CHAPTER 11

Genome Degradation by DNAS1L3 Endonuclease:

A Key PARP-1-Regulated Event in Apoptosis

A. Hamid Boulares, Alexander G. Yakovlev and Mark E. Smulson

Abstract

Degradation of chromatin into internucleosomal fragments, a prevailing hallmark of apoptosis in most cells and tissues, has been tightly associated with a Ca^{2+} and Mg^{2+}-dependent endonuclease activity. Several candidate enzymes have been identified as important players in this process. Several decades ago, murine and bovine Ca^{2+} and Mg^{2+}-dependent endonucleases were observed to be inhibited by poly(ADP-ribosyl)ation in a reaction mediated by PARP-1. PARP-1 is one of the earliest nuclear enzymes to be targeted for degradation by caspases during apoptosis. Such cleavage is believed to prevent energy depletion in response to DNA damage generated as a result of an activation of apoptotic endonucleases. We have recently identified, cloned, and characterized DNAS1L3 as the human homolog of the unidentified bovine poly(ADP-ribosyl)ation-regulated endonuclease. In this review, we will describe the efforts of our and other laboratories in the elucidation of a role for this endonuclease during apoptosis. We will discuss its dependence on Ca^{2+} and Mg^{2+}, its inhibition by poly(ADP-ribosyl)ation, and its requirement for PARP-1 cleavage, and subsequent inactivation of PARP-1, for optimal activity during apoptosis.

Introduction: DNA Fragmentation in Apoptosis

Apoptosis plays important roles in immunity, development, and homeostasis of cells and tissues as well as in the response to cell injury. This process of programmed cell death is characterized by marked changes in cell morphology, including chromatin condensation, membrane blebbing, nuclear breakdown, and the appearance of membrane-associated apoptotic bodies, as well as by internucleosomal DNA fragmentation and the cleavage of several housekeeping proteins including poly(ADP-ribose) polymerase-1 (PARP-1)[1] and lamins. Apoptosis is triggered by various agents, including endogenous cytokines as well as therapeutic and cytotoxic drugs. Its initiation and execution are mediated, in most cases, by activation of members of the caspase family of aspartate-specific cysteine proteases.

Eukaryotic cells are equipped with a sophisticated mechanism to dispose of their genome upon death by apoptosis that has yet to be fully elucidated. Internucleosomal DNA fragmentation represents a terminal step in such a process and is regarded as a prevailing marker of apoptosis. Although chromatin condensation in dying cells was observed over a century ago, its cleavage into nucleosomal fragments was only reported in 1976 by Skalka et al[2] followed by Wyllie in his milestone report in 1980.[3] For many years the scientific community has awaited the discovery of an apoptosis specific endonuclease that is responsible for cleavage of chromatin in such fashion, despite the fact that some Ca^{2+} and Mg^{2+}-dependent

Poly(ADP-Ribosyl)ation, edited by Alexander Bürkle.

endonucleases were already known but not adequately characterized. The various candidate endonucleases identified to date differ in characteristics such as Ca^{2+} and Mg^{2+} dependence, optimal pH, tissue distribution, and requirement for caspase-3 activation. For example, DNA fragmentation factor (DFF), also known as caspase-activated DNase (CAD) and caspase-activated nuclease (CPAN), has been suggested to play a major role in DNA fragmentation during apoptosis (reviewed in refs. 4,5). DFF is composed of two subunits of 40 and 45 kDa, termed DFF40 (CAD) and DFF45 (ICAD), respectively.[6-9] The endonuclease activity of this enzyme, which is intrinsic to DFF40, is induced on cleavage of DFF45 by caspase-3.

We[10-14] have recently cloned and characterized the human homolog, termed DNAS1L3, of a bovine chromatin-bound, Ca^{2+}- and Mg^{2+}-dependent endonuclease[15] that was also thought to contribute to DNA fragmentation during apoptosis. The important point and rationale for this work was that the activity of DNAS1L3,[11] like that of the bovine endonuclease,[15,16] is inhibited by poly(ADP-ribosyl)ation.[11-13]

The cleavage of the DNA repair enzyme PARP-1 between Asp^{214} and Gly^{215} results in separation of the two zinc-finger DNA binding motifs in the NH2-terminal region of the enzyme from the automodification and catalytic domains, thus preventing recruitment of the catalytic domain to sites of DNA damage.[1,17,18] We and others have hypothesized that this cleavage occurs in order to prevent depletion of the energy reserves (NAD and ATP) that are thought to be required for the later stages of apoptosis and also to prevent futile repair of DNA strand breaks during the death program. We have previously shown that expression of a caspase-resistant PARP-1 mutant (mut-PARP-1) in osteosarcoma cells or PARP-$1^{-/-}$ fibroblasts, increases the rate of cell death as a result of excessive NAD depletion.[19] Herceg and Wang[20] also showed that expression of a similar PARP-1 mutant switches the mode of cell death induced by TNF from apoptosis to necrosis. Whereas nonmodified PARP-1 is cleaved primarily by caspase-3,[1,18] automodified PARP-1 is preferentially cleaved by caspase-7,[21] thus emphasizing the exquisite selectivity and necessity for PARP-1 cleavage during apoptosis. In the last several years, our studies have provided evidence for an additional role of poly(ADP-ribosyl)ation and cleavage of PARP-1 during apoptosis. We believe that the role of these events is to allow the smooth continuation of cell destruction at the nuclear level by preventing a persistent inhibition of the Ca^{2+}-Mg^{2+}-dependent endonuclease DNAS1L3 by covalent poly(ADP-ribosyl)ation.

In the present review we will describe our contributions, as well as that of others, to the understanding of the function of DNAS1L3 in catalyzing cleavage of chromatin, its role during apoptosis, and its regulation by poly(ADP-ribosyl)ation.

Poly(ADP-Ribosyl)ation-Regulated Endonuclease: Not Such a New Observation

Although significant progress in unraveling the functions of DNAS1L3 and other Ca^{2+}-Mg^{2+}-dependent endonucleases in the cell has been made only recently, Koide, Yoshihara, and coworkers have pioneered the initial observations on Ca^{2+}-Mg^{2+}-dependent endonucleases and characterization of their function. In 1974, using crude extracts with NAD^+, Mg^{2+}, DNA, and PARP-1, they first demonstrated that a Ca^{2+}-Mg^{2+}-dependent endonuclease present in those extracts was inhibited by poly(ADP-ribosyl)ation.[22] They subsequently purified a 36 kDa endonuclease from bull semen, which had a strong requirement for Ca^{2+}, Mg^{2+}, and a relatively alkaline pH for its optimal activity that could be enhanced by histone H2B.[16] The activity of this endonuclease was also sensitive to SH-blocking reagents, which may suggest a potential role for oxidants in its regulation. The semen-derived endonuclease was later found to be also inhibited by poly(ADP-ribosyl)ation.[22-24] Another endonuclease requiring only Mg^{2+} was reported by another group. It harbored characteristics similar to those observed for the semen-derived endonuclease including its regulation by poly(ADP-ribosyl)ation.[25] However, to the best of our knowledge, no further information on this endonuclease has been reported.

Identification of DNAS1L3 as the Poly(ADP-Ribosyl)ation Regulated Ca^{2+}-Mg^{2+}-Dependent Endonuclease

The long-term aim of our past and current work has been to focus on the role of PARP-1 and the consequences of poly(ADP-ribosyl)ation in regulation of endonuclease activity during cell death in the context of human diseases. We and others have demonstrated a requirement for PARP-1 cleavage and consequent inactivation during apoptosis in response to a variety of stimuli.[12,19,20] These observations suggested that subsequent cleavage of PARP-1 by caspase-3-like proteases might release certain nuclear proteins from poly(ADP-ribosyl)ation-induced inhibition, which might be required for apoptotic DNA fragmentation and cell death. As alluded to earlier, through the work of Yoshihara's group, a significant level approaching 100% of chromatin-bound Ca^{2+}-Mg^{2+}-dependent endonucleases from rat liver and thymus is inhibited in vitro by purified PARP-1 via poly(ADP-ribosyl)ation.[15,22,24] Thus, cleavage of PARP-1 during apoptosis may lead to activation due to the elimination of poly(ADP-ribose) of Ca^{2+}-Mg^{2+}-dependent nucleases required for DNA fragmentation. This led us to attempt to identify and clone a candidate Ca^{2+}/Mg^{2+}-dependent endonuclease and to examine its DNA fragmentation activity during apoptosis as well as test the above hypothesis.

In collaboration with Yoshihara's group, we were able to obtain the bovine Ca^{2+}-Mg^{2+}-endonuclease in a purified form to begin our investigation. According to our in vitro analysis, the purified 36 kDa enzyme possesses properties identical to the characterized Ca^{2+}-Mg^{2+}-dependent endonucleases from bovine thymus, human placenta, and bovine, rat, and mouse liver. It is noteworthy to reiterate that all these enzymes are inhibited by poly(ADP-ribosyl)ation in the presence of PARP-1, NAD^+, Mg^{2+} and DNA.[15,22] Hence, we determined the partial amino acid sequence of the purified bovine endonuclease and identified its human, rat and mouse homologs. Sequence analysis revealed substantial homology of this enzyme to human DNAS1L3,[26] rat DNase-γ,[27,28] and mouse liver and spleen-specific DNase (LSD)[29] (Fig. 1A). Homology analysis indicates that these four proteins are orthologous members of the family of DNase I-related endonucleases. With the use of the TBLASTN 2.0.5 program, we searched all nonredundant peptide sequence databases (nt) for sequences homologous to the determined amino acid sequences. Human DNAS1L3 is 84% identical (92% similar) to rat DNase-γ and 81% identical (90% similar) to mouse LSD; mouse LSD is 91% identical (94% similar) to rat DNase-γ. The extent of sequence homology among these four nucleases is substantially greater than that among other members of the family of DNase I-like enzymes, strongly suggesting that they represent species-specific versions of the same protein.

Catalytic Properties of DNAS1L3

The collective results reported by Yoshihara's group and our laboratories consistently supported the notion that DNAS1L3 mediates Ca^{2+}- and Mg^{2+}-dependent fragmentation of DNA both in vitro and in vivo.[22,26] Our in vitro experiments with purified DNAS1L3 established the dependence of this enzyme on Ca^{2+} and Mg^{2+} for catalytic activity. The optimal concentrations of these cations are similar to those determined for endonuclease activity in rat liver nuclei.[10] The activity of DNAS1L3 can also be supported by Mn^{2+} although it requires ~10 times the concentration shown to be optimal for the rat homolog (DNase γ). The activity of DNAS1L3 in the presence of Ca^{2+} and $Mg,^{2+}$ like that of Dnase,[30] was inhibited by Zn^{2+}. Although Yoshihara's group reported that endonuclease preferentially stimulated the hydrolysis of double-stranded DNA,[15] our experiments with purified enzyme revealed that DNAS1L3 does not preferentially introduce single stranded nicks or double stranded breaks into DNA substrates in vitro. Whether DNAS1L3 preferentially introduces single or double-strand DNA breaks in vivo remains to be determined.

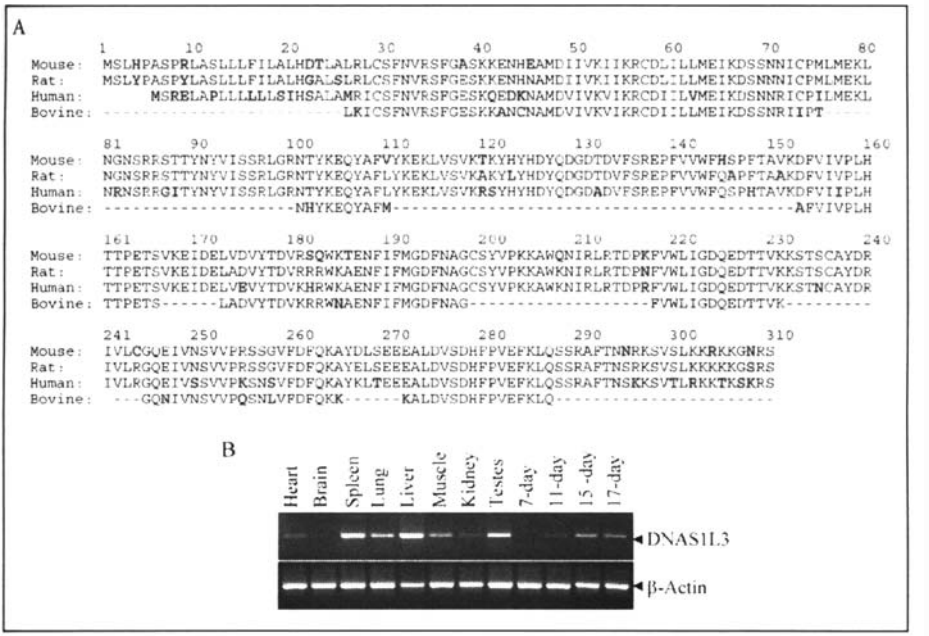

Figure 1. The bovine PARP-1-regulated endonuclease is highly homologous to mouse LSD, rat DNase γ, and human DNAS1L3, with its mouse homolog exhibiting a tissue specific expression pattern. A) Alignment of the amino acid sequences of mouse LSD (GenBank accession no. AF047355), rat DNase [gamma] (GenBank accession no. U75689), human DNAS1L3 (GenBank accession no. U56814) and seven peptides derived from purified bull seminal plasma endonuclease. Residues that are unique to one of the four proteins are highlighted. Residue numbers are indicated above the sequences and the sequences of the peptides from the bovine protein are positioned according to the results of the TBLASTN analysis. Regions of undetermined sequence are indicated with dashes. Reprinted with permission from: Yakovlev et al, Nucleic Acids Res 27(9):1999-2005, ©1999 Oxford University Press. B) RT-PCR analysis of the abundance of transcripts encoding the mouse homolog (LSDNase) of DNAS1L3 in various mouse tissues and during mouse embryogenesis. Total RNA from the indicated mouse tissues and from mouse embryos on the indicated days of embryogenesis was subjected to RT-PCR with primers specific for LSDNase or for β-actin. The PCR products were analyzed by electrophoresis through a 2% agarose gel. Reprinted with permission from: Yakovlev et al, J Biol Chem 275(28):21302-21308, ©2000 The American Society for Biochemistry and Molecular Biology, Inc.

Tissue Distribution of Transcripts Encoding the Mouse Homolog of DNAS1L3

A semi-quantitative RT-PCR analysis of mRNA encoding the mouse homolog (LSDNase) of DNAS1L3 revealed that it is expressed in a variety of tissues, with the highest amounts apparent in spleen, liver, and testes (Fig. 1B). Smaller amounts were detected in heart, lungs, skeletal muscle, and kidney, but LSDNase mRNA was not detected in mouse brain. Similar analysis of mouse embryos at various stages of development revealed that LSDNase mRNA was not detected in 7-days mouse embryos, though small amounts of this mRNA were detected at day 11 and larger amounts were detected at days 15 and 17. A similar pattern of expression was described for rat DNase-γ.[27,28,31] Schsneider's group reported that, in human tissues, DNAS1L3 seems to be highly expressed primarily in the liver and to a lesser extent in the kidney, as assessed by Northern blot analysis.[26] These differences in mRNA expression may be due to the variability in

the techniques utilized to assess the different homologs of DNAS1L3. In general, Ca^{2+} and Mg^{2+}-dependent endonucleases seem to have a tissue specific distribution. In this regard, later in the chapter, we will briefly address an interesting aspect of DNAS1L3 function and its regulation in the context of toxicant-induced cell death as it relates to tissue specific expression.

Poly(ADP-Ribosyl)ation and Inactivation of Recombinant DNAS1L3 by PARP-1 in Vitro

As mentioned earlier, Yoshihara's group showed that bovine chromatin-bound endonucleases and related enzymes are inhibited by poly(ADP-ribosyl)ation in vitro.[22,24] After the identification of DNAS1L3, it became very critical to determine whether this enzyme was indeed susceptible to such modification by PARP-1 in vitro and whether poly(ADP-ribosyl)ation resulted in inhibition of its enzymatic activity. Incubation of purified recombinant DNAS1L3 with recombinant human PARP-1 in the presence of a low concentration of [^{32}P]NAD and high molecular weight DNA resulted in marked poly(ADP-ribosyl)ation of both proteins (Fig. 2A). This effect was blocked in the presence of 3-AB. These results showed that DNAS1L3 activates PARP-1 by introducing breaks into DNA strands, and that PARP-1, in turn, catalyzes the post-translational modification of the nuclease. Increasing NAD concentration of the reaction mixture culminated in an increase in the length of the ADP-ribose chains attached to DNAS1L3 as revealed by the shift of both DNAS1L3 and PARP-1 positions toward the top of the gel. Such shifts in the migration of DNAS1L3 reflected the presence of long chains of ADP-ribose attached to these proteins.[11] Further, using an in vitro DNA degradation assay, it was determined that the DNA fragmentation potential of DNAS1L3 was completely impaired by the addition of PARP-1 and NAD to the reaction mixture (Fig. 2B). The inclusion of 3-AB in, or the omission of NAD or PARP-1 from, the poly(ADP-ribosyl)ation reaction mixture prevented the inhibition of DNAS1L3. This cross-talk between PARP-1 (i.e., DNAS1L3-mediated activation of PARP-1 and PARP-1-mediated inhibition of DNAS1L3) is rather fascinating. It may represent a signaling function of poly(ADP-ribose) to prevent untimely activation of DNAS1L3, which provides the cell with a critical time to undergo a crucial decision of whether to die or not to die. When conditions that favor the activation of DNAS1L3, such as an abnormal increase in intracellular Ca^{2+}, take place in conjunction with caspase activation with a subsequent inactivation of PARP-1 by proteolysis, poly(ADP-ribosyl)ated DNAS1L3 would be released from inhibition by poly(ADP-ribose) glycohydrolase (PARG) (see our model in Fig. 5).

DNAS1L3 Mediates Internucleosomal DNA Fragmentation during Drug or TNF-Induced Apoptosis

During a search for a cell type with which to study the role of DNAS1L3 in DNA fragmentation during apoptosis, we found that human osteosarcoma cells, the cervical carcinoma cell line, HeLa, and fibroblasts do not express DNAS1L3, as revealed by RT-PCR analysis with primers specific for human DNAS1L3 cDNA.[10-12] In contrast, the human monocytic cell line, U-937, expresses relatively large amounts of DNAS1L3 transcripts.[12] Furthermore, unlike U-937 cells, the osteosarcoma cells, HeLa cells, and fibroblasts do not undergo internucleosomal DNA fragmentation in response to the proapoptotic drug etoposide.[12,13] It is noteworthy that HeLa cells and fibroblasts do not undergo such fragmentation of DNA in response to many additional apoptotic inducers including TNF, acetaminophen, or ionomycin.[9-11,14,32] We were thus able to exploit these properties of these cells, along with the use of transfection, to examine the involvement of DNAS1L3 in apoptotic internucleosomal DNA fragmentation. When these different cells, including osteosarcoma cells (Fig. 3A), were transfected with an expression vector that encodes DNAS1L3 and then exposed to the above apoptotic inducers, a marked internucleosomal DNA fragmentation was observed in all cases.[10-14] This effect was completely blocked by the presence in the incubation medium of a cell permeable 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic acid (BAPTA), which inhibits intracellular Ca^{2+} release. These results demonstrated that DNAS1L3 mediates internucleosomal DNA fragmentation in the transfected osteosarcoma

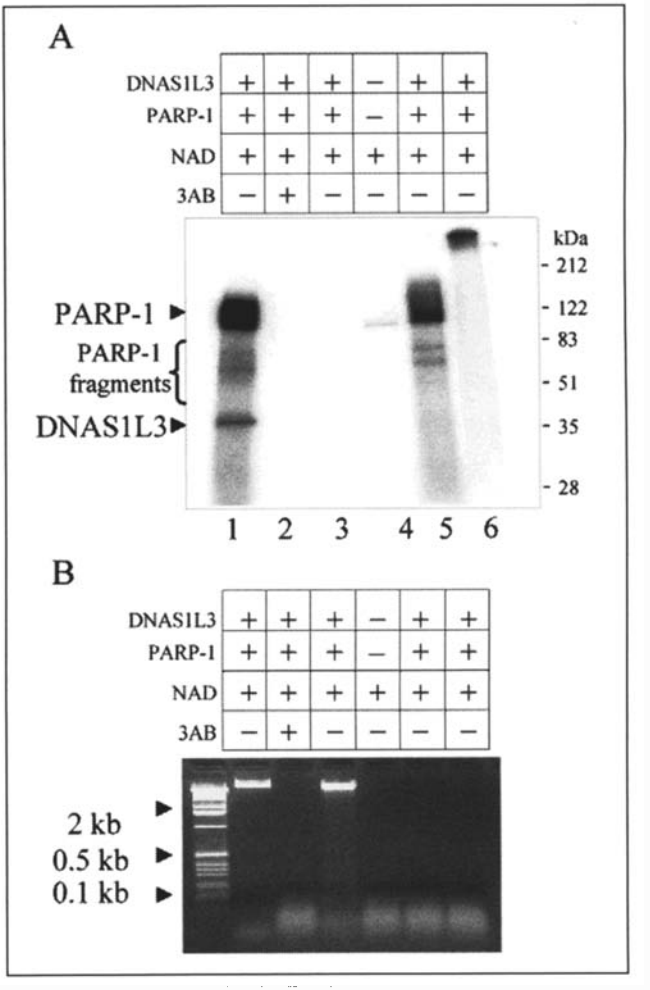

Figure 2. Poly(ADP-ribosyl)ation and inhibition of recombinant DNAS1L3 by PARP-1 in vitro. A) Poly(ADP-ribosyl)ation of DNAS1L3 by PARP-1. The indicated combinations of recombinant human PARP-1, [^{32}P]NAD (lanes 1-5) or nonradioactive NAD (lane 6), recombinant DNAS1L3, and 3-AB were incubated with high molecular weight (lanes 1-4 and 6) or activated (lane 5) DNA. The reaction products were then analyzed by SDS-PAGE and autoradiography. The positions of PARP-1, PARP-1 fragments, and DNAS1L3 are indicated. B) Effect of poly(ADP-ribosyl)ation on DNAS1L3 activity. The indicated combinations of recombinant human PARP-1, nonradioactive NAD, recombinant DNAS1L3, and 3-AB were incubated for 30 min at room temperature in a reaction mixture containing high molecular weight DNA. The mixtures were then placed on ice, and portions were subsequently incubated for 30 min at 37°C with phage DNA in a reaction containing 25 mM Tris-HCl (pH 7.4), 5 mM MgCl2, and 2.5 mM CaCl2. DNA integrity was then analyzed by electrophoresis through a 1.5% agarose gel. The leftmost lane contains 1-kb DNA size markers. Reprinted with permission from: Yakovlev et al, J Biol Chem 275(28):21302-21308, ©2000 The American Society for Biochemistry and Molecular Biology, Inc.

cells and that this activity is dependent on Ca^{2+}, consistent with the nature of the in vitro enzymatic characteristics of DNAS1L3. These results were pivotal toward the elucidation, in the context of the whole cell, of a physiological function for DNAS1L3. Whether DNAS1L3 harbors other enzymatic functions independently of apoptosis remains to be determined.

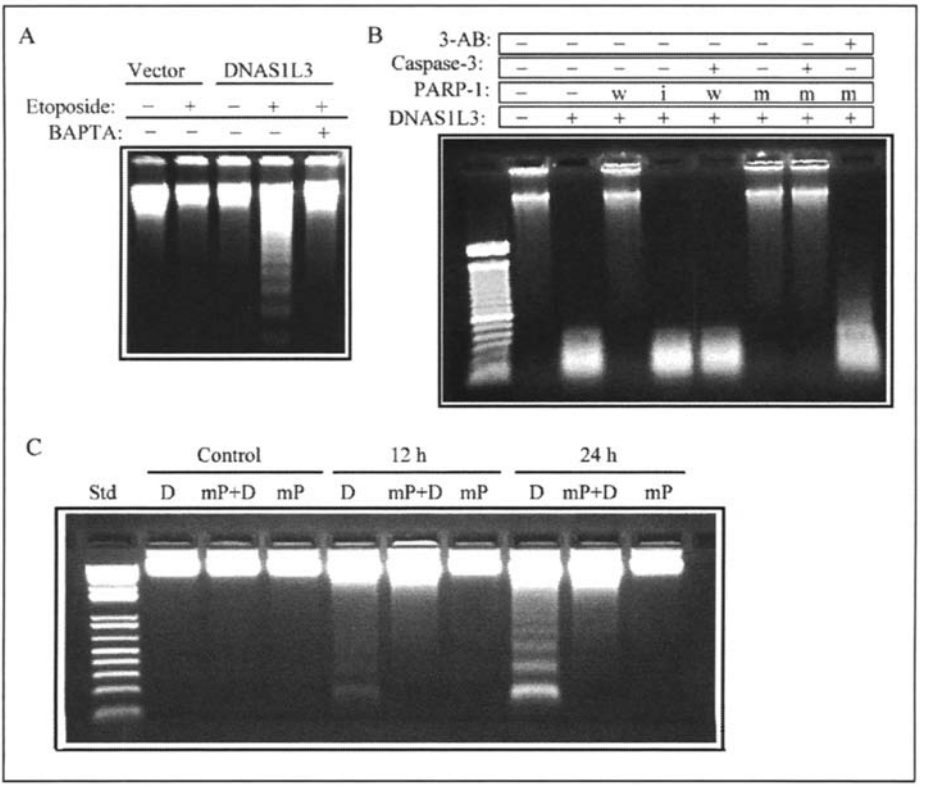

Figure 3. Requirement of PARP-1 cleavage by caspase-3 for DNAS1L3-mediated DNA degradation. A) Effect of DNAS1L3 expression on DNA integrity in etoposide-treated osteosarcoma cells. Osteosarcoma cells transfected with the DNAS1L3 expression vector or the corresponding empty vector were incubated for 24 h in the absence or presence of 70 μM etoposide or 10 μM BAPTA, as indicated. Genomic DNA was then isolated from the cells and analyzed by agarose gel electrophoresis and ethidium bromide staining. B) Requirement of PARP-1 cleavage by caspase-3 for DNAS1L3-mediated DNA degradation in vitro. The indicated combinations of recombinant DNAS1L3 (added last), wild-type (w) PARP-1, catalytically inactive (i) PARP-1, caspase-3-resistant mutant (m) PARP-1, and caspase-3 were incubated in the absence or presence of 3 mM 3aminobenzamide (3-AB) for 60 min at 37°C in a reaction mixture containing genomic DNA and NAD. The integrity of DNA was then analyzed by agarose gel electrophoresis and ethidium bromide staining. The leftmost lane contains DNA size markers (1-kb intervals). C) Effect of mut-PARP-1 expression on DNAS1L3-mediated DNA fragmentation in response to etoposide. Cells expressing recombinant DNAS1L3 (D), mut-PARP-1 (mP), or both proteins (mP+D) were incubated for 12 or 24 h in the presence of 70 μM etoposide or left untreated (Control), after which internucleosomal DNA fragmentation was analyzed by electrophoresis. The leftmost lane (Std) contains molecular size standards (1-kb intervals). Reprinted with permission from: Boulares et al, J Biol Chem 277(1):372-378, ©2002 The American Society for Biochemistry and Molecular Biology, Inc.

It was subsequently demonstrated that DNAS1L3-mediated internucleosomal DNA fragmentation in transfected cells occurs immediately after PARP-1 cleavage during apoptosis,[11,12] suggesting that the cleavage and consequent inactivation of PARP-1 clearly appeared to be required for the induction of DNAS1L3 endonuclease activity in cells. This became very relevant to subsequent investigations into the regulation of DNAS1L3 by poly(ADP-ribosyl)ation.

PARP-1 Cleavage by Caspase Is Required for DNASIL3-Mediated DNA Fragmentation

We have shown that recombinant DNASIL3 mediates the complete degradation of genomic DNA and that this action is blocked by the addition of wild-type PARP-1 and NAD.[12] In contrast, a catalytically inactive mutant of PARP-1 has no effect on DNASIL3-mediated DNA degradation, which demonstrates that poly(ADP-ribosyl)ation per se is required for the inhibition of DNASIL3 endonuclease activity. The presence of recombinant human caspase-3 completely blocks the in vitro inhibition by PARP-1 of DNASIL3 endonuclease activity, suggesting that cleavage and consequent inactivation of PARP-1 by caspase-3 promotes the activation of DNASIL3.[12]

To confirm that the cleavage and consequent inactivation of PARP-1 by caspase-3 are required for DNASIL3 endonuclease activity in vitro, the effect of a partially purified caspase-resistant mutant of PARP-1 (mut-PARP-1), in which the aspartate residue (Asp^{214}) at the caspase-3 cleavage site had been replaced with a glycine residue by site-directed mutagenesis, was investigated. The catalytic activity and structural integrity of this mutant protein were unaffected either by recombinant caspase-3 in vitro or when expressed in osteosarcoma cells induced to undergo apoptosis by staurosporine.[12,19] The caspase-3-resistant mut-PARP-1, which is capable of poly(ADP-ribosyl)ation throughout apoptosis, completely blocked DNASIL3 endonuclease activity in the absence or presence of caspase-3 (Fig. 3B). To demonstrate that inhibition of DNASIL3 by mut-PARP-1 required the catalytic activity of the mutant, the effect of 3-AB was assessed. This drug prevented the inhibition of DNASIL3 endonuclease activity by mut-PARP-1, confirming that poly(ADP-ribosyl)ation is specifically required for the inhibition of DNASIL3. These in vitro results thus indicate that the cleavage and consequent inactivation of PARP-1 by caspase-3 are necessary for the induction of DNASIL3 endonuclease activity.

To examine the role of PARP-1 cleavage in the induction of DNASIL3 endonuclease activity in osteosarcoma cells, we transfected the cells with vectors encoding DNASIL3, mut-PARP-1 (also fused to the His6-FLAG sequence), or both of these proteins. We confirmed the expression of the recombinant proteins in the transfected cells by immunoblot analysis with antibodies to FLAG.[12] This allowed us to next examine the effect of mut-PARP-1 expression on DNASIL3-mediated internucleosomal DNA fragmentation by incubating transfected cells in the presence of etoposide. Expression of mut-PARP-1 together with DNASIL3 blocked etoposide-induced DNA degradation into internucleosomal fragments mediated by the recombinant endonuclease (Fig. 3C), presumably by maintaining it in the poly(ADP-ribosyl)ated (inhibited) state. Expression of mut-PARP-1 alone had no effect on the integrity of DNA in cells cultured in the absence or the presence of etoposide.[12]

PARP-1 Cleavage by Caspases Is Required to Avoid a Stable State of Poly(ADP-Ribosyl)ation and Subsequent Persistent Inactivation of DNASIL3 during Apoptosis

To unambiguously define the relationship between the endonuclease activity and the poly(ADP-ribosyl)ation state of DNASIL3, the endonuclease was precipitated from etoposide-treated osteosarcoma cells expressing the endonuclease in the absence or presence of caspase-resistant mut-PARP-1. The precipitates were then subjected to immunoblot analysis with antibodies to PAR. Cells expressing only DNASIL3 exhibited a transient increase in the extent of poly(ADP-ribosyl)ation of this protein that was apparent after exposure to etoposide for 12 h but not after 24 h (Fig. 4). In contrast, coexpression of mut-PARP-1 was associated with a markedly greater increase in the extent of poly(ADP-ribosyl)ation of DNASIL3 after incubation with etoposide for 12 h, and this level of modification was still apparent at 24 h. These results are totally consistent with the kinetics of DNA fragmentation in the transfected

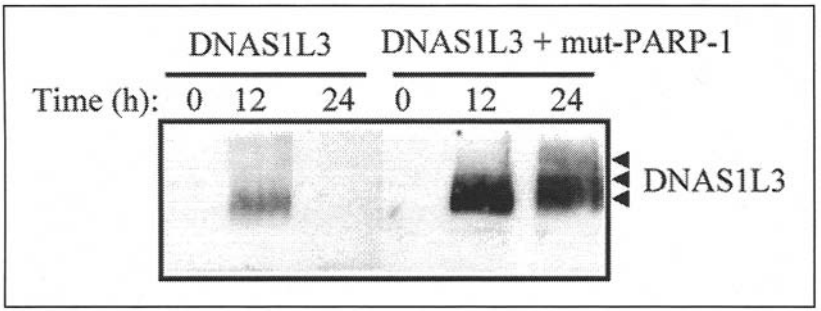

Figure 4. Expression of caspase-resistant mut-PARP-1 induces a persistent poly(ADP-ribosyl)ation of DNAS1L3 during etoposide-induced apoptosis in osteosarcoma cells. Osteosarcoma cells expressing either DNAS1L3 or both mut-PARP-1 and DNAS1L3 were incubated for the indicated times with 70 μM etoposide, after which the recombinant His6-FLAG-tagged proteins were precipitated and then subjected to immunoblot analysis with antibodies to poly(ADP-ribose) polymer (PAR). Reprinted with permission from: Boulares et al, J Biol Chem 277(1):372-378, ©2002 The American Society for Biochemistry and Molecular Biology, Inc.

cells, and confirm, in the context of the intact cell, that hydrolysis of PAR moieties attached to DNAS1L3 by the action of PARP-1 is required for induction of the endonuclease activity of this protein in cells undergoing apoptosis.

This persistent poly(ADP-ribosyl)ation of DNAS1L3 and consequent inhibition of internucleosomal DNA fragmentation in etoposide-treated osteosarcoma cells expressing both DNAS1L3 and mut-PARP-1 was not due to inhibition of the activation of other apoptotic factors, as caspase-3-like activity and resulting cleavage of endogenous PARP-1 were not compromised.[12,13] These results again indicated that the failure of cells expressing both DNAS1L3 and mut-PARP-1 to undergo internucleosomal DNA fragmentation in response to etoposide was strictly related to inhibition of DNAS1L3 by poly(ADP-ribosyl)ation. It is noteworthy, however, that the maximal level of caspase-3-like activity and the extent of cleavage of endogenous PARP-1 were higher in cells expressing only DNAS1L3 than in those expressing mut-PARP-1 alone or both mut-PARP-1 and DNAS1L3. These results are readdressed as they relate to the role of DNAS1L3 in sensitizing cancer cells to etoposide, below.

DNAS1L3 Is Required for Etoposide-Induced Internucleosomal DNA Fragmentation and Increases Etoposide Cytotoxicity in Transfected Osteosarcoma Cells

A seemingly significant and unexpected relationship between DNAS1L3 and the anticancer drug etoposide was observed during a recent investigation with osteosarcoma cells. It was earlier reported that osteosarcoma cells can undergo apoptotic death and internucleosomal DNA fragmentation in response to the protein kinase inhibitor and proapoptotic agent, staurosporine.[13,19] However, when these cells are treated with etoposide, no such DNA fragmentation is observed despite the activation of the apoptotic machinery including activation of caspase-3.[13] The fact that osteosarcoma cells, which do not express DNAS1L3, undergo internucleosomal DNA fragmentation during staurosporine-induced apoptosis indicates the presence of an endonuclease activity that is capable of catalyzing this reaction but which is not responsive to etoposide. These cells gain the ability to degrade their DNA into internucleosomal fragments in response to etoposide only when transfected with an expression vector encoding DNAS1L3, suggesting that this enzyme is required for etoposide-induced internucleosomal DNA fragmentation. Failure to undergo internucleosomal DNA fragmentation was also

observed in other cell lines such as the Daudi, a NonHodgkin's lymphoma cell line, and Raji, a Burkitt's lymphoma cell line (unpublished observations). The reason why etoposide activates DNAS1L3 but not the endonuclease activated by staurosporine is unclear, however, we are actively pursuing this venue as it seems to be quite important for a precise understanding of the relationship between drug action and cellular enzymes.

An example of such intricate relationship between pro-apoptotic drugs and proapoptotic enzymes is the finding that whereas expression of caspase-resistant mut-PARP-1 prevents the induction of internucleosomal DNA fragmentation by etoposide in osteosarcoma cells expressing DNAS1L3, it does not affect that induced by staurosporine in cells not expressing DNAS1L3.[13] These results illustrate that the endonuclease activated during staurosporine-induced apoptosis is distinct from DNAS1L3. Moreover, cleavage of PARP-1 and concomitant cessation of poly(ADP-ribosyl)ation appears to be necessary for DNAS1L3-mediated internucleosomal degradation of DNA. This is consistent with previous in vitro and in vivo observations.

A novel observation concerning the molecular mechanism of an anti-cancer drug is the fact that ectopic expression of DNAS1L3 increased the sensitivity of osteosarcoma cells to etoposide-induced cell death.[13] These results indicate that internucleosomal DNA fragmentation is not merely an end point of apoptosis, but rather contributes to the overall process of cell death. This effect might be attributable to an increased generation of DNA breaks in the transfected cells. These additional DNA breaks may enhance nuclear poly(ADP-ribosyl)ation and thereby result in an increased rate or extent of NAD depletion, which culminates in collapse of the mitochondrial membrane potential and release of cytochrome c. Resistance of cancer cells to chemotherapeutic drugs has been associated with altered drug metabolism (which has been attributed to increased expression of the P-glycoprotein encoded by the *MDR* gene), with increased redox detoxifying action of glutathione, with increased hepatic cytochrome P450 activity, and with mutation of topoisomerase II. Resistance of cells to apoptosis has been attributed to increased expression either of anti-apoptotic factors, such as members of the Bcl-2 family of proteins, or of proliferative factors such as phosphoinositide 3-kinase and the protein kinase Akt. Our results now suggest that loss of expression or inactivation of endonucleases also might contribute to reduced sensitivity of cells to drug-induced apoptosis.

Induction of Internucleosomal DNA Fragmentation by Acetaminophen Treatment Is Associated with DNAS1L3 Expression

DNAS1L3 expression is highly expressed in liver and to a lesser extent in kidney.[10,26] One of our laboratories has maintained a continuous interest in nonmetabolized acetaminophen toxicity in cultured cells and the mechanism by which high concentrations of this drug, mimicking overdose in animal models and humans, induce cell death.[14,33-35] Given the fact that liver and kidney are the major target organs for acetaminophen toxicity and that such toxicity has been associated with a putative but uncharacterized Ca^{2+} and Mg^{2+}-dependent endonuclease,[36-39] we recently investigated a potential role for DNAS1L3 in acetaminophen-induced apoptosis and specifically internucleosomal DNA fragmentation.[14] An opportunity was seized to employ HeLa cells, which do not express DNAS1L3[11] in this investigation. The treatment of HeLa cells with acetaminophen results in internucleosomal DNA fragmentation only after transfection of these cells with a plasmid encoding the DNAS1L3 gene suggesting that this endonuclease is required for acetaminophen-induced internucleosomal DNA fragmentation. Consistent with the results described above utilizing etoposide-treated osteosarcoma cells, DNAS1L3 expression also potentiated the cytotoxic effect of acetaminophen in HeLa cells suggesting an active role in the death process induced by these drugs.[14] Although more studies should be performed directly with liver or kidney cells, the results of these studies strongly suggest that DNAS1L3 may be an important factor in acetaminophen-induced internucleosomal DNA fragmentation and its overall cytotoxicity.

Caspase-3 Activation, Cleavage of DFF45, and Degradation of DNA into 50-kb Fragments Are Insufficient for Induction of Internucleosomal DNA Fragmentation in Etoposide-Treated Osteosarcoma Cells

A number of laboratories, including ours, have demonstrated that activation of caspase-3 and the consequent cleavage of DFF45 are necessary for internucleosomal DNA.[6,9,32,40,41] A key point which required clarification was whether osteosarcoma cells were defective in undergoing key events in the apoptotic process including activation of caspase-3 and DFF40 and cleavage of DNA into large 50 kb fragments in response to treatment with etoposide. Accordingly, these apoptotic events were examined. Etoposide treatment induced a significant activation of caspase-3, which culminated in an actual cleavage of the DFF40 inhibitory subunit and caspase-3 substrate, DFF45, as well as cleavage of chromatin into large fragments comparable to those observed in staurosporine-treated osteosarcoma cells. A similar rationale was followed to examine the reason behind the failure of HeLa cells to undergo internucleosomal DNA fragmentation in response to either acetaminophen[14] or etoposide (unpublished observation). Our studies indicate that neither impaired activation of caspase-3, defective cleavage of DFF45, nor cleavage of chromatin into 50 kb fragments appear to underlie the failure of these cells to undergo internucleosomal DNA fragmentation during apoptosis. Of potential importance, the abundance of the 50 kb DNA fragments accumulates in cells that fail to undergo internucleosomal DNA fragmentation. These results suggest that etoposide-treated osteosarcoma and acetaminophen or etoposide-treated HeLa cells fail to process the 50-kb DNA fragments into internucleosomal fragments. However, these fragments are processed when the cells are transfected with a plasmid encoding DNAS1L3 (unpublished observation). Treatment of cells undergoing apoptosis with the inhibitor of intracellular release of Ca^{2+}, BAPTA, inhibited DNAS1L3-mediated internucleosomal DNA fragmentation, but exhibited little to no effect on the generation of 50 kb DNA breaks (unpublished data) further supporting the notion that the two endonucleases are distinct. In summary, the conclusion of the current status of work in this field supports the hypothesis that chromatin degradation into large 50 kb DNA breaks and 200 pb repeats representing internucleosomal fragments is mediated by the cooperation between two distinct endonucleases. DFF40 is required for both processes while DNAS1L3 mediates internucleosomal DNA fragmentation and is predominantly active in a nonpoly(ADP-ribosyl)ated state after cleavage of PARP-1 by caspases.

Conclusion and Future Directions

The involvement of PARP-1 and poly(ADP-ribosyl)ation in the regulation of Ca^{2+} and Mg^{2+}-dependent endonuclease activity was observed on 1970s by Koide, Yoshihara, and coworkers. The bovine and murine endonucleases were subsequently purified in the 1980s and 1990s. We and others focused on cloning and characterizing the human homolog of this enzyme. Based on the initial observation by Kauffman and coworkers[17] that PARP-1 was cleaved by caspases, which separates the DNA binding domain from its catalytic site, thus destroying its poly(ADP-ribosyl)ation potential, it seemed logical that one key late apoptotic nuclease may be a natural substrate for intact PARP-1 and may be released from catalytic inhibition subsequent to PARP-1 cleavage. Accordingly, a number of groups, including ours, began directly testing this hypothesis. As detailed above, we were able to clone, characterize, and ultimately identify DNAS1L3 as the human homolog of this endonuclease and were thus able clarify and to clearly define its regulation by poly(ADP-ribosyl)ation and how this process plays an active role during apoptosis. The results of a concerted series of investigations in our laboratory as well as contributions on similar endonucleases from sources other than human allow us to postulate a plausible scenario. DNAS1L3, in response to apoptotic stimuli that cause a release of intracellular Ca^{2+}, introduces a number of DNA strand breaks, which progressively and efficiently activate PARP-1. PARP-1, in turn, catalyzes a posttranslational modification of DNAS1L3 leading, at this critical stage of

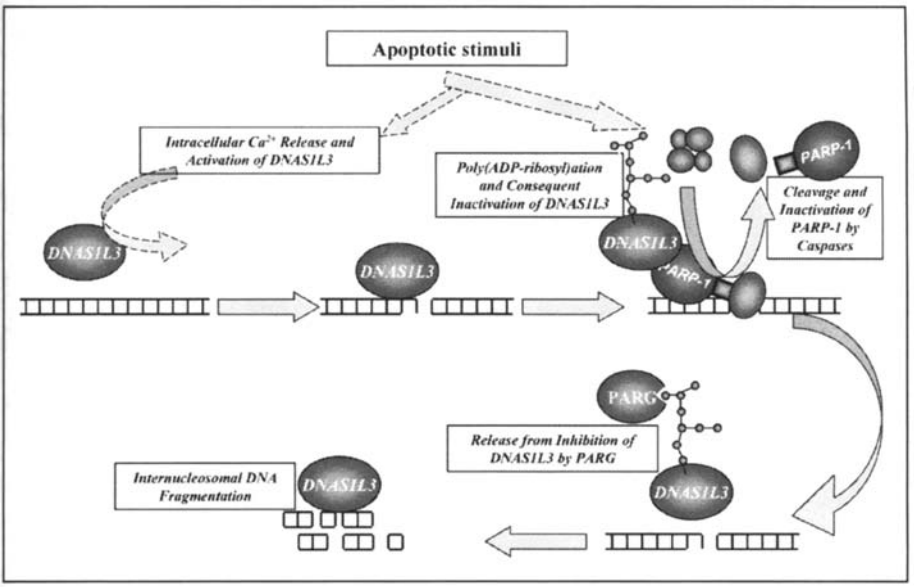

Figure 5. Model for the role of DNAS1L3 in DNA fragmentation during apoptosis and its regulation by poly(ADP-ribosyl)ation. Apoptotic stimuli that cause an increase in the intracellular concentration of Ca^{2+} lead to an initial activation of DNAS1L3, which introduces strand breaks into genomic DNA, consequently triggering the activation of PARP-1. Poly(ADP-ribosyl)ation of DNAS1L3 by PARP-1, in turn, results in inhibition of nuclease activity. Subsequent cleavage and inactivation of PARP by caspases prevent further poly(ADP-ribosyl)ation of nuclear proteins, thereby allowing the activity of PARG to remove polymer from these proteins and thereby release DNAS1L3 from inhibition. The activated nuclease may then catalyze the internucleosomal DNA fragmentation characteristic of the later stages of apoptosis. Dotted arrows indicate multistep and complex processes.

apoptosis, to the inhibition of its endonuclease activity (Fig. 5). This cross-talk between PARP-1 and DNAS1L3 may represent an attempt by PARP-1 to prevent an untimely activation of DNAS1L3 providing the cell with a pause to make a decision to die or to attempt a repair process.

The relationship between DNAS1L3 and PARP-1 is undoubtedly complex since it clearly involves many other factors such as chromatin structure, histones, and other DNA repair enzymes, as well as other endonucleases such as DFF40. In this regard, we recently proposed the existence of an amplification phase of TNF-induced apoptosis, which involves PARP-1, poly(ADP-ribosyl)ation, and DFF40.[9,32] There is also a potential relationship between all of these factors and DNAS1L3 with regard to the involvement of DFF40 in triggering the activation of PARP-1 and the consequent poly(ADP-ribosyl)ation of acceptor proteins which clearly include DNAS1L3. To decipher these complex relationships will undoubtedly require extensive further investigations, which are underway. Overall, our work and that of several other laboratories indicate that the role of PARP-1 is certainly not negligible but rather crucial in the active process of cell death and in regulating pro-apoptotic factors such as DNAS1L3.

Our work also suggests that endonucleases such as DNAS1L3 might play an active role in apoptotic cell death and that loss of expression or inactivation of endonucleases may contribute to reduced sensitivity of cells to drug-induced apoptosis. Defects in endonucleases have been associated with several debilitating diseases including several autoimmune diseases and cancer. Recently, Wilber et al[42] have reported that a loss in DNAS1L3 is the primary reason for the susceptibility of MRL and NZB/W F1 mice to lupus.[42] Many questions remain to be answered with regards to the role of DNAS1L3 during apoptosis and genome degradation both at the cell and tissue level.

Acknowledgements

We would like to thank all the scientists, postdoctoral researchers, and research technicians of the different laboratories who contributed to the work described in this review. This work was supported in part by grant 1P20RR18766 and HL072889 from the National Health Institute (USA) and funds from Louisiana State University Health Sciences Center, New Orleans, LA, (USA) to H. Boulares, and grants from the National Health Institute (PO1CA-74175 and CA25344), the U.S. Air Force Office of Scientific Research (AF-F49620-98-1-042), and the U.S. Army Medical Research and Development Command (DAMD17-95-C-5001) to M. Smulson.

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