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REVIEW

Endonucleases and Apoptosis in Animals

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Abstract—Endonucleases are the main instruments of obligatory DNA degradation in apoptosis. Many endonucleases have marked processive action; initially they split DNA in chromatin into very large domains, and then they perform in it internucleosomal fragmentation of DNA followed by its hydrolysis to small fragments (oligonucleotides). During apoptosis, DNA of chromatin is attacked by many nucleases that are different in activity, specificity, and order of action. The activity of every endonuclease is regulated in the cell through its own regulatory mechanism (metal ions and other effectors, possibly also S-adenosylmethionine). Apoptosis is impossible without endonucleases as far as it leads to accumulation of unnecessary (defective) DNA, disorders in cell differentiation, embryogenesis, the organism's development, and is accompanied by various severe diseases. The interpretation of the structure and functions of endonucleases and of the nature and action of their modulating effectors is important not only for elucidation of mechanisms of apoptosis, but also for regulation and control of programmed cell death, cell differentiation, and development of organisms.

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Endonucleases, enzymes responsible for hydrolysis of nucleic acids, play an important role in the vital activity of cells and of animal organisms. Endonucleases are involved in many genetic processes, such as replication, repair, and recombination, and they are active contributors of nucleic metabolism degrading nucleic acids to low-molecular-weight fragments and mononucleotides, which are utilized for resynthesis of new nucleic acid molecules. Many endonucleases are induced or activated during programmed cell death (PCD). Usually their action is processive with the chromatin decomposing through hydrolysis of DNA (initially it is cleaved into large domains, then internucleosomal fragmentation of DNA occurs, and finally DNA is hydrolyzed to low-molecular-weight fragments to be utilized).

Programmed cell death is an essential and necessary element in the development of animals. Disorders and distortions in apoptosis prevent normal development of the organism or lead to various diseases. At present, three main types of the death and elimination of undesired or damaged cells have been described: apoptosis, autophagy, and necrosis [1]. Apoptosis is accompanied by specific changes in the structure and organization of the cytoplasm, nucleus, chromatin, and DNA. In apoptotic cells, synthesis of nuclear DNA ceases, chromatin is condensed and marginated, DNA is fragmented into nucleosomes, and the cell membrane is wrinkled with subsequent arising of so-called apoptotic bodies, which are produced upon disintegration of the dying cells, and these bodies are engulfed by macrophages or other cells [2, 3]. Autophagy is cell death related with autophagosomes–autolysosomes [4]. Necrosis was considered as a type of spontaneous cell death often caused by an insuperable stress; necrosis is characterized by cell swelling and lysis of cell organelles and of the cell itself. Now programmed necrosis has been proved to exist, and it is termed necroptosis [5-7].

Molecular mechanisms of apoptosis are now studied in significant detail, whereas mechanisms of necrosis and of autophagy-mediated cell death are less clear. The concrete program of the development of an organism, specific features of cell physiology, or momentary environmental conditions suggest the possibility and necessity of interaction of these three forms of cell death.

The apoptotic program can be triggered both by external signals interacting with specific receptors on the cell surface (Fas) and thus activating caspase 8 or 10 [8]

Abbreviations: CAD, caspase-activated DNase; DNA-PK, DNA-dependent protein kinase; DSB, double-stranded break in DNA; EndoG, endonuclease G; GAAD, granzyme A-activated DNase; ICAD, inhibitor of caspase-activated DNase; NLS, nuclear location signal; PCD, programmed cell death.

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and by internal stimuli, such as the release of cytochrome *c* from mitochondria and the associated activation of caspase 9. Cell suicide can be also induced without involvement of caspases; thus, an idea appeared about a caspase-independent cell death, and studies on degradation of specialized cells have revealed other pathways of cell death that are morphologically similar to apoptosis but differ from it in signaling pathways and specific executors (in particular, this is observed on the elimination of epithelial cells, cornification of keratinocytes, death of nervous cells, and cell cannibalism at entosis) [1].

There is a large amount of data on inducers, signaling pathways, and mechanisms of activation and course of apoptosis [9], but we have rather poor knowledge about the terminating stages of apoptosis whose consequences and unique functional role for the organism are still underestimated.

It seems that nothing more can be expected upon the termination of cell apoptosis. However, apoptosis once started has to be terminated "correctly" and be accompanied not only by the observable elimination of the cell and its elements, but also by complete removal of its genetic information represented by its DNA.

Just the destruction of DNA is a necessary terminal stage of PCD: for the dying cell, it serves as a crucial point of return failure, and observed internucleosomal fragmentation of DNA is generally considered to be a molecular marker of apoptosis. Such destruction of DNA is a necessary and obligatory condition for retaining homeostasis of the total organism [10]: the presence of nonhydrolyzed DNA in the extracellular space induces activation of natural immunity and later can promote the development of autoimmune diseases.

The degradation of DNA during PCD is contributed to by many nucleases of different families. These enzymes can be divided into two groups: autonomous cellular DNases, which hydrolyze DNA inside the dying cell, and scavenger nucleases, which hydrolyze DNA inside phagocytes and in the extracellular space [11]. Depending on the type of cells, stage of their differentiation, and/or external stimuli, various nucleases or sets of nucleases are functioning, but the nature, properties, action mechanisms, and regulations of specific endonucleases are still poorly studied [12].

ENDONUCLEASES CLEAVING TO LARGE FRAGMENTS

The degradation of DNA in apoptotic cells is started from cleavage of DNA into long fragments of 50-300 kb at AT-enriched sites (loop domains) in regions of DNA attachment to the nuclear membrane, which manifests itself as chromatin condensation [13, 14]. The nucleases cleaving DNA into high-molecular-weight fragments are called "domain nucleases" [15]. DNase I [16], DNase II [17], cyclophilins [18], and DNase γ [19] were proposed as "domain nucleases", but none of these is fully suited to the parameters of just apoptotic nucleases. Thus, DNase I is a secreted protein, and its expression is organ-specific (kidneys, pancreas, seminal plasma). DNase II is localized in lysosomes, but the primary apoptotic destruction of DNA occurs under conditions of lysosome integrity, and this enzyme is more likely to hydrolyze fragmented DNA in phagocytes.

To date, the only endonuclease activated during apoptosis that cleaves into large fragments that has been identified is granzyme-activated DNase (GAAD), or NM23-H1 [20]. This endonuclease is latently present in the cell as a component of the "SET complex" associated with the endoplasmic reticulum. Under the influence of granzymes A or K, the SET protein is cleaved and the activated endonuclease NM23-H1 is translocated into the nucleus, where it causes single-stranded breaks in the chains of nuclear DNA resulting in appearance of multikilobase fragments [20, 21].

The nuclease LDFF specific for the first stage of DNA degradation in apoptosis induced by exogenous cytochrome *c* has been revealed in extracts from sea urchin oocytes. LDFF cleaves DNA into fragments with length of ~50 kb. Biochemical properties of LDFF are similar to those of the acidic lysosomal DNase II: it is resistant to the specific inhibitor of CAD, depends on Mg^{2+} (but not on Ca^{2+}), and is stable at 45°C and pH 5.0. Moreover, LDFF is activated by caspase 3 [22].

The induction of apoptosis by agents that cause double-stranded breaks (DSB) in DNA, including etoposide and staurosporine, is associated with a rapid mobilization into the DSB zones of proteins involved in the repair by joining nonhomologous ends. The fate of the cell – either the repair of DNA or apoptosis – depends on the degree of damage (dose of the agent). In addition to the DNA-dependent protein kinase (DNA-PK), the repair complex also contains multifunctional enzymes encoded by the family of SNM1 genes (KU70/80, SNM1A, SNM1B/Apollo, and Artemis), which possess nuclease activities. The hypothesis of the possible involvement in this process of the nuclease Artemis (5′-3′ exonuclease) has been confirmed. According to a scheme proposed in [23], DNA-PK, which regulates the availability of DNA to enzymes responsible for processing, is dislocated to DSBs initiated in regions of DNA attachment to the template (a minor fraction of the DNA-PK pool is probably always present in these zones), binds with DNA, and phosphorylates H2AX histones around the DSB. This results in activation of the endonuclease activity of Artemis associated with the catalytic unit of DNA-PK. Moreover, in regions of scaffold attachment to the template, DSBs induce negative "twisting" tension, which initiates single-stranded breaks with subsequent formation of secondary DNA structures, hairpins and stalk loops, which are preferential substrates for Artemis. Thus,

the DNA-PK-dependent activation of Artemis can accelerate region-specific cleavage of DNA into fragments with lengths of 50-300 kb. Recently, the enzymes SNM1A, SNM1B/Apollo, and Artemis were shown to independently participate in etoposide-induced apoptosis [24]. Because Artemis contributes to DNA fragmentation during early stages of apoptosis, when it is still reversible, it is supposed that Artemis might be involved in site-specific cleavage of chromosomes during early stages of apoptosis also induced by other agents (factors).

Apoptosis-inducing factor (AIF), a flavoprotein localized in the intermembrane space of mitochondria, seems to be a possible mediator of "domain nucleases". The induction of apoptosis by agents affecting the transmembrane potential of mitochondria results in disorders in the outer membrane permeability and in the release of AIF, which is subsequently translocated into the nucleus [25, 26]. The adaptogenic properties of AIF are determined by its ability to bind DNA, and not by its activity as an oxidoreductase [27].

Note that under the influence of certain inducers of apoptosis, DNA degradation in neurons is limited by cleavage into high-molecular-weight fragments that is not followed by hydrolysis into oligosomal fragments [28, 29]. It was supposed that topoisomerase II alpha should be involved in this scenario of apoptotic degradation of DNA [30]. Later it was shown that, as discriminated from apoptosis associated with the internucleosomal degradation of DNA, the apoptotic pathway associated with the excision of chromatin loop domains does not depend on caspases and is contributed to by protein kinase p38/JNK from the MAPK family, some apoptotic factors of mitochondria, and topoisomerase II. This pathway of apoptosis is realized independently or in parallel with the caspase-dependent pathway. Thus, the caspase-independent excision of chromatin loop domains is the major mechanism of the DNA disintegration inside staurosporine-treated neuroblasts, whereas in promyelocytes this inducer of apoptosis also causes internucleosomal (oligosomal) fragmentation of DNA [31].

THE MAJOR APOPTOTIC ENDONUCLEASE CAD AND ITS INHIBITOR ICAD

The development of *in vitro*-inducible apoptotic systems allowed apoptotic nucleases to be isolated and characterized, and DNase, or the DNA fragmentation factor (DFF) [32], or the caspase-activated DNase (CAD) [33] that is sometimes termed "professional apoptotic nuclease" [34] was the first one isolated. CAD is a nuclease of the ββα-Me superfamily and is a Mg^{2+}-dependent endonuclease strictly specific to double-stranded DNAs. CAD cannot hydrolyze single-stranded DNAs or single- and double-stranded RNAs or RNA–DNA heteroduplexes. Note that all oligonucleotides that are not hydrolyzed by CAD can inhibit the cleavage of double-stranded DNA [35]. CAD cleaves DNA producing 5′-P and 3′-OH groups, and the two DNA chains are hydrolyzed simultaneously. Moreover, CAD exclusively prefers internucleosomal linker regions of chromatin that are in particular determined by the structure of the scissors-like active site of the nuclease [36]. This conformation of the enzyme makes impossible the cleavage of DNA inside nucleosomes, and therefore the DNA chains are cleaved at internucleosomal regions resulting in the appearance of fragments with the lengths that are multiples of ~180 bp (the size of DNA region on the nucleosome).

In non-apoptotic cells, the 40-kDa peptide CAD is co-expressed with its chaperon-inhibitor ICAD. Upon their synthesis, the heterodimer CAD/ICAD is immediately produced. Each subunit has its own nuclear location signal (NLS) but cannot independently penetrate into the nucleus because the NLS can be exposed only upon the change in the peptide conformation, which occurs on complexing with ICAD [37]. It was thought earlier that the CAD/ICAD complex should be located in the cytoplasm [38, 39], but later this complex was clearly shown to be located in the nucleus [40]. The idea about CAD binding with chromatin only after induction of apoptosis was invalidated: the CAD/ICAD complex is bound with chromatin in non-apoptotic cells, and CAD is activated in the chromatin-bound state [41, 42]. Experiments *in vivo* on the human cell culture [43] and *in vitro* with a recombinant CAD/ICAD complex revealed that apoptotic inactivation of caspase 3 leads to proteolysis of ICAD at two specific sites (aspartate residues D117 and D224) and release of the active endonuclease CAD. Caspase 7 can also hydrolyze ICAD, but less efficiently than caspase 3 [44, 45]. Upon dissociation of the CAD/ICAD complex, the nuclease CAD undergoes conformational changes and produces homooligomers that are enzymatically active forms of CAD and are stabilized depending also on the presence of trace amounts of zinc [43, 46, 47].

The activity of CAD is mainly modified by caspase 3 and its inhibitor ICAD, which performs the double function being concurrently a molecular chaperon responsible for the correct folding of the CAD molecule directly on the ribosome [48]. There are two ICAD isoforms in cells: the long ICAD-L (a 45-kDa peptide) and the short ICAD-S (a 35-kDa peptide), and the CAD activity is mainly regulated by ICAD-L [49]. ICAD-S appears as a result of alternative splicing and is deprived of the C-terminal NLS, which leads to localization of this isoform in the cytoplasm [46]. ICAD-S cannot function as a chaperon for folding CAD: on ICAD-S expression in ICAD(–/–) embryonic mouse fibroblasts, no active CAD is produced [50, 51]. However, ICAD-S can inhibit CAD *in vitro* [52], and it prevents CAD activation in mouse neurons [53]. On inducing apoptosis in DT40 cells *in vivo*, ICAD-S

resistant to caspase 3 due to substitution of the caspase 3 cleavage sites by sites sensitive to TEV protease inhibits the activity of CAD released from the CAD/ICAD-L complex [51]. Nuclei of non-apoptotic cells are shown to contain a small number of free ICAD-L molecules, which together with the cytoplasmic isoform of ICAD-S can constitute a system responsible for protection of the cell against activated CAD molecules. Their appearance in non-apoptotic cells can occur on an accidental or a short-term activation of caspases 3 or 7, rare spontaneous changes in the CAD conformation associated with generation of an autocatalytic form or as a result of an extremely rare dissociation of the CAD/ICAD complex [54].

Some proteins contributing to regulation of CAD activity have been identified [55]. They can be subdivided into three functional groups: factors responsible for the control of correctness of protein folding and cellular localization, nuclease activators, and nuclease inhibitors. During translation, the correctness of assemblage of the CAD/ICAD heterodimer is controlled by chaperons HSC70 and HSP40, whereas the delivery of the complex into the nucleus depends on binding with an importin α/β heterodimer [37].

The cleavage of DNA by the CAD endonuclease is stimulated by chromatin proteins, histones H1 and HMGB1/2 and topoisomerase II; and histones H1 and topoisomerase II directly interact with CAD, whereas HMGB1/2 influences CAD activity indirectly by promoting the availability of the substrate [56-58]. CAD was shown to bind with all known subtypes of histone H1 (H1.1, H1.3, H1.5, and H1.0), but the interaction with a specific H1 subtype depends on the inducer of apoptosis [59]. The histones H1 and topoisomerase II are also likely to contribute to the catalytic specificity of CAD: the histones determine the internucleosomal fragmentation of DNA and formation of characteristic "ladder" of DNA fragments with length being multiples of 180 bp, whereas topoisomerase II stimulates hydrolysis of chromatin loop domains in the early stages of its enzymatic degradation. Phosphorylation of histone H2A.X is also required for successful cleavage of chromatin by CAD. CAD is inhibited by some nuclear proteins: the nucleophosmin/B23 complex with phosphatidylinositol-3-kinase, the Ebp complex with AKT, CIIA [51, 60].

The use of di- and trihybrid yeast systems for screening libraries of cDNA from HeLa cells and human brain tissues revealed some new regulators of CAD activity. One of them, GFAP, binds with ICAD and prevents its cleavage by caspase 3; the other supposed regulator, DRIG, interacts with the CAD/ICAD heterodimer, whereas its antisense RNA influences ICAD hydrolysis and cleavage of chromatin in apoptotic HeLa cells [61]. Moreover, CAD activity is suppressed by heparin, some polyanions, and poly(ADP-riboso)polymerase [62]. The transmembrane protein CD45 and its substrates are shown to retain CAD in the nucleus of apoptotic cells [63].

The manner of DNA cleavage by the CAD endonuclease is similar to the cleavage by micrococcal nuclease, which has long been used to analyze chromatin structure. But, as differentiated from this nuclease, CAD does not cleave DNA inside nucleosomes with production of subnucleosomal fragments; it is more specific to linker regions between nucleosomes, lacks exonuclease activity, is specific to double-stranded DNA, and hydrolyzes H1-containing chromatin more efficiently [62]. All these data suggest CAD as a promising tool to study chromatin structure. Cloning and sequencing of cDNA of CAD and cDNA of ICAD, as well as creating recombinant vectors for co-expression of these proteins in *Escherichia coli*, make this suggestion commercially viable [64].

Recently, CAD has been reported to be involved in processes associated with other vitally important functions of the organism, including cell differentiation. Thus, CAD activated by caspase 3 during early stages of differentiation of skeletal muscles has been shown to induce appearance of transient breaks in DNA throughout the whole genome. Inactivation of CAD terminated differentiation nearly completely. Breaks in DNA chains were also detected in the promoter of the cell cycle regulatory factor p21, and their appearance seemed to be associated with induction of the expression of gene p21 [65]. The sequencing of DNA fragments resulting in actinomycin D-treated leukemic cells HL-60 (the Apoptoseq approach) led to design of a global map of CAD-initiated apoptotic breaks in DNA [66]. The distribution of hydrolysis sites in DNA during apoptosis was shown to be not sporadic, but rather associated with active genes and open regions of chromatin, and a significant fraction of the breaks occurs in the binding sites of transcription factors. Extensive apoptotic cleavage occurs in genes that are translocated more frequently during human carcinogenesis. Thus, the specific fragmentation of DNA during apoptosis is supposed to contribute to gene translocation and development of tumors [66].

ENDONUCLEASE G (ENDO G) IS ANOTHER MAJOR APOPTOTIC DNase

Depending on the conditions inducing apoptosis, on inactivation of CAD/ICAD the nuclear DNA inside the apoptotic cells either remained unfragmented inside the apoptotic cell and was degraded in phagocytes [67], or DNA was fragmented into oligosomes. These studies revealed in HeLa cells another apoptotic DNase, endonuclease G (EndoG) [68], which was initially identified in normal non-apoptotic cells as a nuclear protein [69-71].

The functioning of this nuclease is thought to be associated with a caspase-independent apoptosis [72, 73] induced by oxidative stress [74], hypoxia [75], and ischemia [76].

Homologs of EndoG are described in bacteria (NucA) including *Anabaena* and *Serratia*, yeast (Nuc1p) [77-79], *Leushmania* [80], invertebrates (the nematode *Caenorhabditis elegans*) (CPS-6) [81], the fungus *Aspergillus nidulans* (NucA) [82], and in plants [83]. In the cells of animals, a paralog of EndoG, EXOG, has been found, the appearance of which is believed to be due to duplication of a common ancestral gene [84]. All these enzymes are assigned to the EndoG subfamily within the superfamily of ββα-Me DNA/RNA nonspecific nucleases [36]. Studies on the crystalline structure of the mouse recombinant EndoG (at resolution of 2.8 Å) required for crystallization of the Cys110 residue [85] and CPS-6 (resolution 1.8 Å) have demonstrated mixed αβ topology with two ββα-metal-containing nuclease motifs located distally on two sides of the dimeric enzyme [86].

EndoG and its homologs in eukaryotes are located in the intermembrane space of mitochondria. These nucleases are synthesized in the protoplasm as a 33-kDa propeptide containing the mitochondrial-signaling N-terminal sequence; during the translocation into mitochondria, protein processing occurs accompanied by production of a mature 28-kDa nuclease. EndoG release from mitochondria and its subsequent transfer into the nucleus are associated with damages of the outer mitochondrial membrane.

The human endonuclease EndoG is characterized in most detail. The enzyme is a homodimer lacking specificity to the carbohydrate component of nucleic acids; it can cleave RNA and also single- and double-stranded DNAs. Similarly to CAD, this endonuclease is active at neutral pH values, its activity depends on Mg^{2+}, is stimulated by Mn^{2+}, and is inhibited by millimolar concentrations of Zn^{2+}. On hydrolysis of double-stranded DNAs, the enzyme displays two pH optimums, at 9.0 and 7.0. Similarly to CAD, EndoG cleaves substrates with production of oligonucleotides with 5′-P and 3′-OH-terminal groups, which are substrates for terminal deoxynucleotide transferase (this is the basis for the TUNEL approach). As differentiated from CAD, the action of EndoG results in production of both double- and single-stranded breaks [62, 87]. The enzyme activity is relatively low at physiological values of ionic strength, and it is significantly higher on hydrolysis of single-stranded rather than of double-stranded DNA. In double-stranded DNAs, EndoG the most frequently cleaves the regions $(dG)_n \cdot (dC)_n$, and in single-stranded DNAs the $(dC)_n$ regions [69]. Just here breaks preferentially occur in the S regions of DNA (regions of DNA recombination on switching over Ig classes) and V(D)J regions of DNA (regions of somatic hypermutations). In these GC-enriched regions of DNA R-, G-loops, and stalk–loop structures are formed, which are markedly preferred by EndoG. This depicts the enzyme as a structure-specific nuclease [88]. In "naked" DNA, EndoG preferentially detaches the 5′-terminal G. The substitution of conserved residues of histidine, asparagine, and arginine by alanine has shown that for catalytic and substrate specificity of EndoG the His141, Asn163, and Asn172 residues in the H–N–N motif are essential, as well as the Asn251 residue located in the C-terminal part; residue Glu271 is also important for binding metal ions [89].

Recombinant EndoG, EXOG, Nuc1p, and CPS-6 are similar in their biochemical features. Except for CPS-6, these nucleases are characterized by a wide range of optimal concentrations of Mg^{2+} (0.2-5.0 mM). In the presence of physiological concentrations of Mg^{2+} (0.8-1.0 mM), all of these enzymes can catalyze cleavage of the phosphodiester bond with nearly maximal activity. Only EXOG is noticeably active in the presence of Ca^{2+} (1.0 mM). Despite similar structure of the active center of nucleases of the ββα-Me superfamily, these nucleases are different in affinities to individual metal ions, and none of them need univalent cations for manifesting the maximal activity. At physiological concentrations of NaCl (100-150 mM), all these enzymes demonstrate up to 50% of their maximal activity. These nucleases contact their substrates mainly through electrostatic interaction with their phosphodiester skeleton. At weakly acidic pH values, these enzymes cleave single-stranded but not double-stranded DNAs [90].

Comparative analysis of recombinant mitochondrial endo/exonucleases of the EndoG subfamily has revealed their differences in the ability of endo- and exonucleolytic processing different DNA substrates: EndoG of mammals has only endonuclease activity, whereas its homologs also have the exonuclease activity [90].

On treatment with recombinant EndoG of nuclei from HeLa cells and separation of the hydrolysis products by pulsing-field or two-dimensional electrophoresis, at the very start of hydrolysis the picture corresponded to the first stage of DNA degradation in an apoptotic cell: DNA was hydrolyzed into elongated fragments with the length of ~50 kb. It seemed that the enzyme initially attacked hypersensitive sites on the boundary between chromatin domains. The further hydrolysis occurred by DNA linkers with production of single-stranded breaks [87] and appearance of characteristic "ladders" of oligonucleosomal DNA fragments and their deeper hydrolysis as a result of the combined action of EndoG with other nucleases.

Until recently, the manner of EndoG binding and hydrolyzing DNA was unclear. The CPS-6 enzyme from the nematode *C. elegans* exemplified preferential binding with the G-sulcus of DNA in a low-salt buffer at pH 7.0. The structural model of the CPS-6–DNA complex suggested that a positively-charged DNA-binding "sulcus" should exist near the Mg^{2+}-binding active site. Mutations of the Phe122, Arg146, Arg156, and Phe166 residues noticeably decreased the GPS-6 binding and its nuclease activity, and consequently these amino acid residues are crucial for the interaction of the enzyme with DNA [86].

Effectors involved in the functioning of EndoG homologs are insufficiently studied. The apoptosis-inducing factor (AIF) is mainly responsible for the effect of EndoG. This protein is translocated into the cell nucleus upon its releasing from mitochondria, and this triggers the initial stage of chromatin condensation associated with the DNA cleavage into long fragments [91]. The AIF-related protein WAH-1 activated in *C. elegans* GPS-6 is homologous to EndoG [92]. Later, EndoG was shown to form complexes with AIF and endonuclease FEN1 [93]. But earlier hydrolysis of nuclear DNA was shown to occur as a result of the combined action of EndoG, exonuclease ExoIII, and DNase I in the presence of specific effectors [87]. By means of confocal fluorescence microscopy, EndoG was shown to interact with histone H2B and DNA topoisomerase-alpha during apoptotic cell death, and corresponding mathematical models were designed. EndoG and DNA topoisomerase-alpha are supposed to form a degradation complex, which also includes AIF and cyclophilin A [94].

Endonucleases similar in features with EndoG are involved in apoptosis in plants [95-97]. Their action is modulated by histones (histone H1) [98] and also by short biologically active peptides [99]. These Ca^{2+}/Mg^{2+}-dependent plant endonucleases have a pronounced processive effect, they are markedly site-specific in early stages of DNA hydrolysis [100], sensitive to the state of DNA methylation, and are differently controlled by S-adenosyl methionine (SAM) [101, 102].

The new data allow us to speak about the discovery of a new regulatory mechanism of eukaryotic endonuclease activities. This partially suggests their relation with typical bacterial restriction endonucleases and indicates that higher eukaryotes (plants) can possess at least elements of the host's restriction–modification (R-M) system of the genome. But up to now it is unclear whether animal endonucleases can similarly to the described plant enzymes possess pronounced site-specific activities, be modulated by S-adenosyl methionine, and recognize substrate DNAs by the methylation state. Our preliminary data (unpublished) suggest that the action of EndoG from rabbit liver mitochondria can be modulated by SAM.

Only scarce evidence also existed about the mechanism for protection its own DNA in healthy cells against EndoG in the case of its slight "leakage" from mitochondria. It seemed that the localization of EndoG in the intermembrane space of mitochondria should suggest the isolation of the nuclease and the absence of need for other mechanisms for protection of both mitochondrial and nuclear DNAs. However, a specific inhibitor of EndoG, EndoGI, has been detected in the cells of *Drosophila melanogaster*. This inhibitor is encoded by the gene *cg4930*, and its micromolar concentrations are present in the nuclei of normal cells. EndoG and EndoGI form a 2 : 1 complex where the dimer EndoG is bound with two tandemly repeated homologous domains of the monomer EndoGI. On induction of apoptosis, the content of the inhibitor EndoGI in the nucleus is sharply decreased, and it is released into the cytoplasm. It was shown that *cg4930* mutants are characterized by a markedly decreased viability [103]. Analysis of the three-dimensional crystalline structure of the EndoG/EndoGI complex confirmed the supposed mechanism of nuclease inhibition: EndoGI blocks the active sites of two EndoG monomers and the nucleotide-binding sulcus. The EndoGI structure is different from the structure of the earlier described nuclease inhibitors, and it seems to have been formed during evolution independently of other ancestral bacterial inhibitors [104].

EndoG is functionally the most important and active nuclease in mitochondria of all eukaryotes, and it is well presented in extracts from eukaryotic cells. EndoG is expressed in all eukaryotes, and its gene is highly conserved in various organisms from yeast to primates. It was reasonable to suppose that EndoG should be also involved in other vitally important processes [72].

Functions of the mitochondrial enzyme Nuc1p from budding yeast are the best studied. In addition to lethal function in the course of cell death, the Nuc1p nuclease was shown to be also needed for normal cell proliferation [78, 79]. A similar function was shown to be inherent in a CPS-6 homolog from *C. elegans* [105, 106]. Double functions of these enzymes can be due to their possessing two enzymatic activities: the endonuclease activity with respect to DNA/RNA and the 5′-3′-endonuclease activity that allows them to create single-stranded breaks in double stranded DNA during replication or repair [77].

The discovery of an EndoG paralog, a new EndoG-like mitochondrial endo/exonuclease "EXOG" in mammals, has induced an idea about the possible separation of vital and lethal functions of conserved mitochondrial endo/exonucleases of lower eukaryotes among the enzymatic activities of EndoG and EXOG of vertebrates [84].

A report about the involvement of EndoG in the processing of primers for replication of mtDNA [107] remained without further development. However, the absence of EndoG was shown to have no influence on the number of copies of mitochondrial DNA, its structure, or the rate of mutations during at least the first five generations of EndoG(–/–) mice [72]. However, EndoG was recently shown to be associated with biogenesis of mitochondria: the chronic expression of EndoG in the HEK cells and superexpression in a culture of cardiomyocytes was associated with an increase in the mitochondrial mass. In ChIP-PCR experiments, the interaction between EndoG and mtDNA was demonstrated, whereas earlier EndoG binding was observed with mitochondrial transcription factor TFAM, which is very important for the synthesis and repair of mtDNA [108]. It was suggested that EndoG should modulate the synthesis of mtDNA and influence its stability and/or transcription [109]. The

idea about an important role of EndoG in the biogenesis of mitochondria is supported by data on the correlation of EndoG with non-adaptive hypertrophy of cardiac muscle. The inhibition of EndoG in a culture of cardiomyocytes led to an increase in the cell size and appearance of hypertrophy biomarkers in the absence of pro-hypertrophic stimuli. Cardiomyocytes of mice with EndoG deletion contained exhausted mitochondria with pronounced dysfunction, which resulted in an increased content of reactive oxygen species correlated with the increase in the size of cardiomyocytes and steatosis. Moreover, EndoG was found to be directly controlled by major regulators of mitochondrial cardiac functions, ERR-α and PGC1α. This clearly indicated that an interrelationship should exist between mitochondrial dysfunction, reactive oxygen species, cardiac pathologies, and the endonuclease EndoG [109].

Another alternative function of EndoG is especially interesting. In mutant EndoG(–/–) mice and mutant EndoG(–/–) B cells, EndoG was shown to play a noticeable role in the generation of double-stranded breaks in S regions of DNA during its recombination on the switching of Ig classes (CSR): in the mutants the CSR was twofold decreased. This was manifested by decreased contents of surface IgG1, IgG2a, IgG3, and IgA, secreted IgG1, circle transcripts Iγ3-Cμ, Iε-Cμ, and Iα-Cμ, and post-recombinant transcripts Iμ-Cγ1, Iμ-Cγ3, Iμ-Cε, and Iμ-Cα. The mutant mice also had a markedly changed set of mutations in the IgH locus. The enzyme deficiency did not influence the proliferation and the course of induced apoptosis in the EndoG(–/–) B cells [88].

NUCLEASES OF THE DNase I FAMILY

The question about the involvement of Ca^{2+}/Mg^{2+}-dependent endonucleases from the DNase I family in apoptotic degradation of DNA is still under consideration, and sometimes interesting results appear [110-113].

In addition to DNase I itself, which is known to be a secreted pancreatic DNase, this family also includes three recently isolated DNase I-like nucleases, DNase1L1-3 [114]. These nucleases are thought to be related based on similarities in the structure of their encoding genes and in the predicted amino acid sequences of their products [115]. All these nucleases contain a signaling hydrophobic peptide, and two His residues that are important for the catalysis [116]. The endonuclease DNase1L1 was first isolated from human muscles [117], then DNase1L2 was described [118], and DNase1L3, which is the most alike DNase I, was isolated from nuclei of rat thymocytes and became the third and best studied member of the DNase I family [119, 120].

The majority of nucleases of the DNase I family is characterized by synergic activation with Ca^{2+} and Mg^{2+}, pH optimum close to neutral, preferential use of double-stranded DNAs as substrates, endonucleolytic mechanisms of DNA hydrolysis with production of 3′-P and 5′-OH termini, and inhibition in the presence of millimolar concentrations of Zn^{2+} and of chelators of bivalent ions. However, these enzymes also have some unique features: they are different in respect to G-actin, aurine tricarboxylic acids, and metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}), and the pH optimum of DNase1L2 is in the acidic region.

These nucleases are different in their extra- and endocellular localization: DNase I and DNase1L2 are secreted enzymes, whereas DNase1L1 and DNase1L3 are situated inside the cell, which is due to the presence of the C-terminal extradomains preventing their secretion. The ancestral forms of these nucleases are thought to be initially secreted proteins, and the C-terminal extradomains to have been gained during evolution.

The genes of these nucleases are expressed with pronounced tissue specificity, and this seems to indicate the unique physiological role of these enzymes: most frequently they are found in apoptosis associated with cell differentiation [116].

DNase I is expressed in the spleen and secreted into the gastrointestinal tract and blood, where the hydrolyzed DNA is a source of oligonucleotides. Moreover, utilization of DNA of its own during their destruction suppresses anti-DNA autoimmunity. However, a possibility is also considered of the involvement of DNase I in the degradation of 50-300-kb DNA fragments produced under the influence of endonucleases cleaving into large fragments during the early stages of apoptosis. Analysis of the substrate specificity of recombinant DNase I has shown that the enzyme efficiently cleaves "naked" plasmid DNA and also can cleave nDNA by internucleosomal linkers, but proteases hydrolyzing chromatin proteins are also present. Removal of the signaling peptide from the N-terminus results in accumulation of the enzyme in the cytoplasm and nucleus [111].

Being incorporated into the cell membrane, DNase1L1 functions in skeletal muscles and myocardium: it protects the cells against penetration of heterologous genetic material [112].

DNase1L2 is expressed in brain and epidermis cells [113]. Nuclear DNA is degraded during terminal differentiation of epidermal keratinocytes under the influence of endonuclease DNase1L2, and if the expression of DNase1L2 siRNA is knocked-out, this does not occur and the nuclei are retained [113]. In transgenic mice with a directed deletion of endonuclease DNase1L2 specific for keratinocytes, DNA contents were increased in the cells of hair, claws, tongue, and esophagus epithelium, and the DNA excess in hair corneocytes decreased their mechanical resistance. As differentiated from human keratinocytes, removal of DNase1L2 did not influence the state of the epidermal cornified layer in mouse epithelium cells. However, the main function of DNase1L2 is the

elimination of heterogeneous and exogenous DNAs from the surface layer of skin [121].

DNase1L3, which is more frequently termed DNase γ, is expressed in lymphoid organs – spleen, liver, thymus, lymph nodes in the B cells of the lymphoid node embryonic center, and also in brain tissues, kidneys, and small intestine [122]. As differentiated from other members of the DNase I family, DNase γ is capable of cleaving DNA into oligonucleosomal fragments during apoptosis [116, 123]. DNase γ has a molecular weight of 32 kDa, p*I* 9.5, and its pH optimum is about 7.0. The enzyme possesses endonuclease activity and hydrolyzes single- [124] and double-stranded DNAs [116] producing 3′-OH and 5′-P ends; it is Ca^{2+}/Mg^{2+}-dependent, can be also activated with Mn^{2+} and Co^{2+}, and is inhibited with Zn^{2+}, but is not suppressed by monomeric actins. During apoptosis, its activity is regulated by poly(ADP-riboso)polymerase. Although DNase γ contains an N-terminal signaling peptide, which determines its transfer into the rough endoplasmic reticulum, there are also data on the enzyme binding with chromatin in the nuclei of non-apoptotic cells [124, 125]. This is explained by the presence of two nuclear localization signals (NLS) in the mature protein [126].

A scheme of biosynthesis and activation of DNase γ has been proposed: the newly synthesized molecule of the protein is transferred into the endoplasmic reticulum due to the existence of a hydrophobic precursor peptide. Upon elimination of the peptide by a signaling peptidase, the "mature" DNase γ is translocated and fixed on the outer side of the nuclear envelope. The appearance of certain apoptotic stimuli induces the release of the enzyme, and the presence of the NLS determines its entrance into the nucleus [116]. However, the penetration of DNase γ into the nucleus is believed to be realized only in the case of pronounced damage to the nuclear membrane during the terminal stages of apoptosis [127].

However, conditions and apoptotic stimuli responsible for activation of DNase γ are studied insufficiently. DNase γ is known to occur in the active state in normal thymocytes, and its activity does not change during apoptosis induced by X-rays or dexamethasone. This was the first indication of the different status of the enzyme in non-apoptotic cells and possible various pathways of its posttranslational activation [126].

Induction of DNase γ is obligatory for DNA fragmentation on apoptosis initiated by spontaneous myogenic differentiation of C2C12 line myoblasts [128]. In proliferating neuroblasts of the N1E-115 and PC12 lines, the induction of apoptosis with staurosporine leads to appearance of "ladders" of DNA fragments, and this process is inhibited when a caspase-resistant form of ICAD is expressed. However, on triggering, differentiation the CAD activity is not observed but DNase γ is induced. Thus, this was the first demonstration that cells could selectively use different apoptotic DNases depending on the stage of differentiation. DNase γ has also been shown to be involved in apoptosis that accompanies the development of nervous cells [129]. In immature B-cells of the WEHI-231 line, induction of apoptosis with cytotoxic compounds is associated with activation of CAD, and this is accompanied by subsequent internucleosomal fragmentation of DNA and destruction of the nucleus. However, when apoptosis is induced by ligation of the B-cell receptor without the activation of CAD, both DNA and the nucleus are similarly destroyed. This is associated with an increase in the expression of DNase γ. A specific inhibitor of DNase γ, DR396 suppresses the fragmentation of DNA and of the nucleus. DNase γ is thought to "serve" as an alternative mechanism of destruction of the nucleus and nDNA on disorders in the CAD-activated apoptotic cascade [130].

On staurosporine-induced apoptosis in human lymphoid cell culture Ramos, the initial degradation of DNA during the first three hours after induction is performed by CAD, whereas in the terminal stages of destruction of the nucleus and its contents 48 h after induction the activity of DNase γ was increased. It seems that DNase γ can play a subsidiary role during the destruction of nDNA in the terminal stages of cell death in classic apoptosis, providing for more complete and rapid hydrolysis of nDNA [127].

The terminal stages of apoptosis associated with destruction of membranes are sometimes called "secondary necrosis". DNase γ can also be involved in such necrotic death of cells. Thus, DNase γ can be responsible for the most complete elimination of nDNA in dying cells, although the mechanism of activation of this enzyme and its true functions *in vivo* are not sufficiently clear.

As to alternative functions of DNase γ, some data suggest its contribution to generation of immunity. The enzyme activity correlates with the rate of somatic hypermutations, including point mutations and nucleotide insertions/deletions, and also with production of stepwise double-stranded breaks in the reorganized V region [131]. There are also correlations between the fragmentation of DNA in staurosporine-induced apoptosis in HeLa S3/γ cells, which are stably expressing DNase γ and the secretion into the extracellular space of a nonhistone chromatin protein HMGB1 that is involved in the development of inflammatory response [132].

NUCLEASES OF THE DNase II FAMILY

An essential role of DNase II in higher eukaryotes was clearly demonstrated in transgenic mice incapable of expressing the CAD endonuclease [133]. In apoptotic cells of these mice, fragmentation of DNA was either absent or poorly expressed, but nevertheless these mice grew and developed normally. Thus, a preliminary frag-

mentation of DNA on apoptosis is not obligatory for its complete degradation, and DNA can be cleaved in phagocytes under the influence of DNase II.

The family of DNases II includes nucleases that, as differentiated from the majority of other nucleases, have pH optimum in the acidic region, do not depend on bivalent cations, and hydrolyze double-stranded DNA into short oligonucleotides with predominant production of 3′-P terminal groups [134, 135]. These enzymes are synthesized in the endoplasmic reticulum and then translocated into lysosomes, but they are found in the nuclei of both normal and apoptotic cells, and moreover they are also present in secreted fluids [134]. During apoptosis, the content of an apoptotic cell is acidified, which can activate acidic nucleases including DNase II, and these enzymes can contribute to the degradation of chromosomal DNA [136].

Of all the described acidic nucleases, the degradation of DNA during programmed cell death is thought to be associated with DNase II α (DNase 2), DNase II β, and L-DNase II.

DNase II α is an obligatory component of lysosomes, which hydrolyzes DNA of apoptotic bodies engulfed by phagocytes or DNA occurring inside cells due to endocytosis [18, 19]. The enzyme was isolated from swine and human liver and then cloned and sequenced concurrently by two groups of researchers [137, 138]. Later, amino acid sequences were determined for DNase II α from cattle, pigs, mice, rats, and human, as well as for the enzyme from *D. melanogaster* and *C. elegans* [134]. The enzyme is expressed in all mammalian tissues studied [133] and is synthesized as a propeptide with subsequent processing. The active enzyme is formed by two pathways. Two of three peptides (35 and 10 kDa), which appeared as a result of cleavage of the propeptide molecule, are bound with bisulfite bridges, and the enzyme is glycosylated at six sites [137, 140]. The other structural model is a monomeric peptide resulting upon the detachment from the propeptide of a region encoded by 91 bp, which is glycosylated by four sites [141].

DNase II α is involved in differentiation of erythrocytes. In macrophages of mouse embryos carrying a mutation in the DNase II α gene, undamaged DNA is accumulated, leading to termination of erythropoiesis and finally to death of the embryos because of anemia [142, 143]. The development of the thymus in mice also depends on DNase II α: in embryos of mice deprived of the DNase II α gene, the development of the thymus is blocked at the pro-T-stage [144].

DNase II β ((β)/DLAD, DNase2-like acidic DNase) could be identified after it had been cloned and the gene encoding it had been sequenced because its biochemical features remind those of DNase II α. Amino acid sequences of DNase II β homologs in human, rodents, drosophila, and *C. elegans* include a 22-member signaling peptide and contain many conserved regions [145, 146]. DNase II β is an intracellular protein but can be secreted similarly to DNase II α. DNase II β does not need cofactors and is maximally active at acidic pH values; however, the enzyme is rather active also at neutral pH. The hydrolysis of DNA by this enzyme results in fragments with 3′-P and 5′-OH termini.

In humans this enzyme is expressed in trachea, lungs, prostate, and lymph nodes, but its highest expression is observed in the salivary glands. In rats and mice, DNase II β is intensively expressed in liver. DNase II β is especially interesting in connection with the differentiation of the lens. The enzyme is specifically involved in the degradation of DNA of the lens cells in mice, which was shown on mice with the knocked-out gene of DNase II β. An increased expression of this enzyme was observed in fibers of the developing lens in mice and humans. The inactivation of the DNase II β gene in mice was associated with accumulation of non-hydrolyzed DNA in fibers of the developing lens, which led to formation of cataract [147, 148]. Thus, DNase II β is responsible for autonomous destruction of DNA in the nuclei of the developing lens fiber cells that essentially provided for the transparency of the lens. The question about mechanisms of DNA degradation in the lens was under discussion for a rather a long time, and the involvement of apoptosis, cytosolic degradation, and autophagy was considered. However, the absence of macrophages and activation of CAD in the fiber cells as well as the normal development of the lens in mice with the inactivated gene of CAD excluded apoptosis [147], whereas the normal degradation of the organelles in the lens and erythroid cells of mice with Atg5 deficiency (a protein required for formation of autophagosome) prevented autophagy [149]. Markedly activated transcription of the genes of DNase II β and other lysosomal enzymes was observed in cortical cells of developing lens fibers. These enzymes seemed to play the major role in the degradation of the cellular organelles during lens differentiation [150]. DNase II β also contributes to degradation of the interfinger mesenchymal cells during the final stages of formation of the extremities in embryos [151].

In the model of differentiating cells of the chicken's lens, a nuclease was shown to cleave DNA to oligonucleosomal fragments; however, the known TUNEL-labeling was negative. The authors supposed that a nuclease from the DNase II family should be involved in this process [152]. This nuclease was purified and sequenced. Its sequence occurred to be identical to that of a conserved protein serpin, which is a leukocytic elastase inhibitor (LEI). Proteolysis of LEI results in appearance of L-DNase II [153]. Mechanisms of the LEI proteolysis and conformational changes of the hydrolysis products are described in detail in works [154, 155]. The enzyme is synthesized as a precursor with protease-inhibiting activity and is localized in the cytoplasm. Due to a posttranslational modification – hydrolysis with elastase in the

region of the active center loop – the enzyme gets nuclease activity while loosing the previous proteolytic activity, and a site is exposed that determines its nuclear localization [154]. L-DNase II, similarly to DNase II β, is differentially expressed during the morphogenesis of extremities and determines the internucleosomal fragmentation of nDNA in dying cells of the interfinger mesenchyme during inactivation of endonuclease CAD [151].

The epidermal growth factor (EGF) in GH4C1 pituitary cells induces cell death with signs of apoptosis and paraptosis. The observed internucleosomal fragmentation of DNA is a result of the L-DNase II activity [156].

OTHER APOPTOTIC NUCLEASES

At first the search for nucleases involved in apoptotic degradation of DNA was limited by establishment of correlation between the nuclease activities and DNA fragmentation. A number of Ca^{2+}/Mg^{2+}-dependent DNases were revealed that were identified only by molecular weight. Thus, in CTLL cells the DNA fragmentation during apoptosis induced by removal from the medium of IL-2 was thought to be associated with 40- and 58-kDa nucleases (respectively, NUC40 and NUC58). The activities of these enzymes depended on Ca^{2+} and Mg^{2+}. And NUC40 and NUC58 were shown to be localized in the nucleus and cytoplasm, respectively; the two enzymes have activity optima at neutral pH values and are inhibited by Zn^{2+} [157].

On glucocorticoid-induced apoptosis in rat thymocytes, nuclease activity of NUC18 (18 kDa) associated with DNA fragmentation was found. The enzymatic activity of the isolated NUC18 nuclease depended on Ca^{2+} and was suppressed by Zn^{2+} and aurine tricarboxylic acids [158]. The structure of NUC18 is similar to that of proteins of the cyclophilin family. Cyclophilins A, B, and C possess Ca^{2+}/Mg^{2+}-dependent nuclease activity similar to that of NUC18 and seem to be involved in the apoptotic degradation of DNA in lymphocytes [18]. Recombinant proteins cyclophilin A (CypA) and AIF are jointly involved in the *in vitro* degradation of plasmid DNA, and they induce destruction of DNA in purified nuclei. The apoptotic cooperation of CypA and AIF does not depend on the peptidyl-prolyl *cis-trans*-isomerase activity of CypA. In CypA-expressing cells, the superexpression of AIF enhanced apoptotic chromatolysis. The AIF-dependent hydrolysis of DNA into long fragments was less expressed in CypA-knocked-out cells than in the control. AIF mutants lacking the domain of CypA binding are inefficient as activator of apoptosis in experiments with transfection. Moreover, AIF is unable to activate apoptosis in CypA-knocked-out cells, but this effect is restored on introduction into the cells of the intact *cypA* gene. Thus, during hydrolysis of chromatin, CypA and AIF interact between themselves [159].

In nuclei of rat hepatoma cells 5123tc, a two-stage degradation of DNA was observed. The first stage of DNA hydrolysis into long fragments (50-300 kb) was stimulated only by Mg^{2+}, whereas the second stage required both Mg^{2+} and Ca^{2+} to be present. Endonucleolytic activities involved in these two stages of the DNA cleavage can be separated under definite conditions: the Mg^{2+}-modulated activity is tightly associated with chromatin, whereas the Ca^{2+}/Mg^{2+}-dependent activity can be easily extracted with buffers with low ionic strength. The Ca^{2+}/Mg^{2+}-dependent activity belonged to a new 97-kDa endonuclease, which was also activated by Mn^{2+} and Co^{2+} and inhibited in the presence of millimolar concentrations of Zn^{2+}. Moreover, this activity was not extracted with a low salt buffer from the nuclei of apoptotic cells. Isoelectrofocusing revealed that the p97 protein was present as multiple forms with different isoelectric points, which suggests its posttranslational modification. The enzyme p97 was constitutively present in different cultures of rat cells and tissues. The enzyme was active over a wide range of pH values (6.0-9.0) and was suppressed by reducing agents. Under *in vitro* conditions, the enzyme possessed both the endo- and exonuclease activities and could hydrolyze both single- and double-stranded DNAs [160].

A crude nuclear extract from rat apoptotic thymocytes induced the cleavage of nuclear DNA of HeLa cells into oligonucleosomal and long fragments (50-300 kb). By gel filtration, from this extract new nuclease activities were isolated and identified with molecular weights of 260 and 25 kDa, the first one hydrolyzing DNA into elongated fragments (50-300 kb) and the other cleaving DNA into both long and oligonucleosomal fragments. Similarly to other nucleases involved in apoptosis, these enzymes hydrolyzed DNA with production of 3′-OH terminal groups, depended on Ca^{2+} and Mg^{2+}, and were inhibited by N-methylmaleimide, sodium tetraionate, aurine tricarboxylic acids, and NaCl. They could be also inhibited by some inhibitors of serine proteases, calpain inhibitors, and caspase inhibitors. Apoptosis is thought to be also contributed by non-caspase proteases, which seem to change the chromatin structure promoting the availability of DNA for nucleolytic attack [161].

During apoptosis in leukemic cells, DNA fragmentation is also contributed by apurine/apyrimidine nuclease APE1 known as the nuclease of excision repair [162]. APE1 is a multifunctional enzyme, which in addition to the endonucleolytic activity also has 3′-phosphodiesterase, 3′-5′-exonuclease, and 3′-phosphatase activities [163]. A shortened from the N-end form of APE1 (AN34) is also involved in the degradation of DNA in leukemic cells [164].

On the PBOX-6-induced caspase-independent apoptosis of myelogenous leukemic cells (MLC), DNA was fragmented into oligosomes in the absence of the CAD activation. In this case the destruction of DNA

occurred under the influence of acidic endonuclease activated due to the presence of a chymotrypsin-like serine protease [165].

The SET complex, which was mentioned earlier in relation to the NM23-H endonuclease cleaving to large fragments, also includes exonuclease TREX1 that eliminates nucleosides from the 3′-OH ends produced under the influence of NM23-H1. This results in an irreversible destruction of DNA and cell death. The SET complex components, including the exonuclease TREX1, can contribute to the repair of DNA depending on the corresponding intracellular context [166, 167]. TREX2 homologous to TREX1 is involved in the caspase-dependent degradation of DNA, inducing in it 3′-OH breaks that cannot be repaired under conditions of genotoxic stress [168].

NUCLEASES RESPONSIBLE FOR AUTOPHAGY AND NECROPTOSIS

Fragmentation of DNA on non-apoptotic cell deaths, such as necroptosis and autophagic cell death, is characterized very poorly. Cell death in the *D. melanogaster* ovaries is associated with removal by autophagy of these cells as a source of nutrients for the developing oocyte in the late stages of oogenesis, and DNase II plays the major role in the degradation of nuclear DNA. Mutants with deficient synthesis of DNase II are characterized by serious disorders in removal of the feeder cells. In this case DNase II acts directly inside the dying cell, and the presence of autophagosomes in these cells suggests that autophagy is a mechanism of cell death in the oogenesis stage under consideration [169]. Inactivation of the autophagy genes *atg1*, *atg13*, and *vps34* during the late stage of oogenesis leads to appearance of cavities containing undamaged nuclei of subsidiary feeder cells [170]. Necroptosis, necrosis, and secondary necrosis are characterized by the same sequence of subcellular events: oxidative burst, hyperpolarization of mitochondrial membranes, and permeabilization of lysosomal and plasma membranes with the markedly different kinetics [171]. A concentrated destruction of cytoplasmic membranes and of membranes of cell organelles on necroptosis suggests that the hydrolysis of DNA inside the cell can be contributed by both EndoG and DNase II of lysosomes. Thus, H_2O_2-induced necroptosis in primary cortical neurons of C57/Black 6J mice is accompanied by releasing EndoG and fragmentation of nDNA [172].

COUPLING OF ENDONUCLEASE ACTIVITIES

The degradation of DNA accompanying programmed cell death is rather complicated. The involvement of different DNases seems to increase the efficiency of this process due to specific hydrolysis of definite structures in DNA and strengthening the nuclease activities due to their interaction. It seems that type of tissue or cell line, differentiation stage, type of pro-apoptotic stimulus, stress intensity, and also specificity of a pathogen can determine differential expression of cellular endonucleases during apoptosis. In principle, several scenarios of DNA degradation can be realized in the same cell. This is clearly demonstrated by data on the existence in *C. elegans* of two different pathways of apoptotic degradation of DNA performed by different autonomous cellular nucleases [11].

The unique features of this model allowed researchers to follow the death of individual cells of the nematode during its development, and now it is known not only what invariant lines of somatic cells die, but also when and where the apoptosis occurs. In 2002, this work [173] was awarded the Nobel Prize in medicine. Programmed cell death in *C. elegans* occurs on two stages of the nematode's development, during embryogenesis and after it and in different tissues. The following genes involved in the cleavage of DNA during apoptosis were revealed: *nuc-1* and *crn6* (homologs of DNase II), *cps-6* (homolog of EndoG), *wah-1* (homolog of AIF), *crn1* (homolog of flap endonuclease FEN-1), *crn2* (homolog of TatD), *crn3* and *crn5* (homologs of ribonuclease components of exosomes of multi-exonuclease complex), *crn4* (homolog of nucleases of the family of 3′-5′-exonucleases including RNase T), and *cyp-13*, which is similar to the gene of cyclophilin E in animals. The loss or decrease in activity of any of these genes results in accumulation of TUNEL-positive cells in embryos of *C. elegans*. These genes form two groups: (1) *cps-6*, *wah-1*, *crn1*, *crn4*, *crn-5*, and *cyp-13* and (2) *crn2* and *crn3*. Mutations in the genes of these two groups not only lead to serious disorders in the degradation of nDNA, but also cause disorders in the engulfment of dead cell fragments. The proteins CPS-6, CRN-1, CRN-4, CRN-5, and CYP-13 form a complex called the degradosome. This complex also includes protein WAH-1 interacting with CPS-6 and increasing its nuclease activity. Nucleases encoded by *crn2* and *crn3* are not components of the degradosome, and genetic analysis has shown that they can function concurrently and independently of this complex of nucleases [174].

The genes *nuc-1* and *crn-6* and also the homologous gene *crn-7* encode acidic DNases II. Products of these genes were thought to have no influence on the activation and dynamics of apoptosis, but they more likely act during the late stages of the apoptotic degradation of DNA in lysosomal compartments of the engulfing cells. To elucidate the role of the genes, the three DNases II, their expressions in *C. elegans* during apoptosis and development, specific features of the mutant phenotypes, enzymatic activities of their products in experiments with the exchange of promoters were analyzed, as well as the significance of their secretion for other functions associated

with the degradation of DNA. Homologs of DNase II were found to be differently associated with the degradation of DNA on apoptosis in *C. elegans*. The major role belongs to nuclease NUC-1; CRN-6 is likely to perform an auxiliary role, whereas CRN-7 is not essential for this process. During the apoptotic degradation of DNA, CRN-6 but not CRN-7 can partially substitute for NUC-1, but neither nuclease is able to substitute for NUC-1 in the elimination of undesired bacterial DNA in the intestine. It was shown for the first time that the secreted nuclease NUC-1 could be transferred over long distances beyond the limits of its expression and be involved in the degradation of DNA directly inside apoptotic cells. However, this "wandering" nuclease can also be captured by phagocytes, where the degradation of DNA of the apoptotic bodies is terminated [175]. The involvement of DNase II in DNA degradation inside apoptotic cells is also confirmed by detecting in them of short DNA fragments with length of $\geqslant$20 nucleotides separated with the interval of 10 nucleotides. These fragments can be labeled with terminal deoxynucleotidyl transferase only upon treatment with phosphatase and appearance of 3′-P ends, which suggests their being generated under the influence of DNase II. The observed "ladders" of short DNA intermediates is identical to the "ladders" resulting upon the treatment with nucleases of DNA bound with the core of nucleosomal proteins. Consequently, the target of DNase II in the apoptotic cell is DNA bound with nucleosome, because DNA is hydrolyzed in phagocytes in the presence of proteases cleaving nucleosomal proteins [176].

However, one of the most important problems in cell death regulation is still unclear. How are some components important for cell growth and survival transformed on apoptosis into proapoptotic molecules? Studies on the fine structural organization of CRN proteins homologous to components of important cell processes, such as the control of protein folding, RNA processing, and replication and repair of DNA, which are essential for survival and correct development of the nematode, can be of help for answering this question. Thus, studies on the crystalline structure of CRN-4, besides characterizing the catalytic DEDDh nuclease domain, have revealed the presence of an additional C-terminal DNA-binding Zn-domain responsible for the interaction with the substrate and/or for reorganization of the molecule. This additional domain is thought to determine different variants of the oligomeric assemblage of the enzyme and/or the interaction with new cofactors that can lead to changes in the substrate specificity of the enzyme and manifestation of the proapoptotic function [177]. Analysis of the crystalline structure and biochemical properties of CRN-5 [178] indicates that normally the monomeric protein is included in the exosome, whereas in the apoptotic cell a homodimer is formed that is deprived of nuclease activity but capable of binding to DNA and interacting with apoptotic nuclease CRN-4, enhancing its activity.

Endonucleases are the major agents of the obligatory degradation of DNA during apoptosis. Many nucleases display marked processive action: initially, they cleave DNA inside chromatin into very large domains, and then fragment it to internucleosomes with subsequent hydrolysis into small fragments (oligonucleotides). During apoptosis, chromatin is attacked by many nucleases having different activities and specificities; some of them possess both endo- and exonuclease activity; some nucleases are capable of hydrolyzing only DNA, whereas others hydrolyze both DNA and RNA; they can produce in DNA point breaks and elongated gaps or processively hydrolyze only single- or double-stranded DNAs or both. No doubt, there is a regular order in the actions of different nucleases in the apoptotic cell, but it is still unclear. The activity of every endonuclease is controlled by specific regulatory mechanisms, such as metal ions and other effectors, including, in particular, S-adenosylmethionine. The activities of nucleases can also be modulated by histones and short peptides. It seems that the set and state of all these effectors are different in normal and apoptotic cells.

It seems that the multiplicity of endonucleases is a kind of insurance for the efficient and complete degradation of DNA in the apoptotic cell. It should be kept in mind that endonucleases in the cell must recognize and find in chromatin the regions of DNA available for hydrolysis. Chromatin and its protein components (histones) can influence endonucleases and modulate their action. This is, in particular, supported by data on the modulation of plant endonucleases by histones and short peptides [179]. Perhaps this regulatory mechanism of activities of endonucleases also exists in animals.

In any case, apoptosis cannot occur without the action of nucleases: disturbances in this process lead to accumulation of unnecessary or even harmful DNA, disorders in cell differentiation, embryogenesis, and development of whole organisms, and are accompanied by many severe diseases. All this explains the currently increased interest in studies on apoptotic nucleases as they are. Knowledge of their features, the further interpretation of their structure and functions, as well as of the nature and influences of the modulating effectors are important not only for comprehension of fine mechanisms of apoptosis, but also for monitoring programmed cell death together with cell differentiation and development of different organisms.

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