linearization and unwinding should provide access to the polymerase. A cell-free system containing either supercoiled plasmid with mouse EndoG gene inserted in pET29b plasmid or linearized pTRI with mouse β -actin gene was exposed with recEndoG or bleomycin to induce breaks. Two different plasmids were used to reduce the possibility of false-positive results based on the same primary structure of the DNA substrate. DNA degradation was controlled by gel electrophoresis. The experiment outcome clearly showed that recEndoG produced significant relaxation of supercoiled plasmid coinciding with the significant peak of transcription (Fig. 9A, B). A peak was also observed with bleomycin but it was not statistically significant since the cleavage of plasmid was deemed negligible (Fig. 9C). Importantly, the linearized plasmid did not produce any elevation of transcription (Fig. 9D–F), suggesting that supercoiling is important for the transcription induction by DNA breaks.

FIG. 8. Both EndoG and bleomycin induce DNase I and DNase X transcription in isolated rat brain cell nuclei. (A, B) *In vitro* transcription of DNase I and DNase X genes in isolated rat brain nuclei exposed with varying concentrations of recombinant EndoG (recEndoG) after 1 h incubation at 37°C as measured by real-time RT-PCR ($n=4$, $*p \leq 0.05$ vs. nontreated control). (C, D) *In vitro* transcription measured by real-time RT-PCR of DNase I and DNase X genes in isolated rat brain nuclei exposed with varying concentrations of bleomycin for 1 h at 37°C ($n=4$, $*p \leq 0.05$ vs. nontreated control).

FIG. 9. *In vitro* transcription of plasmids cleaved with recEndoG and bleomycin. (A) Agarose electrophoresis of digested supercoiled mouse-EndoG/pET29b plasmid. (B, C) Expression results of pET29b-EndoG plasmid cleaved with recEndoG or bleomycin measured by real-time RT-PCR ($n=4$, $*p \leq 0.05$ vs. expression of nondigested plasmid). (D) Agarose electrophoresis of digested linearized pTRI-β-actin plasmid. (E, F) Results of expression of pTRI-β-actin plasmid cleaved with recEndoG and bleomycin measured by real-time RT-PCR ($n=4$, $*p \leq 0.05$ vs. expression of nondigested plasmid).

Discussion

This study, for the first time, demonstrated a link between EndoG and other endonucleases. It showed that EndoG overexpression in NRK-52E cells causes apoptosis associated with the induction of several apoptotic endonucleases of DNase I family and CAD. Since the timing of CAD induction was different from that of DNase I family induction, it is tempting to speculate that CAD was induced by a different mechanism compared to the DNase I family. The DNase 2 family of endonucleases was not induced. The induction was temporary; it preceded cell death and was associated with some DNA degradation of promoter/exon 1 regions of the endonuclease genes. The experiments with isolated nuclei and supercoiled plasmids suggest that EndoG may upregulate other endonucleases by producing a limited number of DNA breaks and causing relaxation of DNA. This may increase the access of DNA to transcription proteins. At later stages, EndoG overexpression was associated with degradation of RNA. Therefore, EndoG seemingly exhibited both DNase and RNase activities.

EndoG is a regulatory protein that is upstream of other pathways in cell death as indicated by our findings. Before this study, EndoG was considered only as an executioner of apoptosis, a downstream effector that degrades DNA at the end of cell death pathways. Apoptosis induced by EndoG was shown to be caspase-independent (Uren *et al.*, 2005; Zanna *et al.*, 2005). EndoG was shown to have numerous regulators, including reactive oxygen species (Kim *et al.*, 2008), apoptosis inducing factor (Wang *et al.*, 2002; Cregan *et al.*, 2004), heat shock protein HSP70 (Kalinowska *et al.*, 2005), FEN-1 (Parrish *et al.*, 2003) and ATP (Kalinowska *et al.*, 2005). Although inactivation of EndoG does protect against injury in several models [reviewed in Apostolov *et al.* (2007)], it remains unclear if EndoG can also regulate other proteins. Our data show that it regulates other endonucleases.

Our study confirmed that EndoG is moderately cytotoxic. It was previously shown that overexpression of extramitochondrial active EndoG in HeLa and CV1-cells induced cell death by acting alone, while the expression of an inactive mutant of EndoG failed to induce cell death (Schafer *et al.*, 2004). Likewise, the loss of EndoG activity in

Caenorhabditis elegans resulted in increased cell survival (Hengartner, 2001). However, the fact that EndoG, unlike other endonucleases, can be localized in the nucleus during apoptosis may indicate that EndoG's DNA-degrading activity and cytotoxicity through DNA degradation are limited compared to other endonucleases.

Endonucleases are known to destroy the host DNA by acting simultaneously or sequentially. For example, Montero *et al.* (2010) showed that even though CAD was the main endonuclease at an initial phase of interdigit regression in the embryonic development of birds at peak stages of the degenerative process, the two other endonucleases, L-DNase II and DNase 2B, became predominant. An overexpression of DNase 2B in avian embryonic limb mesoderm was accompanied by upregulation and activation of L-DNase 2. Apoptotic DNA fragmentation induced by staurosporine in Ramos Burkitt's lymphoma cell line showed that CAD was activated 3 h later, whereas DNase γ was activated 48 h after the impact (Mizuta *et al.*, 2009). Apoptosis in N1E-115 neuroblastoma cells was catalyzed by either CAD or DNase γ depending on the differentiation state (Shiokawa and Tanuma, 2004). CAD induced DNA fragmentation during staurosporine-induced apoptosis in undifferentiated cells. However, after induction of neuronal differentiation, CAD disappeared and DNA fragmentation was caused by DNase γ . Our previous study (Yin *et al.*, 2007) demonstrated that EndoG is induced by DNase I during cisplatin kidney injury.

Taken together, it may be suggested that endonucleases could be engaged in a vicious cycle causing activation of all endonucleases. As EndoG was shown to cooperate with DNase I (Widlak *et al.*, 2001), it is becoming clear that endonucleases activate other members of their group and cumulatively destroy DNA of the host cell during and after cell death. Our results also show that bleomycin that cuts DNA via oxidative breaks, also induced endonucleases. This suggests that DNA breaks of nonenzymatic nature, act as endonuclease inducers similar to EndoG. Therefore, it is possible that the vicious cycle can start from an endonuclease action, DNA repair or DNA breaks initiated by a physical, chemical agent or oxygen radicals.

The specificity of other endonuclease induction is unclear at this time and will require additional studies. The fact that DNase 2 group of enzymes were not induced by EndoG suggests that there is at least some specificity. The latter, especially at initial stages of DNA cleavage, can be in oligo-G sequences, which constitute the initial substrate of EndoG. The finding that promoter/exon 1 regions of activated DNases but not GAPDH again points at the specificity of cleavage. Cell death is not a single-moment event. In most cases, cell death is prolonged in time and can take several hours or even days. This offers a significant therapeutic window to slow, prevent, or even reverse the course of injury. Future studies will also need to determine which EndoG activities, DNase or RNase, is more important in its role as a regulator of apoptosis. Based on our data, it appears that DNase activity, at least initially, acts as an activator of other endonucleases, while RNase inactivates them.

In conclusion, this study shows that EndoG contributes as an upstream regulator to several other apoptotic endonucleases by activating and then inactivating them. Therefore, EndoG should be considered as a potential target for drugs

to protect against tissue injuries, or to promote tissue injury in cancer cells.

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Disclosure Statement

No competing financial interests exist.

References

- Apostolov, E.O., Ray, D., Alobuia, W.M., Mikhailova, M.V., Wang, X., Basnakian, A.G., *et al.* (2011). Endonuclease G mediates endothelial cell death induced by carbamylated LDL. *Am J Physiol Heart Circ Physiol* **300**, H1997–H2004.
- Apostolov, E.O., Soultanova, I., Savenka, A., Bagandov, O.O., Yin, X., Stewart, A.G., *et al.* (2009). Deoxyribonuclease I is essential for DNA fragmentation induced by gamma radiation in mice. *Radiat Res* **172**, 481–492.
- Apostolov, E.O., Wang, X., Shah, S.V., and Basnakian, A.G. (2007). Role of EndoG in development and cell injury. *Cell Death Differ* **14**, 1971–1974.
- Basnakian, A.G., Apostolov, E.O., Yin, X., Abiri, S.O., Stewart, A.G., Singh, A.B., *et al.* (2006). Endonuclease G promotes cell death of non-invasive human breast cancer cells. *Exp Cell Res* **312**, 4139–4149.
- Basnakian, A.G., Apostolov, E.O., Yin, X., Napirei, M., Mannherz, H.G., and Shah, S.V. (2005). Cisplatin nephrotoxicity is mediated by deoxyribonuclease I. *J Am Soc Nephrol* **16**, 697–702.
- Chahory, S., Padron, L., Courtois, Y., and Torriglia, A. (2004). The LEI/L-DNase II pathway is activated in light-induced retinal degeneration in rats. *Neurosci Lett* **367**, 205–209.
- Cote, J., and Ruiz-Carrillo A. (1993). Primers for mitochondrial DNA replication generated by endonuclease G. *Science* **261**, 765–769.
- Cregan, S.P., Dawson, V.L., and Slack, R.S. (2004). Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23**, 2785–2796.
- Ermakov, A.V., Kostyuk, S.V., Konkova, M.S., Egorina, N.A., Malinovskaya, E.M., and Veiko, N.N. (2008). Extracellular DNA fragments. *Ann N Y Acad Sci* **1137**, 41–46.
- Evans, C.J., and Aguilera, R.J. (2003). DNase II: genes, enzymes and function. *Gene* **322**, 1–15.
- Hamada, M., Sumi, T., Iwai, S., Nakazawa, M., and Yura, Y. (2006). Induction of endonuclease G-mediated apoptosis in human oral squamous cell carcinoma cells by protein kinase C inhibitor safinol. *Apoptosis* **11**, 47–56.
- Hengartner MO. (2001). Apoptosis. DNA destroyers. *Nature* **412**, 27, 9.
- Hofnagel, O., Luechtenborg, B., Stolle, K., Lorkowski, S., Eschert, H., Plenz, G., *et al.* (2004). Proinflammatory cytokines regulate LOX-1 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24**, 1789–1795.
- Ikeda, S., and Kawasaki, N. (2001). Isolation and characterization of the *Schizosaccharomyces pombe* cDNA encoding the mitochondrial endonuclease(1). *Biochim Biophys Acta* **1519**, 111–116.
- Kalinowska, M., Garncarz, W., Pietrowska, M., Garrard, W.T., and Widlak, P. (2005). Regulation of the human apoptotic

- DNase/RNase Endonuclease G: involvement of Hsp70 and ATP. *Apoptosis* **10**, 821–830.
- Kim, J.S., Lee, J.H., Jeong, W.W., Choi, D.H., Cha, H.J., Kim do H, *et al.* (2008). Reactive oxygen species-dependent EndoG release mediates cisplatin-induced caspase-independent apoptosis in human head and neck squamous carcinoma cells. *Int J Cancer* **122**, 672–680.
- Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, B.I., Lee, D.J., Cho, K.J., and Kim, G.W. (2005). Early nuclear translocation of endonuclease G and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. *Neurosci Lett* **386**, 23–27.
- Li, L.Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95–99.
- Manders EMM, Verbeek, F.J., and Aten, J.A. (1993). Measurement of co-localization of objects in dual-colour confocal images. *J Microsc* **169**, 375–382.
- Mercer, K.E., Apostolov, E.O., da Costa, G.G., Yu, X., Lang, P., Roberts, D.W., *et al.* (2010). Expression of sulfotransferase isoform 1A1 (SULT1A1) in breast cancer cells significantly increases 4-hydroxytamoxifen-induced apoptosis. *Int J Mol Epidemiol Genet* **1**, 92–103.
- Mizuta, R., Mizuta, M., Araki, S., Suzuki, K., Ebara, S., Furukawa, Y., *et al.* (2009). DNase gamma-dependent and -independent apoptotic DNA fragmentations in Ramos Burkitt's lymphoma cell line. *Biomed Res* **30**, 165–170.
- Montero, J.A., Lorda-Diez, C.I., Certal, A.C., Moreno, N., Rodriguez-Leon, J., Torriglia, A., *et al.* (2010). Coordinated and sequential activation of neutral and acidic DNases during interdigital cell death in the embryonic limb. *Apoptosis* **15**, 1197–1210.
- Nagata S. (2000). Apoptotic DNA fragmentation. *Exp Cell Res* **256**, 12–18.
- Napirei, M., Basnakian, A.G., Apostolov, E.O., and Mannherz, H.G. (2006). Deoxyribonuclease 1 aggravates acetaminophen-induced liver necrosis in male CD-1 mice. *Hepatology* **43**, 297–305.
- Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H.G., and Moroy, T. (2000). Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet* **25**, 177–181.
- Ohsato, T., Ishihara, N., Muta, T., Umeda, S., Ikeda, S., Mihara, K., *et al.* (2002). Mammalian mitochondrial endonuclease G. Digestion of R-loops and localization in intermembrane space. *Eur J Biochem* **269**, 5765–5770.
- Parrish, J.Z., Yang, C., Shen, B., and Xue, D. (2003). CRN-1, a *Caenorhabditis elegans* FEN-1 homologue, cooperates with CPS-6/EndoG to promote apoptotic DNA degradation. *EMBO J* **22**, 3451–3460.
- Prats, E., Noel, M., Letourneau, J., Tiranti, V., Vaque, J., Debon, R., *et al.* (1997). Characterization and expression of the mouse endonuclease G gene. *DNA Cell Biol* **16**, 1111–1122.
- Pravdenkova, S.V., Basnakian, A.G., James, S.J., and Andersen, B.J. (1996). DNA fragmentation and nuclear endonuclease activity in rat brain after severe closed head injury. *Brain Res* **729**, 151–155.
- Roszer, T., Kappelmayer, J., Nagy, G.G., Szentmiklosi, A.J., Basnakian, A.G., and Banfalvi, G. (2006). The neuropeptide FMRFamide can protect cells against apoptosis in the snail digestive gland. *Apoptosis* **11**, 173–182.
- Ruiz-Carrillo, A., and Renaud, J. (1987). Endonuclease G: a (dG)n X (dC)n-specific DNase from higher eukaryotes. *EMBO J* **6**, 401–407.
- Schafer, P., Scholz, S.R., Gimadutdinov, O., Cymerman, I.A., Bujnicki, J.M., Ruiz-Carrillo, A., *et al.* (2004). Structural and functional characterization of mitochondrial EndoG, a sugar non-specific nuclease which plays an important role during apoptosis. *J Mol Biol* **338**, 217–228.
- Schneiders, U.M., Schyschka, L., Rudy, A., and Vollmar, A.M. (2009). BH3-only proteins Mcl-1 and Bim as well as endonuclease G are targeted in spongistatin 1-induced apoptosis in breast cancer cells. *Mol Cancer Ther* **8**, 2914–2925.
- Shiokawa, D., and Tanuma, S. (2004). Differential DNases are selectively used in neuronal apoptosis depending on the differentiation state. *Cell Death Differ* **11**, 1112–1120.
- Uren, R.T., Dewson, G., Bonzon, C., Lithgow, T., Newmeyer, D.D., and Kluck, R.M. (2005). Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. *J Biol Chem* **280**, 2266–2274.
- van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., *et al.* (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ* **8**, 1136–1142.
- Wang, X., Tryndyak, V., Apostolov, E.O., Yin, X., Shah, S.V., Pogribny, I.P., *et al.* (2008). Sensitivity of human prostate cancer cells to chemotherapeutic drugs depends on EndoG expression regulated by promoter methylation. *Cancer Lett* **270**, 132–143.
- Wang, X., Yang, C., Chai, J., Shi, Y., and Xue, D. (2002). Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. *Science* **298**, 1587–1592.
- Widlak, P., Li, L.Y., Wang, X., and Garrard, W.T. (2001). Action of recombinant human apoptotic endonuclease G on naked DNA and chromatin substrates: cooperation with exonuclease and DNase I. *J Biol Chem* **276**, 48404–48409.
- Yin, X., Apostolov, E.O., Shah, S.V., Wang, X., Bogdanov, K.V., Buzder, T., *et al.* (2007). Induction of renal endonuclease G by cisplatin is reduced in DNase I-deficient mice. *J Am Soc Nephrol* **18**, 2544–2553.
- Zanna, C., Ghelli, A., Porcelli, A.M., Martinuzzi, A., Carelli, V., and Rugolo, M. (2005). Caspase-independent death of Leber's hereditary optic neuropathy cybrids is driven by energetic failure and mediated by AIF and Endonuclease G. *Apoptosis* **10**, 997–1007.

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