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MULTIPLE PATHWAYS OF DNA DISINTEGRATION DURING NEURONAL APOPTOSIS

Doctoral dissertation

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ABSTRACT

Apoptotic cell death has been shown to contribute to neurodegeneration. Two biochemical processes are thought to be obligatory in apoptosis, namely, the activation of the caspases and the fragmentation of nuclear DNA. The purpose of this study was to investigate the mechanisms of DNA disintegration during neuronal apoptosis and to clarify whether HMW- and internucleosomal DNA cleavage represent distinct and separate types of apoptotic DNA disintegration. It has been shown that the treatment of NB 2A neuroblastoma cells with genotoxic insults (etoposide, Ara-C) led to the formation of HMW-DNA fragments of about 50-100 kb and 300 kb without the formation of an oligonucleosomal DNA ladder. Apoptosis induced by nongenotoxic insults (serum deprivation, OKA) was associated with disintegration of nuclear DNA into 50-100 kb DNA fragments and oligonucleosomal DNA ladder. ATA, Zn^{2+}-ions, cycloheximide and suramin effectively prevented internucleosomal and HMW-DNA cleavage in serum-deprived and OKA treated cells, but had no inhibitory effect on DNA disintegration in etoposide or Ara-C treated cells. The cell free system experiments allowed further to separate nuclease activities, responsible for these two types of DNA fragmentation. Our results suggest that distinct pathways underlay DNA disintegration during apoptosis induced by genotoxic and nongenotoxic stimuli, and formation of HMW-DNA fragments may proceed independently of the internucleosomal DNA cleavage. We further showed that treatment of CGCs with OKA or L-glutamate both resulted in cell death. The cytotoxicity induced by OKA was associated with activation of caspase-3-like proteases and formation of oligonucleosomal and HMW-DNA fragments. In contrast to OKA, neither activation of caspase-3- like proteases, nor formation of oligonucleosomal DNA ladder was observed during L-glutamate induced excitotoxicity. In OKA-treated cells, both internucleosomal- and HMW-DNA fragmentation were effectively inhibited by caspase inhibitors. However, in L-glutamate treated cells, these inhibitors had no any apparent inhibitory effect on the formation of HMW-DNA fragments. Our further experiments showed that addition of staurosporine to a serum-deficient medium induced in NB 2A cells apoptosis without formation of oligonucleosomal DNA cleavage. Caspase inhibitors effectively prevented activation of caspase-3-like proteases in staurosporine treated neuroblastoma cells without inhibition of HMW-DNA fragmentation. These results suggest that caspase activation is not essential for HMW-DNA fragmentation in cells induced to undergo apoptosis by staurosporine in serum-deficient medium. In summary, this study demonstrates that HMW- and internucleosomal DNA cleavage represent independent programmes of apoptotic DNA disintegration. Different mechanisms are involved in the formation of HMW-DNA cleavage that can be induced in both caspase-dependent and -independent manners. Suramin was demonstrated to protect neuronal cells against apoptotic and excitotoxic cell death.

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Medical Subject Headings: neuronal apoptosis, excitotoxicity, cell death, neurodegeneration, NB 2A neuroblastoma cells, cerebellar granule cells, serum deprivation, etoposide, L-glutamate, staurosporine, okadaic acid, caspases, nuclear apoptotic morphology, HMW- and internucleosomal DNA fragmentation, nuclease activities, caspase inhibitors, suramin.

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I worship the memory of my sister Elena.

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Kuopio, June 2001

Zinayida Bezvenyuk

ABBREVIATIONS

Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
Ac-LEHD-AFC	Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin
Ac-WEHD-AFC	Acetyl-Trp-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin
Ac-YVAD-AMC	Acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin
AIF	apoptosis inducing factor
Ara-C	cytosine arabinoside
ATA	aurintricarboxylic acid
BME	β-mercaptoethanol
BPB	bromophenol blue
BSA	bovine serum albumin
CAD/ICAD	caspase-activated DNase/inhibitor of CAD
Caspases	cysteinyl aspartate-specific proteinases
CGCs	cerebellar granule cells
DFA	dimethylformamide
DFF	DNA fragmentation factor
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid
FACS	fluorescence activated cell sorter
FIGE	field inversion gel electrophoresis
GrB	granzyme B
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
HMW	high molecular weight
LDH	lactate dehydrogenase activity
MK-801	dizocilpine maleate
MTT	(3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide
MPT	mitochondrial permeability transition
M.W.	molecular weight
NB 2A	Neuro 2A neuroblastoma cells
OKA	okadaic acid
PARP	poly(ADP-ribose)polymerase
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	propidium iodide
PIPES	1,4-piperazinediethanesulphonic acid

PMSF	phenylmethylsulphonyl fluoride
RT	room temperature
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBE	tris-boric acid-EDTA buffer
Z-DEVD-fmk	Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK
Z-IETD-fmk	Z-lle-Glu(OMe)-Thr-Asp(OMe)-CH_2F
Z-VAD-fmk	Z-Val-Ala-Asp(OMe)-CH_2F
Z-VDVAD-AFC	Z-Val-Asp-Val-Ala-Asp-7-Amino-4-trifluoromethylcoumarin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V.**

I Solovyan V., Bezvenyuk Z., Huotari V., Tapiola T., Suuronen T., Salminen A. (1998) Distinct mode of apoptosis induced by genotoxic agent etoposide and serum withdrawal in neuroblastoma cells. Mol. Brain Res., 62, 43-55.

II Solovyan V., Bezvenyuk Z., Huotari V., Tapiola T., Salminen A. (1999) Distinct mechanisms underlay DNA disintegration during apoptosis induced by genotoxic and nongenotoxic agents in neuroblastoma cells. Neurochem. Int., 34, 465-472.

III Bezvenyuk Z., Salminen A., Solovyan V. (2000) Excision of DNA loop domains as a common step in caspase-dependent and -independent types of neuronal cell death. Mol. Brain Res., 81, 191-196.

IV Bezvenyuk Z., Solovyan V. Caspases are not essential for the excision of DNA loop domains during apoptosis in NB 2A cells (manuscript).

V Bezvenyuk Z., Suuronen T., Salminen A., Solovyan V. (2000) Protective effect of suramin against cell death in rat cerebellar granule neurons and mouse neuroblastoma cells. Neurosci. Lett., 292, 111-114.

CONTENTS

1. INTRODUCTION

Apoptosis is a genetically regulated form of cell death, in which superfluous or abnormal cells are eliminated from an organism, ensuring normal development of multicellular organisms and maintenance of tissue homeostasis (Wyllie et al., 1980). Disregulation of apoptosis is widely viewed as a principal event involved in a number of pathological paradigms ranging from cancer to neurodegeneration (White, 1996). Therefore, an understanding of the molecular mechanisms of apoptosis is not only of theoretical importance but may also have some other, especially, therapeutic value.

Apoptotic cell death has been shown to occur in diverse cell types and is triggered by a number of extracellular and intracellular signals. Apoptosis is associated with typical morphological changes in the cells, such as plasma membrane blebbing, chromatin condensation and fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by neighbouring cells (Kerr et al., 1972). Extensive investigations into the molecular mechanisms underlying apoptotic cell death pointed to the presence of two biochemical markers of apoptosis, namely, the activation of the caspases (cysteinyl aspartate-specific proteinases) (Patel et al., 1996) and the fragmentation of nuclear DNA into oligonucleosome-sized DNA fragments by an apoptotic nuclease(s) (Wyllie et al., 1980). Recently, a direct link between caspase activation and internucleosomal DNA fragmentation has been demonstrated by the observation that caspase 3 directly activates at least one of the apoptotic nucleases, DFF40/CAD, by proteolytic cleavage of its inhibitor, DFF45/ICAD (Liu et al., 1997; Enari et al., 1998) leading to the formation of the characteristic oligonucleosomal DNA ladder in apoptotic cells.

Activation of an endonuclease, which preferentially cleaves DNA at the internucleosomal regions, has been considered as the biochemical hallmark of apoptosis for many of years (Wyllie et al., 1980). Another type of DNA cleavage during apoptosis has been described to yield a set of the HMW-DNA fragments sized about 50-100 kb (Solovian and Kunakh, 1991; Cohen et al., 1992). The cleavage of nuclear DNA into HMW fragments has been shown to precede the formation of the oligonucleosomal DNA ladder in many cell types, in several cell lines this can occur in the absence of internucleosomal DNA fragmentation (Oberhammer et al., 1993; Brown et al., 1993). The formation of HMW-DNA fragments is considered as an early critical event during apoptotic DNA disintegration (Cohen et al., 1992; Oberhammer et al., 1993; Brown et al., 1993), and is widely thought to result from the disassembly of the higher-level chromatin structural domains (Lagarkova et al., 1995; Li et al., 1999).

While the pathways leading to the internucleosomal DNA disintegration during apoptosis are starting to be elucidated, the mechanisms involved in the higher-level chromatin disassembly remain poorly understood. Meanwhile, the understanding of the pathway that regulates the initial steps of nuclear DNA disintegration during apoptosis may have important clinical implications, and the nuclease(s) that is involved in the disassembly of the higher-level chromatin structural domains could be a useful therapeutic target in several pathological paradigms including cancer and neurodegenerative/trauma situations.

2. REVIEW OF THE LITERATURE

2.1 Apoptosis: a general overview

Apoptosis is a genetically regulated form of cell death, which plays an important role in tissue homeostasis, differentiation and development (Wyllie et al., 1980) and may be also involved in many degenerative diseases. Apoptosis is an active form of cell death. It requires energy and protein synthesis (Wyllie et al., 1995). It has been shown that apoptotic cell death is triggered by different intracellular and extracellular stimuli and proceeds in two phases: an initial commitment phase and an execution phase, which results in typical apoptotic morphological changes in the cells such as plasma membrane blebbing, shrinkage of the cytoplasm, dilation of endoplasmic reticulum, nuclear chromatin condensation and fragmentation into apoptotic bodies that are phagocytosed by neighbouring cells (Takahashi and Earnshaw, 1996; White, 1996). During apoptosis cells reduce their volume, pump out ions and there is contraction of the cytoskeleton, forming a cage-like structure around the nucleus. At the same time, the dying cells activate their own proteolytic enzymes in addition to those acting in the phagolysosomes (Zamzami and Kroemer, 1999).

In addition to "classical" apoptosis, another type of active cell death, associated with autophagy, has been recently described. Autophagy is a mechanism, by which cells degrade their own organelles and cytoplasm (Dunn, 1994). The morphological changes, which occur at the beginning of this process, comprise appearance of a double membrane-bound vacuole (autophagosome) around portions of the cytoplasm, which is formed from the endoplasmic reticulum (Dunn, 1990 a). During the next step, the autophagosome is converted into a single membrane-bound degradative vacuole (autolysosome) due to fusion with endosomes, lysosomes and Golgi elements containing acid hydrolases (Dunn, 1990 b).

Apoptotic and autophagic cell death represent normal physiological processes that occur during embryonic development (Oppenheim, 1991; Hinchliffe, 1981), formation and sculpting of body parts (Haanen and Vermes, 1996), mitochondrial turn-over in non-proliferating tissues (Dunn, 1990 a). However, both processes can be triggered by different apoptotic signals, such as trophic factor deprivation, drug and hormone treatments (Villa et al., 1998; Xue et al., 1999). It has been shown that onset of the mitochondrial permeability transition (MPT) in an increasing number of mitochondria within the cell leads either to autophagy or apoptosis (Lemasters et al., 1998). Both of these events were inhibited by the anti-autophagic drug 3-methyladenine (Jia et al., 1997; Xue et al., 1999). Autophagy, induced in sympathetic neurons from the superior cervical ganglion by NGF-deprivation or Ara-C treatment, during its late stage was associated with typical apoptotic events: nuclear condensation and DNA fragmentation, mitochondrial swelling, cytoplasm vacuolisation, disruption of the nuclear envelope and plasma membrane (Xue et al., 1999). All these results support the concept that autophagy and apoptosis are overlapping processes (Zakeri et al., 1995). There is one hypothesis which accounts for the transition from autophagy to apoptosis and involves the process of MPT (Lemasters et al., 1998). Autophagy is initiated in the cells when the stimulating signals activate MPT in only a few mitochondria and this leads to the degradation of these leaking mitochondria by lysosomes. When more mitochondria start to undergo MPT, pro-apoptotic factors, such as cytochrome c and apoptosis inducing factor (AIF), which are released from the mitochondria, may trigger apoptosis. When all of the mitochondria are undergoing MPT,

ATP becomes depleted because of uncoupling of oxidative phosphorylation and ATP hydrolysis by the mitochondrial ATPase. This results in another type of cell death - necrotic cell death.

Necrotic cell death occurs very rapidly due to the acute disruption of cellular metabolism, it has been defined, as unprogrammed or accidental cell death. The main features of necrosis are: ATP depletion, ionic disregulation, swelling of mitochondria and endoplasmic reticulum, ultimately whole cell swells, and this eventually leads to disruption of the plasma membrane and cell lysis (Trump et al., 1965). Chemically induced hypoxia depending on the intensity of the insult produces apoptosis or aponecrosis, a form which shares the molecular and morphological features of both apoptosis and necrosis (Formigli et al., 2000). These data suggest that in some cases apoptosis may be reprogrammed to the alternative type of cell death - necrosis. The crucial point in this transition from apoptosis to necrosis is suggested to be the inhibition of caspase-3 protease activity, which is activated in apoptotic cells but remains to be nonactive in necrotic cells (Formigli et al., 2000; Lemaire et al., 1998).

2.2 Molecular mechanisms of apoptosis

2.2.1 Activation of proteolytic system

Apoptosis is characterised by activation of proteolytic enzymes, including proteasomes, serine and cysteine proteases (Patel et al., 1996; Villa et al., 1998). Cysteine proteases may be divided into two superfamilies: interleukin-1β-converting enzymes (ICE) or caspases and the papain superfamily of cysteine proteases (Berti and Storer, 1995).

2.2.1.1 Role of caspases in apoptosis

There are currently 14 members of the caspase (cysteinyl aspartate-specific proteinases) family. They all possess sequence identity with CED-3, a protein, required for developmental cell death in the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Yuan et al., 1993). It is evident that caspases play a critical role in many biochemical events controlling apoptosis in nematodes and mammals (Ellis et al., 1991; Thornberry et al., 1992). This conclusion is based on several observations: first, caspases hydrolyse proteins that are known to be selectively cleaved at the beginning of apoptosis: lamins (Lazebnik et al., 1995), inhibitors of caspase-activated DNAse (DFF45/ICAD) (Liu et al., 1997; Enari et al., 1998), PARP (Lazebnik et al., 1994). Secondly, post-translational activation of several caspases correlates with the main apoptotic events: plasma membrane blebbing, shrinkage of the cytoplasm, dilation of endoplasmic reticulum, nuclear chromatin condensation and DNA fragmentation (Takahashi and Earnshaw, 1996; White, 1996). Thirdly, naturally occurring protease inhibitors such as viral proteins Crm A (cytokine response modifier A) (Ray et al., 1992) and p35 (Clem et al., 1991) as well as the IAP (the inhibitor of apoptosis) family of proteins (Roy et al., 1995) and Bcl-2/Bcl-x_L (inhibitors of cell death, induced by many stimuli) (Vaux et al., 1988) all can prevent apoptosis. Finally, mice deficient in caspase-3 suffer a severe defect in apoptosis during brain development (Nicholson, Thornberry, 1997).

The family of caspases can be divided into three subfamilies based on their biological functions: the ICE subfamily (caspases-1, -4, -5), which activate cytokines during inflammation, initiators of apoptosis (caspases-2, -8, -9, -10) and executioners of apoptosis (caspases-3,-6, -7) (Nicholson and Thornberry, 1997; Cohen, 1997; Stennicke and Salvesen, 1998).

All caspases have four amino acids in their substrate cleavage site and aspartic acid in the P_1 position (Alnemri et al., 1996). Caspases are synthesised as inactive proenzymes, comprising an N-terminal peptide (prodomain), one large and one small subunit, and they contain in their structure a conserved pentapeptide active-site motif with the catalytic cysteine (Cohen, 1997). The difference in structure of various proenzymes may be due to the length of the prodomain and the presence of a linkage peptide between the two subunits. The length of the N-peptides varies from 22 amino acid residues for caspase-3 and -6 to over 200 in caspases -8 and -10, whereas the mature enzymes are quite similar in their sequence and length (Stennicke and Salvesen, 1998). The maturation of caspases consists of cleavage at the Asp sites with formation of two subunits. Both subunits contain residues that are essential for the function of the mature enzyme. Active caspases are heterotetramers of two large and two small subunits (Cohen, 1997).

2.2.1.2 Mechanisms of caspase activation

In general, there are several ways by which caspases can be activated. The first is as a result of cleavage by autolysis and by upstream proteases, such as other caspases or granzyme B (GrB), which is the only mammalian serine protease that shares the caspase specificity for the Asp residue at the P_1 position. A common feature of the ICE family proteases is the presence of a prodomain that has been hypothesized to keep the enzyme in an inactive form. Prodomain is necessary also for dimerization, which occurs prior to autoprocessing (Van Criekinge et al., 1996). GrB *in vivo* processes initially caspase-3, which in turn can remove the prodomain from caspase-7, and, finally, caspase-7 is fully processed by GrB (Yang et al., 1998). In a heterologous expression system it has been shown that recombinant caspase proenzymes are autolytically processed to their mature forms when synthesised at sufficiently high levels. A mutation in catalytic cysteine residue prevents this processing. Isolated caspase proenzymes can be autoactivated when they are concentrated by ultrafiltration (Nicholson and Thornberry, 1997).

A second mechanism of caspase activation is through death-inducing receptors of the tumour necrosis factor receptor family (TNFR/NGFR), including Fas/CD95/Apo1, TNFR1, TNFR2, DR3/Wsl-1/Tramp, DR4/TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor-1), DR5/TRAIL-R2/TRICK2/Killer and DR6. These receptors contain both cysteine-rich extracellular domains and intracellular cytoplasmic death domain (DD) (Bratton et al., 2000). The interaction of the appropriate ligand with the receptor results in its trimerization and recruitment of adapter molecules (Ashkenazi and Dixit, 1998). In the case of Fas-induced apoptosis, adapter protein FADD (Fas-associated death domain or Mort 1) contains C-terminal DD, which promote the interaction with the same DD in trimerized Fas-receptor, and N-terminal DED (death effector domain), which associates with DED in the large N-terminal peptides of caspase-8 (Fig. 1, A) (Boldin et al., 1996). This complex is called DISC (death-inducing signalling complex), and it is thought that as more procaspase-8 molecules become involved in this complex, they start to be activated, probably by autocleavage (Medema et al., 1997). Similar mechanisms have been demonstrated for the tumour necrosis factor receptor (TNF-R1), which seems to require an additional adapter molecule TRADD (TNFR-associated death domain), which recruits FADD and procaspase-8 (Fig. 1, A). Furthermore, TRADD can recruit serine-threonine kinase RIP (receptor interacting protein) and an adapter molecule RAIDD, which has sequence similarity with the prodomains of caspases-2, -9 and CED-3 and can activate procaspase-2 (Duan and Dixit, 1997). At the same time, the physiological role of caspase-2 in the activation cascade of caspases remains

unclear. It is likely that caspases-8 and -10 are the major apical proteases in TNF and anti-Fas induced apoptosis, which activate the downstream caspases (Medema et al., 1997). In the next step, activated downstream caspases can activate themselves and other caspases, leading to the amplification cascade of caspase activation. The recruitment of caspases-8 and -10 may be inhibited by viral inhibitors of the apoptotic signals (FLIPs) (Tschopp et al., 1998). These inhibitors contain two N-terminal DEDs in their structure. They interfere with FADD-caspase-8 binding and inhibit both caspase-8 activation and apoptosis. Caspases -1, -2, -4, -5, -9 and CED-3 contain another "caspase recruitment domain" (CARD), required for assembly of activation complexes (Hofmann et al., 1997).

The third way in which the caspase cascade can be initiated involves translocation of cytochrome c from the mitochondria to the cytoplasm (non-receptor-mediated pathway or stress-induced apoptosis) (Fig. 1, B) (Green and Reed, 1998). The mechanism of this translocation remains unclear and may be due to opening of a mitochondrial permeability transition pore, rupture of the outer membrane or the presence of specific channels for cytochrome c (Green and Reed, 1998). Cytochrome c release from mitochondria is under the control of the Bcl-2 family of proteins, that either inhibit (Bcl-2, Bcl-x_L) or promote (Bax, Bak, Bik, Bid) apoptosis (Antonsson and Martinou, 2000). In the cytoplasm, cytochrome c interacts with Apaf-1 (the human homolog of C. elegans protein CED-4).

Apaf-1 recruits pro-caspase-9 leading to the active form of the enzyme, which can activate pro-caspases-3 and -7, thus initiate the proteolytic cascade essential for apoptosis (Zou et al., 1997; Seshagiri and Miller, 1997; Gorman et al., 1998). In C. elegans, CED-3 (homolog of caspase 9) is activated following binding to the CED-4. Apaf-1 and CED-4 proteins both contain ATP binding motifs, highlighting the importance of ATP for activation of caspases.

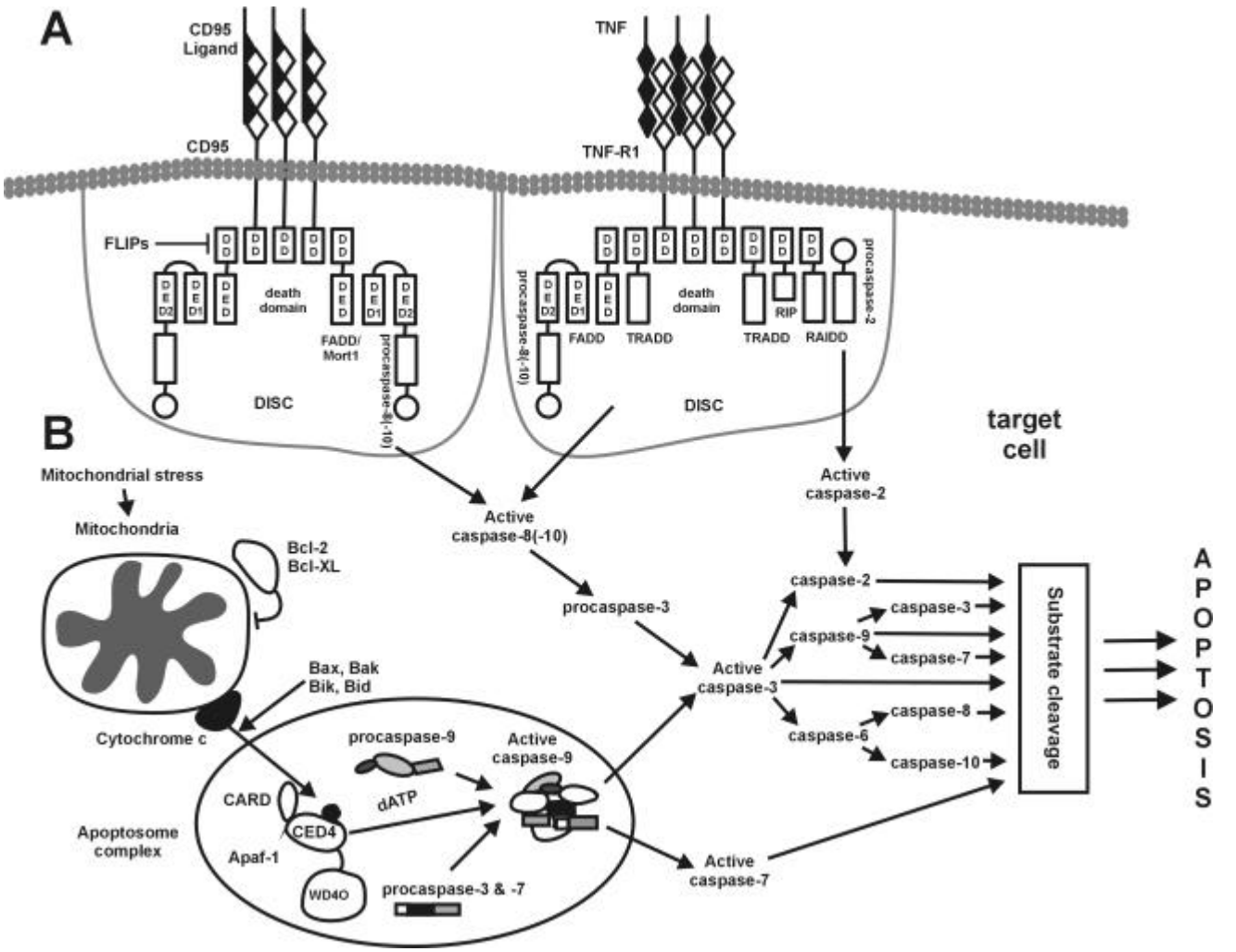

Fig. 1. Receptor-mediated (A) and stress-induced (B) caspase activation pathways (see text for details)

In contrast to CED-4, Apaf-1 possesses two additional domains: a CARD and series of 12 WD40 repeats (Zou et al., 1997). Nevertheless, the mechanism of caspase activation in non-receptor-induced apoptosis is similar in both C. elegans and humans and may be considered as mechanism of homo-activation (Stennicke et al., 1998). The mechanism of hetero-activation is related to receptor-induced apoptosis and involves caspases (-2,-3,-6,-7) that form proteolytic cascade with caspases -8 and -10 at the beginning (Srinivasula et al., 1996; Zou et al., 1997). It is interesting that receptor- and stress-induced apoptotic pathways are overlapping, for example, activated by the mechanism of homo-activation caspase-3 processes caspase-6, which, in turn, activates caspases-8 and -10. These caspases are at the beginning of caspase activation cascade that proceeds by the mechanism of hetero-activation (Slee et al., 1999). Simultaneously, caspase-3 following its activation by caspase-8 and/or -10 (mechanism of hetero-activation) may process also caspase-9, which is known to be activated by the mechanism of homo-activation via the apoptosome (the activating complex formed by cytochrome c, Apaf-1, dATP/ATP and procaspase-9).

It should be noted that all these results have been obtained *in vitro*. It is rather difficult to determine the order, in which caspases activate each other *in vivo*. However, in the light of these data, caspases-8, -10 and -9 appear to be the apical caspases in receptor- and stress-induced apoptosis (Sun et al., 1999).

2.2.1.3 Substrates cleaved by caspases during the execution phase of apoptosis

During apoptosis, more than 100 proteins and enzymes are cleaved by caspases-executioners (Stroh and Schulze-Osthoff, 1998; Nicholson and Thornberry, 1997). The ultimate result of all these caspase-mediated cleavage events is: 1) stop of cell-cycle progression, 2) breakdown of the structural components of the cell, 3) impairment of homeostatic and repair mechanisms, 4) cell detachment from the surrounding tissue, – processes which make the cells recognisable by macrophages and facilitate their engulfment.

Some caspases have common substrates (caspase-3 and caspase-7 both cleave PARP), whereas others are more selective, e. g. protein kinase C δ is cleaved by caspase-3, but not by caspase-7. Other caspases may have unique substrate specificities (caspase-6 is the only caspase known to cleave lamins).

Cleavage of a definite number of key proteins is very important for the development of apoptotic events. Proteins which are substrates for caspases may be divided into three groups:

Table 1. **Substrates specifically cleaved by caspases**

Cytoskeletal and nuclear proteins, critical for maintenance of cell structure:
β-actin (Mashima et al., 1995)
α- fodrin/non-erythroid spectrin (Martin et al., 1995)
lamins (Lazebnik et al., 1995)
Gas 2, which is important for microfilament stability (Brancolini et al., 1995)

Proteins, involved in metabolism:
protein kinase C δ (Emoto et al., 1995)
retinoblastoma protein (An and Dou, 1996)
cellular phospholipase A2 (Voelkel-Johnson et al., 1995)
inhibitor of caspase-activated DNAse (DFF45/ICAD) (Liu et al., 1997; Enari et al., 1998),
Bcl-2, Bcl-x_L (Vaux et al., 1988)
460 kDa catalytic subunit of DNA-dependent protein kinase (Casciola-Rosen et al., 1995)
70 kDa protein component of the U1 small ribonucleoprotein (Casciola-Rosen et al., 1994)
heteronuclear ribonucleoproteins C (Waterhouse et al., 1996)
140 kDa component of the DNA replication complex C (Song et al., 1997)

Repair enzymes: poly-(ADP-ribose)polymerase (Lazebnik et al., 1994)

2.2.1.4 Role of non-caspase proteases in apoptosis

2.2.1.4.1 Calpains

The papain superfamily of cystein proteases includes a group of cytoplasmic enzymes, calpains, bleomycin hydrolases, papain itself and lysosomal proteases, cathepsins (Berti and Storer, 1995). There is an increasing body of evidence indicating that calpains, cathepsins and proteasome play an important role in apoptosis, acting either upstream or downstream of the caspases (Saito et al., 1993; Villa et al., 1998; Squier and Cohen, 1997; Waterhouse et al., 1998).

Calpains are calcium-dependent neutral thiol-proteases that cleave transcription factors, kinases, phosphatases, membrane associated, cytoskeletal and regulatory proteins (the plasma membrane Ca^{2+}-ATPase, spectrin, fodrin, protein kinase C, actin-binding proteins) (Croall and DeMartino, 1991). There are two isoforms of the enzyme: calpain I (μ-form) and calpain II (m-form). Both isoforms have the same substrate specificity. Calpains are heterodimers comprising of 80 and 30 kDa subunits. The 80 kDa subunit encoded by a separate gene is unique to each isoform and consists of four domains (one of them, the protease domain, is similar to that of papain) and a 30 kDa subunit, which is common to both isoforms and consists of two domains (Sorimachi et al., 1994). Disturbances in calcium homeostasis during different pathological processes in the central nervous system (ischemia, Alzheimer's and Parkinson's diseases, trauma) lead to the activation of several calcium-dependent enzymes, including calpains (Saito et al., 1993). In addition to regulation by calcium levels, calpain activity is controlled by an endogenous protein inhibitor calpastatin (Waxman and Krebs, 1978).

In some cases calpains may play a regulatory role during apoptosis (Debiasi et al., 1999; Waterhouse et al., 1998; Villa et al., 1998; Hiwasa, 1996; Squier et al., 1997; Squier et al., 1999). Induction of calpain activity by the depletion of calpastatin was sufficient to initiate apoptosis in neutrophils (Squier et al., 1999). After induction of apoptosis with anti-CD95 monoclonal antibody, staurosporine or TNF in Jurkat T-lymphocytes and U937 monocytic leukemia cells, calpastatin was cleaved, and this cleavage was inhibited by the caspase inhibitors z-VAD-cmk and z-IETD-fmk, thus indicating, that caspases may regulate calpain activity during apoptotic cell death via calpastatin cleavage (Pörn-Ares et al., 1998). During drug-induced

apoptosis in HL-60 cells, it has been shown that calpain activity is downstream of caspases (Wood and Newcomb, 1999).

There is an increasing body of evidence to suggest that in many forms of apoptosis calpains may act upstream of caspases, for example, in calcium ionophore-stimulated apoptosis during platelet activation (Wolf et al., 1999 a), cycloheximide- (Squier et al., 1999), radiation- (Waterhouse et al., 1998) and trophic factor deprivation-induced apoptosis (Villa et al., 1998). McGinnis et al. (1999) demonstrated that procaspase-3 and PARP are substrates for calpains in maitotoxin-treated cells. These data were confirmed also by *in vitro* experiments, where purified μ-calpain cleaved procaspase-3 and PARP to 30 kDa and 40 kDa fragments, respectively. Furthermore, calpain and caspase-3 have common apoptotic substrates such as calpastatin (Pörn-Ares et al., 1998), calcium/calmodulin-dependent protein kinase IV (CaMK TV) (McGinnis et al., 1998), fodrin (Martin et al., 1995; Waterhouse et al., 1998), alpha-spectrin (Nath et al., 1996), actin (Villa et al., 1998) and lamins (Traub et al., 1988), suggesting that there may be overlapping of calpain and caspase activities during apoptosis.

It has become evident that calpains are involved in many types of apoptosis, but several aspects of their role in apoptosis remain to be clarified, for example, their position in the proteolytic cascade and their specific substrates.

2.2.1.4.2 Cathepsins

Lysosomal cysteine proteases have traditionally been viewed as mediators of terminal protein degradation and their primary function was to degrade the proteins nonselectively inside the lysosomes. However, cathepsins can be also found outside lysosomes under special conditions, suggesting that these enzymes are involved in a range of specific cellular tasks (Turk et al., 2000). Nowadays 11 human cathepsins have been identified, which can be divided into two families according to their sequence homology: cathepsin L-like (L, V, S, K, F, H, W) and cathepsin B-like (B, C, O and X) (Berti and Storer, 1995; Barrett et al., 1998). The mature forms of the enzymes belonging to these families are rather similar, but the proregions differ significantly in their length (Berti and Storer, 1995). Lysosomal cystein proteases are small proteins of 20 -30 kDa (with the exception of cathepsin C, which is a 200 kDa protein) (Turk et al., 2000).

Cathepsins are active in a slightly acidic environment and most are endopeptidases, although some of them may act as dipeptidyl carboxypeptidase (cathepsin B), aminopeptidase (cathepsin H) and dipeptidyl aminopeptidase (cathepsin C) (Barrett et al., 1998). Cathepsins are synthesised as preproenzymes, which are translocated to the endoplasmatic reticulum, and proteolytic processing of the enzymes to the active form occurs in the acidic conditions of late endosomes or lysosomes (Nishimura et al., 1988). Maturation of lysosomal cathepsins occurs by the process of autoactivation or by other proteases, such as pepsin, neutrophil elastase and cysteine proteases (Nishimura et al., 1988).

There are several ways that regulate cathepsin activity:

1) pH. Most of the lysosomal cysteine proteases are unstable and weakly active at neutral pH (cathepsins L, B, H, K, V, F), their activation optimum is in the acidic conditions (pH 5.5). During maturation of lysosomes, the internal pH drops to 3.8, sufficient for the denaturation of cathepsins

B, S and L (Turk et al., 1999). At lower pH all ligands, including substrates, inhibitors and propeptides bind weakly to cathepsins (Turk et al., 2000). Denaturated cathepsin L is proteolytically degraded by aspartic proteinase cathepsin D, which is highly active at acidic pH (Turk et al., 1999).

2) Redox potential. The active site cysteine is readily oxidised and the enzymes are predominantly active in a reducing environment.

3) Targeting of enzymes to endosomes and lysosomes. For the lysosomal targeting, cathepsins should be mannosylated followed by the interaction of phosphomannosyl residues with mannose-6-phosphate lysosomal receptors.

4) Cysteine protease inhibitors. The main inhibitors of lysosomal cysteine proteases are the superfamily of cystatins, consisting from intracellular stefins (type I, cystatin A and B), extracellular cystatin C (type II) and kininogens (type III) (Chapman et al., 1997), thyropins and the general protease inhibitor α_2-macroglobulin (Turk et al., 2000). Another set of inhibitors are serpin squamous cell carcinoma antigen 1 (Suminami et al., 1991) and the cytotoxic T-lymphocyte antigen-2β (Delaria et al., 1994). These inhibitors appear to inhibit active enzymes, that have escaped compartmentalisation.

In addition to their degradative function, cathepsins process many of the proteins outside of lysosomes. They are involved also in the immune responses and extracellular matrix remodelling important in bone development and have been implicated in a number of diseases, including cancer, rheumatoid arthritis, multiple sclerosis and Alzheimer's disease (Chapman et al., 1997; Shuja et al., 1991; Mort et al., 1984; Cataldo and Nixon, 1990; Bever and Garver, 1995).

A growing body of evidence stresses the role of cathepsins as positive mediators of apoptosis. One piece of indirect evidence for the involvement of lysosomal cysteine proteases in neuronal apoptosis was provided by the experiments with stefin B knock-out mice (Pennacchio et al., 1998), in which stefin B deficiency resulted in cerebellar apoptosis. This inhibitor does not inhibit caspases, the major apoptotic proteases (Turk et al., 2000), but inhibits lysosomal cysteine proteases, thus suggesting their possible role in apoptosis. Apoptosis induced by serum deprivation in dorsal root ganglion neurons and in PC 12 cells was associated not only with activation of caspase-3, but also activation of aspartic proteinase cathepsin D (Isahara et al., 1999). Further experiments with cathepsin inhibitors have revealed a novel pathway for initiating cell death, which is regulated by lysosomal cathepsins and in which cathepsin D acts as a death factor. This death-inducing activity may be normally suppressed by cathepsin B (Isahara et al., 1999). Overexpression of cathepsin D by ectopic expression has been shown to induce cell death even in the absence of any external stimulus (Deiss et al., 1996). In some cases, the apoptosis-stimulating effect of cathepsin D occurs upstream of caspase activation (Öllinger, 2000).

Recently the capacity of cathepsins to process caspases has been demonstrated. In HeLa and L929 cells cathepsin B is a caspase-1 and caspase-11-processing proteinase (Vancompernolle et al., 1998) and cathepsin G can generate active caspase-7 (Zhou and Salvesen, 1997). Cathepsins B and L are potential processing proteinases for caspase-3 during induction of apoptosis in erythroid progenitor cells (Gregoli and Bondurant, 1999). In a cell free system, cathepsin L liberated from the digitonin-treated fraction of rat liver lysosomes, activated procaspase-3 in rat

liver cytosol (Ishisaka et al., 1999). Cathepsin C is indirectly involved in the activation of caspases through its action on the cytotoxic T cell serine protease granzyme B (Pham and Ley, 1999).

In summary, these data lead to the conclusion that cathepsins are involved in the development of apoptosis and they can act either upstream or downstream of the caspases.

2.2.1.4.3 Proteasomes

Proteasomes are large multicatalytic protease complexes that selectively degrade intracellular proteins. The 26 S proteasome consists of the 20 S (M.W. 700 kDa) proteasome with peptidase activities and regulatory complex, composed of multiple ATPases (Rivett, 1993). A protein, which is destined for degradation, is attached to multiple molecules of ubiquitin, a 76-amino acid protein. This ubiquitin-proteasome degradation pathway is very important for the degradation of short-lived proteins in eukaryotic cells. Ubiquitin is activated by an activating enzyme and is transferred to the conjugating enzyme, which helps to bind ubiquitin to the ε-amino groups of lysine residues of substrate protein (Ciechanover, 1994). Ubiquitinated protein is transferred to the proteasome, where it undergoes final degradation, and the ubiquitin is recycled. Proteasome is involved in cell cycle regulation and cell proliferation (various cyclins and proto-oncogenic proteins are proteolytically degraded by proteasome) (King et al., 1996), in antigen presentation (Rock et al., 1994), in the regulation of gene expression (Palombella et al., 1994) and can be also involved in apoptosis (Dallaporta et al., 2000).

The role of proteasomes in apoptosis remains unclear. Some reports have suggested that proteasome might be an upstream regulator of caspases (Grimm et al., 1996; Sadoul et al., 1996; Hirsch et al., 1998) and of ubiquitin gene expression (Delic et al., 1993) during apoptosis. It has been shown that functionally active proteasomes are necessary for induction of apoptosis in quiescent cells, such as thymocytes and sympathetic neurons (Grimm et al., 1996; Sadoul et al., 1996). Apoptosis in thymocytes, induced by glucocorticoids, topoisomerase II inhibitor, etoposide (Dallaporta et al., 2000; Hirsch et al., 1998; Grimm et al., 1996; Stefanelli et al., 1998), ionising radiation or phorbol ester (Grimm et al., 1996) was associated with proteasome activation. The same results have been obtained also in sympathetic neurons undergoing programmed cell death during nerve growth factor (NGF) deprivation (Sadoul et al., 1996). Specific proteasome inhibitors lactacystin and MG 123 or MG 132 prevented all of the manifestations of apoptosis.

In contrast, in proliferating cells (tumour cell lines) proteasome mediated proteolysis is known to be important for cell survival and cell cycle progression, and proteasome inhibition by specific proteasome inhibitors induces apoptosis (Kitagawa et al., 1999; Chandra et al., 1998; Glockzin et al., 1999). It appears that the proteasome can perform diverse, even opposite, functions when cells are in different growth conditions (Grassilli et al., 1998).

In summary, the results demonstrate that activation of caspases plays a crucial role in the development of apoptosis. However, in addition to caspases, other components of the proteolytic machinery involving cathepsins, calpains and proteasomes can also mediate apoptosis. Non-caspase proteases can operate either downstream of caspase and thus participate in the amplification of proteolytic cascade, or operate upstream of caspases thus triggering this proteolytic cascade.

2.2.2 Activation of apoptotic nucleases and chromatin disintegration

2.2.2.1 Role of caspase activated DNase (CAD) in apoptotic DNA fragmentation

Disintegration of nuclear DNA into oligonucleosomal fragments represents a classical manifestation of apoptosis (Wyllie et al., 1980; Kerr et al., 1972). The discovery of caspase-activated DNase (CAD) and its inhibitor ICAD (Enari et al., 1998) has provided a direct link between caspases and apoptotic DNA disintegration.

The CAD and its inhibitor ICAD have been purified from mouse lymphoid cells, the respective M.W. of these proteins is 40 kDa and 35 kDa (Enari et al., 1998). ICAD was identified only in the extracts of proliferating cells and not of the apoptotic cells. At the same time, Liu et al. (1997) and Halenbeck et al. (1998) independently purified DNA fragmentation factor (DFF) or CPAN, the proform of CAD, from human HeLa and Jurkat cells, respectively. DFF consists of two subunits of 45- and 40 kDa proteins (DFF-45 and DFF-40). Treatment with caspase-3 led to the cleavage of DFF-45 and induction of the DNA fragmentation activity. Both mouse and human CADs are very similar, their amino acid sequences are conserved (75.9% identity), but they do not have any homology with other DNases, such as DNases I and II (Enari et al., 1998; Halenbeck et al., 1998). Mouse and human CADs have a series of basic amino acids at the C-terminus of the molecule. CAD, which does not have this C-terminal stretch of 15 basic amino acids, still possesses DNase activity, but does not cause DNA fragmentation in nuclei, indicating, that amino acids 3-329 are sufficient for the DNase activity, and amino acids 330-344 are important for nuclear transport. The full-length form of ICAD (ICAD-L) is a 45-kDa protein. A short form (ICAD-S) consists of amino acids 1-265 of ICAD-L. Human DFF-45 purified in a complex with DFF-40 (CAD) and mouse ICAD have been identified as a CAD inhibitors and correspond to ICAD-L and ICAD-S respectively (Enari et al., 1998). Both ICAD-L and ICAD-S possess of two caspase-3-recognition sites (amino acid positions 117 and 224) and can efficiently bind to CAD, inhibiting its DNase and DNA fragmentation activity (Sakahira et al., 1999 a). Both of the ICAD caspase recognition sites must be cleaved by caspases-3 and -7 (but not with caspases 1, 2, 4-6, and 8) to activate CAD (Sakahira et al., 1998; Mcllroy et al., 1999; Wolf et al., 1999 b). ICAD-L, but not ICAD-S, has a specific chaperone-like activity for CAD, therefore, ICAD-L (DFF-45) is complexed with CAD (DFF-40) in the cells. As soon as apoptosis is initiated, activated caspase-3 cleaves ICAD-L and releases CAD, which is capable of degrading the chromosomal DNA. ICAD-S regulates the CAD activity by binding to the activated CAD (Nagata, 2000).

The expression of CAD and ICAD mRNAs and their proteins depends on the type of tissues or cell lines. There is a positive correlation between levels of CAD and ICAD proteins and the intensity of DNA degradation in cells induced to undergo apoptosis. If cells with low levels of CAD and ICAD proteins (fibroblasts, nerve cells) were transfected with CAD and ICAD expression vectors, they start to show extensive DNA fragmentation upon the induction of apoptosis (Mukae et al., 1998).

To understand the *in vivo* role of DFF45 in programmed cell death, Zhang et al. (1998) generated DFF45 mutant mice. The experiments revealed that in response to apoptotic stimuli, splenocytes, thymocytes, and granulocytes from DFF45 mutant mice were resistant to DNA fragmentation, and splenocytes and thymocytes were also resistant to chromatin condensation. Similar results have been obtained with lymphoid and myeloid cell lines, expressing caspase-resistant ICAD (Mcllroy et

al., 1999; Sakahira et al., 1998). When these cell lines were induced to undergo apoptosis, they did not show DNA fragmentation, because ICAD remained intact. These data confirm the concept, that apoptotic DNA fragmentation is regulated by the CAD/ICAD system, which is downstream of caspase-3 (Nagata, 2000).

Activation of an endonuclease which preferentially cleaves DNA at the internucleosomal regions, has been considered as a biochemical hallmark of apoptosis for many of years (Wyllie et al., 1980; Liu et al., 1997). Another type of DNA cleavage during apoptosis has been described to yield a set of HMW-DNA fragments sized about 50-100 kb (Cohen et al., 1992; Oberhammer et al., 1993). The cleavage of nuclear DNA into HMW fragments has been shown to precede the formation of the oligonucleosomal DNA ladder in many cell types, and in several cell lines it can even occur in the absence of internucleosomal DNA fragmentation (Brown et al., 1993). The formation of HMW-DNA fragments is considered to be an early critical event during apoptotic DNA disintegration (Oberhammer et al., 1993), and is widely thought to result from the disassembly of higher-level chromatin structural domains (Lagarkova et al., 1995; Li et al., 1999).

Compactization of DNA in the cell nucleus is achieved by the several hierarchical levels of chromatin folding. At the lower level of chromatin folding nuclear DNA is tightly bound to disc-like structures of histones forming a repeating order of DNA-protein particles called nucleosomes (Fig. 2, A, B, Alberts et al., 1989).

The nucleosome is a disc-shaped particle with a diameter of about 11 nm, consisting of an octameric histone core (2 copies of nucleosomal histones H2A, H2B, H3, H4) with two full turns of double-stranded DNA helix (83 nucleotide pairs per turn) wound around, plus adjacent “linker DNA”. The length of the linker DNA, the region between nucleosomes, can vary in length from 0 to 80 base pairs. Nucleosomes are spaced at intervals of about 200 base pairs along the DNA, resulting in the formation of “beads-on-a-string” chromatin structure. The nucleosome “bead” can be removed from the DNA “string” by the DNA degrading enzymes.

The next order of DNA organisation in the chromatin is the packing of nucleosomes in the 30- nm chromatin fibers (Fig. 2, C). This type of packing requires one molecule of histone H1 per nucleosome. The molecule of H1 has a central conserved globular region connected to the extended amino-terminal and carboxyl-terminal “arms”. Every H1 molecule binds to the specific site on a nucleosome by its globular region and the arms reach other sites on the histone cores of adjacent nucleosomes thus providing regular arrays in which the DNA is more highly condensed.

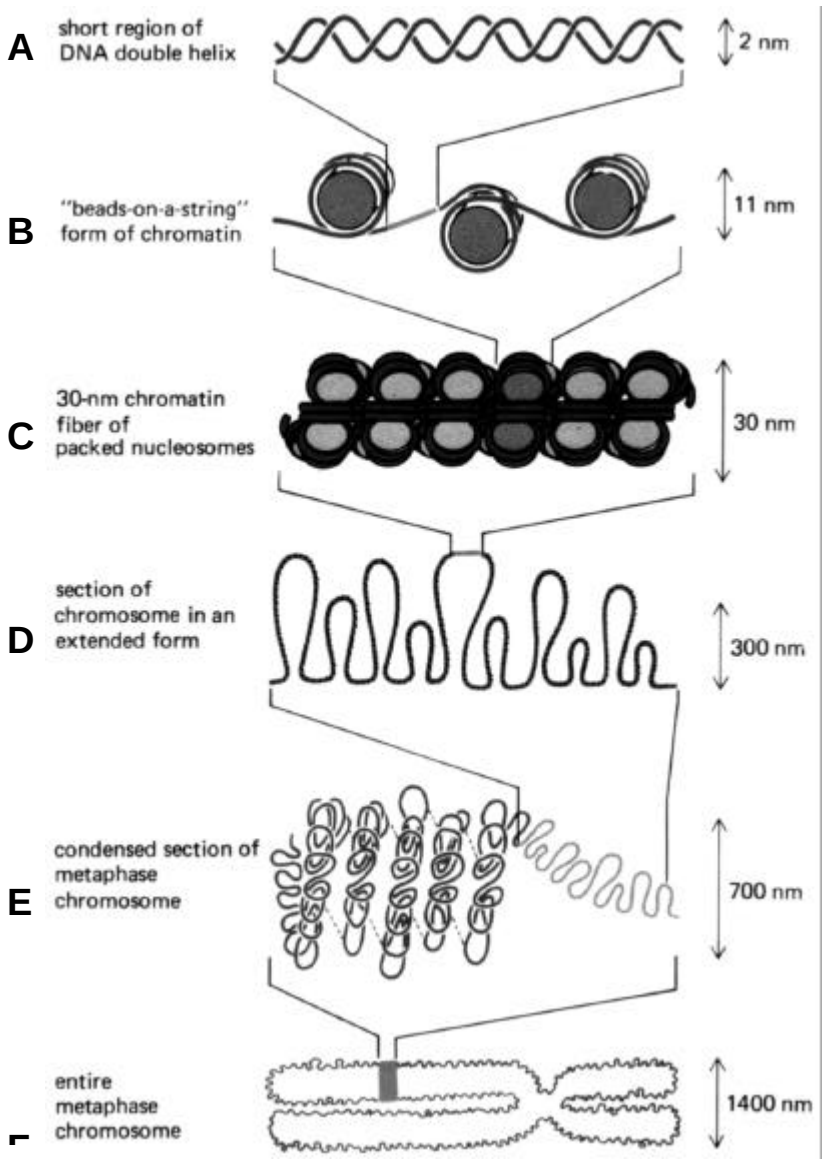

Fig. 2. Schematic illustration of some of the many orders of chromatin packing (by Alberts et al., 1989)

The higher-order structure of chromatin is provided by the formation of looped domains, loops of chromatin that extend from the chromosome axis (Fig. 2, D, E; 3). These looped domains may be formed and maintained by DNA-binding proteins that recognise the regions of specific nucleotide sequences and clamp 30 nm chromatin fibers together (Fig. 3, A). Another way for the formation of chromatin loops is binding of the DNA at the base of the loop to the chromatin axis (Fig. 3, B). Cleavage of the chromatin superstructure into 300 kb fragments corresponds to rosette-like formations that contain six individual α-helical loops and 50 kb fragments correspond to individual loop domains (Filipski et al., 1990).

The axial regions where the ends of DNA loops are located, are highly enriched for DNA topoisomerase II, an enzyme that forms covalent bonds to both strands of the DNA helix, making a transient double-strand break in the helix. Topoisomerase I and II play important roles during DNA replication, forming a "swivel" in the DNA helix. Taking into account the ability of topoisomerase II to cleave the double-strand DNA helix and the position of the enzyme on the ends of DNA loops,

one can hypothesise that topoisomerase II may be involved in the HMW-DNA cleavage which occurs during apoptosis.

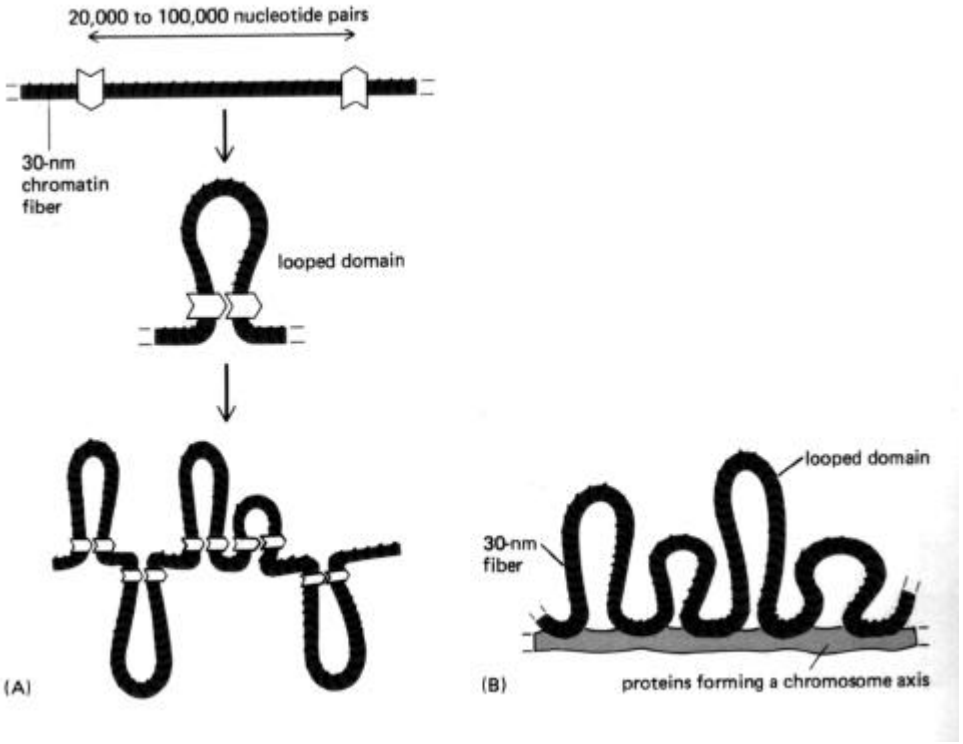

Fig. 3. Schematic views of a section of a chromosome folded into a series of looped domains, each containing perhaps 20,000 to 100,000 nucleotide pairs of double-stranded DNA condensed in a 30-nm chromatin fiber. (A) A folding model requiring sequence-specific DNA-binding proteins at each end of a loop. (B) A folding model requiring a chromosome axis. The axial region of isolated mitotic chromosomes (where the ends of the loops are located) is highly enriched for the abundant enzyme DNA topoisomerase II (by Alberts et al., 1989)

2.2.2.2 Role of non-CADs in nuclear apoptosis

In most mammalian models of apoptosis, the inhibition of activated caspases does not prevent cell death. In some cases, caspase inhibitors may only retard the cell death. This is the case for Fas/Apo-1/CD95-induced apoptosis in L929 cells (Vercammen et al., 1998). The cell death following the inhibition of caspase activity may occur either in the presence (Dong et al., 1998) or in the absence of internucleosomal DNA fragmentation (McCarthy et al., 1997), thus suggesting that caspase/CAD pathway is redundant in apoptosis.

Recently new data concerning a mammalian death effector called apoptosis inducing factor (AIF) have been reported (Lorenzo et al., 1999). AIF bears a highly significant homology with oxidoreductases from eukaryotes and prokaryotes in its C-terminal part (Susin et al., 1999). AIF is localised in the cell in mitochondrial intermembrane space. Within the oxidoreductase-like domain

of AIF, there are nuclear localisation signals which are compatible with translocation of AIF to the nucleus during induction of apoptosis. This nuclear relocalisation of AIF leads to the minor peripheral chromatin condensation (stage I) and DNA disintegration into HMW-DNA fragments of ~50 kbp without the formation of an oligonucleosomal DNA ladder (Lorenzo et al., 1999). As shown in experiments on mouse embryonic fibroblasts obtained from caspase $3^{-/-}$ or Apaf-$1^{-/-}$ mice, microinjection of such cells with recombinant AIF causes only peripheral chromatin condensation (stage I), whereas microinjection with activated caspase 3 or its downstream target CAD causes more profound chromatin condensation (stage II). The same results have been obtained also in cell free system experiments on purified HeLa nuclei (Susin et al., 2000). AIF and CAD act independently from each other during the induction of nuclear apoptosis. Inhibition of CAD by ICAD or immunodepletion of AIF failed to block nuclear DNA loss in purified HeLa nuclei treated with apoptotic cytosols. At the same time, when AIF immunodepletion was combined with ICAD treatment, apoptotic DNA loss was prevented (Susin et al., 2000). Thus AIF and CAD belongs to distinct pathways of nuclear apoptosis, acting in a redundant fashion.

Microinjections of recombinant AIF into the cytoplasm of live cells lead to the morphological and biochemical changes typical of apoptosis. None of these changes is prevented by the broad spectrum caspase inhibitor z-VAD-fmk, suggesting that they are caspase-independent (Susin et al., 1999). To summarise these data, one could propose that AIF is a possible effector of a caspase-independent cell death that causes changes associated with apoptosis. Taking into consideration the fact that translocation of AIF from mitochondria to the nucleus leads to the formation of HMW-DNA fragmentation, but not to an oligonucleosomal ladder, one would predict that AIF must activate some nuclease(s) distinct from CAD. These DNase(s) may represent domain nuclease(s) and cleave nuclear DNA into HMW-DNA fragments in a caspase-independent manner.

2.3 Role of apoptosis in neuronal development and degeneration

The programmed cell death that occurs during development was firstly described for nervous system (Oppenheim, 1991). Neurons seem to be produced during developmental stage in much greater numbers than those found in the adults and these excess neurons are eliminated (reviewed by Oppenheim, 1991). The reason for this abundant overproduction of neurons is unknown, but the most common hypothesis is that neurons compete for contacts with their cellular partners and thus diminish their numbers to those sufficient for innervation of their targets (Pettmann and Henderson, 1998). The reason of neuronal death during development may be competition for access to limiting quantities of neurotrophic factors produced by their target cells and unsuccessful cells then die (Oppenheim, 1991). In C. elegans, neurons destined to die may express specific genes that predispose them to cell death (Ellis and Horvitz, 1986).

Abnormal neuronal cell death may occur after stroke, trauma and in the neurodegenerative diseases (ND): amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) etc. In the majority of cases, ND are sporadic and their causal events have rarely been identified. Familial forms of ND are similar, but not identical to the sporadic diseases. Familial ND are traced back to mutations or deletions of genes, for example, familial PD is associated with loss of dopaminergic midbrain neurons as a result of a mutation in the gene for α-synuclein, which is normally expressed in presynaptic nerve terminals (Polymeropoulos et al., 1997). Mutations in genes coding presenilins 1 (PS-1) and 2 (PS-2) are associated with AD (Hardy, 1996) and, finally,

mutations in Cu, Zn-superoxide dismutase (SOD) seems to cause the loss of cortical and spinal motoneurons in patients with familial ALS (Brown, 1997).

Neuronal cell death after trauma, stroke and some ND may occur as a result of excitotoxicity caused by enhanced levels of excitatory amino acids, in particular, glutamate. During ischemic brain injury, glutamate accumulation leads to overstimulation of postsynaptic glutamate receptors, influx of Ca^{2+}, Na^{+}, Cl^{-} and H_2O, resulting in marked cell body swelling and cell death with subsequent release of endogenous glutamate (reviewed by Choi, 1995) that eventually provokes "secondary lesions" during days and weeks after the insult. The acute cell death related to hypoxia or to injury may be similar to necrosis, whereas a secondary lesion may occur due to an apoptotic type of cell death (Bonfoco et al., 1995). In cultured CGCs, glutamate can induce either early necrosis or delayed apoptosis (Ankarcrona et al., 1995). One subpopulation of cells die by necrosis during and shortly after exposure to neurotoxic concentrations of glutamate. Dying neurons rapidly lose their mitochondrial membrane potential and energy charge. Neurons that survive in this necrotic phase recover their mitochondrial activity but later undergo apoptosis. Similar results have been obtained with hippocampal cells: neurons surviving the initial necrotic stage of glutamate-induced cell death started to undergo DNA cleavage typical of apoptosis (Lauritzen et al., 1997). These results confirm that excitotoxicity induced in neuronal cells by glutamate treatment occurs by both necrotic and apoptotic routes of cell death.

AD is characterised by a progressive cognitive impairment that is a consequence of extensive neuronal loss (Berg et al., 1993). The principal pathological feature of the disease is the extracellular deposition of fibrillar amyloid-β peptide (Aβ) and its compaction on into insoluble senile plaques (Hyman, 1998). Mutations in the gene coding for the Aβ precursor protein (APP) and in the presenilin genes mediating proteolytic processing of APP are associated with increased production of the 42-amino-acid form of the peptide ($A\beta_{1-42}$), which is the predominant form found in the amyloid plaques of AD (Hardy, 1996; Scheuner et al., 1994). This insoluble form of amyloid is produced as a result of cleavage of APP by β- and γ-secretases, whereas the soluble or diffuse form of amyloid $A\beta_{17-42}$ (P3) is produced by α-secretase (Halliday et al., 2000). Only $A\beta_{1-42}$, but not $A\beta_{17-42}$, is able to bind to complement and activate microglia which then release the membrane attack complexes (MAC) and free radicals that are involved in the deposition of Aβ and neurodegeneration of neurons (Halliday et al., 2000). Caspases are other candidate enzymes to cleave APP to Aβ peptide and these enzymes are activated during apoptosis (Marin et al., 2000).

Incubation of cultured cells with Aβ promotes the cells to die by apoptosis. As shown by Marin et al. (2000), $A\beta_{25-35}$ induced apoptosis in cerebellar neurons from rat pups by activation of caspase-3. Aβ-induced apoptosis in differentiated SH-SY-5Y human neuroblastoma cells was associated with calcium influx and increased generation of reactive oxygen species (ROS) (Ekinci et al., 2000). Cell culture experiments revealed that incubation of cortical neurons with antioxidants B27, vitamin E and U83836 E, a vitamin E analog, effectively protected the cultured neurons against $A\beta_{25-35}$-induced cytotoxicity (Huang et al., 2000).

The ROS may be required as intermediates in Aβ-induced activation of the transcription factor NF-κB as shown on CGCs and in neurons and astroglia of brain sections from AD patients (Kaltschmidt et al., 1997). These results suggest that abnormal gene expression in diseased nervous tissue is at least in part due to Aβ-induced activation of NF-κB, an early transcriptional regulator of numerous proinflammatory genes. All of these findings point to some link between deposition of

Aβ, oxidative stress, disruption of ion homeostasis and an apoptotic form of neuronal death in AD. Expression of the human PS-1 L286V mutation in PC12 cells increased their susceptibility to apoptosis induced by trophic factor withdrawal and Aβ. Increases in oxidative stress and intracellular calcium levels induced by the apoptotic stimuli were exacerbated greatly in cells expressing the PS-1 mutation, as compared with control cell lines and lines overexpressing wild-type PS-1 (Guo et al., 1997; Furukawa et al., 1998). Expression of PS-1 mutation in PC12 cells results also in aberrant differentiation responses to nerve growth factor (NGF) and suppression of DNA binding activity of the transcription factor AP-1, induced by NGF (Furukawa et al., 1998).

The PSs are membrane proteins that are localized mainly in the endoplasmic reticulum (ER). Their normal function and pathogenic roles in AD are not known. PSs are thought to be involved in APP processing, expression of mutant PSs in cultured cells and transgenic mice results in increased production of an amyloidogenic-cytotoxic form of Aβ (Mattson and Guo, 1997). Defective presenilin function causes chromosome mis-segregation during mitosis resulting in apoptosis (Li et al., 1997). Thus, there is evidence that PSs mutations are involved in the pathogenesis of neuronal degeneration in AD and sensitise the cells to apoptosis in cell culture models (Li et al., 1997; Mattson et al., 1998).

Caspases, the main executioners of apoptosis, actively contribute to the molecular pathogenesis of neurological diseases. For example, the presence of activated caspase 8 has been shown in the insoluble fraction of affected brain regions from Huntington's disease (HD) patients, but not in those from control patients (Sanchez et al., 1999). Caspases appear to be involved in the molecular pathology of HD by directly cleaving huntingtin and generation toxic protein fragments containing the polyglutamine tract (Wellington and Hayden, 2000). Several proteins involved in AD including APP and PSs are also cleaved by caspases (Wellington and Hayden, 2000). Activated caspase 3 and DNA fragmentation have been detected in both neurons and astrocytes in frontotemporal dementia (FTD) cases and cerebral ischemia models (Su et al., 2000; Wiessner et al., 2000). Some therapeutic approaches may be linked with inhibition of caspase activation during apoptotic neuronal death in ALS, cerebral ischemia, HD and AD (Li et al. 2000; Wiessner et al., 2000; Wellington and Hayden, 2000). At the same time, there is some evidence that neuronal apoptosis may proceed in a caspase-independent manner (Nicotera, 2000; Ray, 2000), which poses the question whether caspase inhibitors could potentially be used to prevent excess apoptosis in disease states. Other approaches may be undertaken, especially, the possible use of trophic factors to regulate plasticity in the adult nervous system, which represents potential drug target in neurodegeneration associated human diseases (Froestl, 2000).

3. AIMS OF THE STUDY

The aim of the present study was to investigate the mechanisms of DNA disintegration during neuronal apoptosis and to clarify whether HMW- and internucleosomal DNA cleavage represent separate types of apoptotic DNA disintegration.

The specific aims were as follows:

1. To determine whether apoptosis induced by different insults in neuronal cells can proceed with a distinct pattern of DNA disintegration (**I**)

2. To elucidate whether HMW- and internucleosomal DNA cleavage represent independent programmes of apoptotic DNA disintegration (**I, II**)

3. To investigate the role of caspases in the activation of HMW-DNA fragmentation (**III, IV**)

4. To explore whether suramin a multitargeted antiproliferative drug prevents the apoptosis associated with distinct pattern of DNA disintegration (**V**)

4. MATERIALS AND METHODS

4.1 Reagents

The substrates for caspase-1, Ac-YVAD-AMC (Cat. #400020); caspase-2, Z-VDVAD-AFC (Cat. #218740); caspase-5, Ac-WEHD-AFC (Cat. #218754); caspase-9, Ac-LEHD-AFC (Cat.# 218765); cathepsin B, Z-Arg-Arg- AMC (Cat.#219392) and calpain I and II (Cat.#208723), as well as etoposide, OKA, Ara-C, staurosporine and suramin were purchased from Calbiochem. Ac-DEVD-AMC (Cat. #66081T), a caspase-3 substrate, and recombinant caspase-3 were from Pharmingen. Anti-poly-(ADP-ribose)-polymerase polyclonal antibody and Complete^{TM} proteinase inhibitors were supplied by Boehringer. Anti-c-Jun, anti-c-Fos, anti-p53 antibodies were provided by Santa Cruz Biotechnology, Inc. ECL-nitrocellulose membrane (0.45μm) and horse radish peroxidase-conjugated donkey anti-rabbit immunoglobulin F(ab)^2 fragment were purchased from Amersham. ECL-solution (Pierce), Hoechst 33258, MTT reagent, DMEM medium with 1,000 and 4,500 mg/l glucose, L-glutamate , ATA, MK-801 were provided by Sigma, IMMU-MOUNT ^{TM} by Shandon Inc., CytoTox 96^R Non-Radioactive Cytotoxicity Assay by Promega. Fetal calf serum and trypsin-EDTA were from Gibco BRL. Protein Assay Kit was from Bio- Rad Laboratories and 1 kb-DNA ladder and midrange II PFG molecular weight markers from New England Biolabs.

4.2 Cell culture

Mouse NB 2A neuroblastoma cells, obtained from the American Type Culture Collection (CCL 131), were cultured in an atmosphere of 10% CO_2 at +37^{0}C in DMEM medium with 1,000 mg glucose/l, supplemented with 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum.

The suspension of CGCs was prepared from 7-day old rats as described previously (Korhonen et al., 1997). Cells were plated to a desired density (2.0-2.5x10^{5}cells/cm^2) to the poly-D-lysine coated Nunc dishes (90 mm diameter) or 12-well Nunc plates, containing coverslips inside. CGCs cultures were maintained in an atmosphere of 10% CO_2 at +37^{0}C in DMEM, containing 4,500 mg glucose/l, supplemented with 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and inactivated 10% fetal calf serum. The total amount of medium was 0.2-0.25 ml/cm^2.

4.3 Induction of apoptosis

Apoptosis in NB 2A cells was induced in exponentially growing cells either by serum withdrawal or by the treatment of cells with etoposide (5 - 40 μM), staurosporine (40 nM), OKA (10 - 100 nM), or Ara-C (0.5-2 μM). Treatments were performed up to 3 days. The CGCs cultures 8- to 9-day old were treated with OKA (40 nM) (0-48 h) or 50 μM L-glutamate (15 min). The treatment of CGCs with L-glutamate was carried out in Locke's salt solution as described previously (Korhonen et al., 1997) followed by washing with the same solution containing 1mM $MgSO_4$ and reincubation in the old culture medium up to 36 h. The control cells were subjected to the same procedure without L-glutamate. In experiments with MK-801 (10 μM), ATA (100 μM) and suramin (100 μM, 200 μM) the chemicals were added 1 or 2 h before cell treatment and were

present during and after L-glutamate treatment. In CGCs, apoptosis was induced also by incubation of the cells for 0-48 h in serum-deficient medium, containing 5 mM KCl (Ser^-/K^-).

4.4 Cell harvesting

After removing of cell culture medium, the cells were washed once in ice-cold PBS. Cells were scraped in 1 ml of ice-cold PBS, followed by washing of culture plates with 0.5 ml ice-cold PBS. Cells were combined and collected by centrifugation at 1,500 rpm for 5 min. The gathered cells were either immediately used for analysis or stored at -70^{0}C until required.

4.5 Measurement of cell viability

4.5.1 LDH assay

The assessment of cytotoxicity was performed according to CytoTox 96^R Non-Radioactive Cytotoxicity Assay Kit, which quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis (study III). Cell culture medium (500 µl) was taken from different samples and centrifuged at 1,000 rpm for 4 min. Then, 50 µl of each supernatant in triplicate were transferred to a 96-well enzymatic assay plate. To evaluate the target cell maximum LDH release, cells were incubated with 0.8% Triton X-100 (final concentration) in an atmosphere of 5% CO_2 at 37^{0}C for 45 minutes to yield complete lysis of cells. Then, 50 µl of the reconstituted substrate mix was added to each well. After incubation for 30 minutes at room temperature in the dark, the reaction was stopped by addition of 50 µl of stop solution to each well. After removal of bubbles, the absorbance was measured at 490 nm in an microplate reader (Microplate Manager^R 4.0 Bio-Rad Laboratories, Inc.) within one hour after the addition of stop solution.

4.5.2 MTT test

Conversion of MTT to coloured formazan catalyzed by the viable, but not dead cells, was used for measurement of cell viability (studies I, V). Cells ($7.2x10^4$/well) were plated in 12-well plates and cultured for 24 h in culture medium. Apoptosis was induced as described above, and 100 µl of MTT reagent (5 mg/ml in PBS) was added to the cell samples in 900 µl of medium followed by incubation for 1 h at 37^{0}C. After removal of culture medium, 1.0-1.5 ml of lysis buffer (10% SDS, 50% DMF, 2% acetic acid, 2.5% 1N HCI) were added and left for at least 24 h at 37^{0}C. Then, 200 µl of each sample in triplicate were transferred to 96-well Costar plates and absorbance was measured at 595 nm in microplate reader. The results are presented as per cent of the control.

4.5.3 Cytofluorometric analysis

For identification of cells at the late stages of apoptosis or those that have already died, we used cytofluorometric analysis based on the DNA staining by fluorescent dye PI, which cross plasma membrane only after loss of its integrity (Bezvenyuk et al., 2000). Cells (1.0×10^6) were resuspended in 500 µl PBS. The cell suspension was added (drop by drop) into continuously rotating tube with 70% EtOH (5 ml of EtOH pre-cooled at - 20^{0}C). After incubation at -20^{0}C for 2 h, the cell suspension was centrifuged 1,500 rpm for 5 minutes and EtOH was removed. Cells were resuspended in 1 ml PBS containing RNase (0.15 mg/ml) and incubated over night at +37^{0}C in a shaker followed by PI staining (75µg/ml) at +37^{0}C for 2 h. The DNA content was measured at

575 nm on a FACS (Becton Dickinson, USA). Data were analysed with CellQuest software (Becton Dickinson, USA). In all experiments, 10,000 cells were collected.

4.6 Analysis of apoptosis

4.6.1 Nuclear morphology (Hoechst staining)

One of the main features of apoptosis is chromatin condensation and fragmentation of the nuclei into apoptotic bodies. Nuclear morphology was analysed by staining of DNA with Hoechst 33258 dye (studies I-V). To examine nuclear morphology, the coverslips with attached cells were transferred to the 12-well culture plate, washed once in 2 ml PBS and fixed with 1ml 4.0% PFA in PEM buffer, pH 6.8 (100 mM PIPES, 5.0 mM EGTA, 2.0 mM $MgCl_2$) for 10 min at room temperature. After permeabilisation with 0.2% Triton X-100 for 10 min, cells were stained with 500 µl of the Hoechst 33258 (0.2 µg/ml in PBS) nuclear dye-solution for 10 min in dark at RT and washed 3 times with 2 ml PBS. Coverslips were mounted cell side down in IMMU-MOUNT ^{TM} on the objective slides and apoptotic nuclear morphology was analysed with a fluorescent microscope (Nikon, Japan). Nuclei bearing three or more pieces of fragmented chromatin were scored as apoptotic. In each experimental setting, 10-15 independent fields, comprising all together about 1,000 nuclei, were monitored.

4.6.2 Western blot

Western blot was used for detection of proteolytic cleavage of PARP, a nuclear enzyme involved in DNA repair. PARP cleavage by an ICE-like protease represents an early biochemical event in apoptosis (study I). Cells were resuspended in 40 µl of 60 mM Tris-HCl (pH 6.8) with 10% glycerol. SDS to the final concentration of 2.0% was added to each sample, and samples were boiled for 10 min. After centrifugation (12,000 rpm for 12 min at room temperature) the protein in the supernatant was measured according to the Bio- Rad Protein Assay. Then, 30 µg of extracted polypeptides in loading buffer (60 mM Tris-HCl, pH 6.8, 2.0% SDS, 0.2 % BFB, 10% glycerol, 5.0% BME) were resolved on 10% SDS-PAGE in running buffer (24.8 mM Tris (base), 200 mM glycine, 0.1% SDS) at 200 V for 1.5 h. The gel was soaked in transfer buffer (24 mM Tris (base), 192 mM glycine, 20% methanol) for 30 min and electrophoretically transferred in the same buffer onto nitro-cellulose membrane at 20 V overnight. The membranes were stained with Ponceau S for 5 min and washed twice for 5 min in PBS. After washing, the membranes were blocked for 1 h in blocking solution (1% BSA, 0.5% dry milk, PBS-0.05% Tween-20) at RT and washed three times for 5 min with PBS-0.05% Tween-20, followed by incubation for 2 h at RT with the anti-poly-(ADP-ribose)-polymerase polyclonal antibody (1:2000 in blocking buffer). After incubation, the membranes were washed as described above and incubated with the secondary antibody (horseradish peroxidase-conjugated anti- rabbit immunoglobulin $F(ab)^2$ fragment) (1:4000 in blocking buffer) for 1 h at RT. After washing in PBS+0.05% Tween-20, membranes were finally washed with PBS without Tween-20 for 15 min, incubated with ECL-solution (Pierce) (10 -30 sec.) and exposed against ECL-film.

4.6.3 Analysis of protease activity

4.6.3.1 Caspases

Activation of caspases is early marker of the cells undergoing apoptosis. Measurement of caspase activity is one of the obligatory parameters in analysis of apoptotic cell death (studies I-V). Cells were lysed in 10 volumes of ice-cold hypotonic buffer (1.0 mM DTT, 10 mM KCI, 2.0 mM $MgCI_2$, 0.1 mM EDTA, 0.1 mM EGTA, 20 mM HEPES, pH 7.5, 0.1 mM PMSF) on ice for 20 min. After centrifugation (4,500 rpm for 5 min at + 4^0C), the supernatant was further clarified by centrifugation at 13,000 rpm for 20 min at 4^0C. After measurement of protein concentration (Bio-Rad Protein Assay), the activity of caspases in cytosolic extracts (protein concentration 0.2 mg/ml) was assayed in the protease assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2.0 mM DTT) containing 20 μM of fluorogenic caspase substrates (Nicholson et al., 1995). After incubation at 37^0C for 2 h, activity of caspases was measured by Wallac 1420 Victor^{TM} Multilabel Counter with an excitation wavelength of 355 nm and an emission wavelength of 460 nm for caspase-1 and -3 substrates and, correspondingly, 405/535 nm for caspase-2, -5 and -9 substrates. Caspase activities are presented in arbitrary units.

4.6.3.2 Cathepsin B and calpains

An increasing number of evidence suggests that non-caspase proteases may also be involved in apoptosis. Cathepsin B activity was measured in cell lysates diluted with the protease assay buffer (0.4 M Na-acetate (pH 5.5), 4.0 mM EDTA). After centrifugation (1,250 rpm, 20 min) samples were analysed using cathepsin B fluorogenic substrate Z-Arg-Arg-AMC as described previously (Barret and Kirschke, 1981).

Calpain activity was analysed in cytosolic extracts after incubation at 30^0C for 5-10 min with 60 mM imidazole-HCl buffer (pH 7.3), 5.0 mM L-cysteine, 2.5 mM BME, 5.0 mM $CaCl_2$, 4% Me_2SO and 20 μM of calpain fluorogenic substrate. Fluorescence of AMC was monitored using a spectrofluorometer (Wallac 1420 Victor^{TM} Multilabel Counter). The results are presented in arbitrary units.

4.6.4 Analysis of DNA integrity

One of the later steps in apoptosis is disintegration of nuclear DNA into fragments of 50 to 300 kb (HMW-DNA fragmentation) and subsequently into smaller DNA pieces of about 200 bp length (oligonucleosomal DNA ladder). To analyse both types of DNA fragmentation we used conventional and field inversion gel electrophoresis (FIGE) (study I-V). Cells were collected, resuspended in 15 μl of PBS and pre-heated at 42^0C for 5 s. For purification of DNA an equal volume of 1.5% low-melting point agarose pre-heated at 42^0C (in PBS buffer containing 5.0 mM EDTA) was added to the cell suspention and mixed well. The cell suspension was placed into the wells of pre-cooled 24-well cell culture plate and left to solidify for 5 min at 4^0C. Agarose-embedded cells were incubated for 1 h at 37^0C with 500 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 50 mM NaCl) containing 0.5% sodium sarcosyl, 50 μg/ml RNase A, 100 μg/ml proteinase K. Agarose plugs, containing deproteinised DNA, were washed three times with 0.5X TBE buffer (0.045 M Tris, 0.045 M boric acid, 1.0 mM EDTA, pH 8.0) and quantitatively

loaded to wells of 1% agarose gel. The wells were covered with 1.5% low-melting point agarose. The integrity of DNA was analysed in the samples by the conventional gel electrophoresis at 5.0 V/cm for 2-3 h, using 0.5X TBE buffer or by FIGE at 4.0 V/cm for 18 h in 0.5 TBE buffer under constant pulses of electric field (24 s forward and 8 s backward) as previously described (Solovyan and Salminen, 1999).

4.7 Cell free apoptosis assay

4.7.1 Isolation of nuclei

Isolated nuclei (substrate nuclei) were used in cell free apoptosis assay (studies I, II, IV, V). Cells were scraped and harvested by centrifugation at 500 x g for 5 min followed by washing once with PBS and once with ice-cold nuclei isolation buffer (NIB) (10 mM PIPES pH 7.4, 10 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.2 mM PMSF). The cells were resuspended in 10 volumes of NIB and allowed to swell on ice for 20 min, followed by lysis in Dounce homogeniser with 100 gentle strokes of a B-type pestle. The crude nuclear pellet was obtained by centrifugation of cell lysate at 4,500 rpm for 5 min, nuclei were layered over 1 M sucrose, prepared in NIB, and centrifuged at 4,500 rpm for 10 min, followed by washing once in nuclear isolation buffer and resuspension in nuclei storage buffer (10mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5.0 mM EGTA, 1.0 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 50% glycerol, 1.0 mM PMSF) at $2.0x10^8$ nuclei/ml. Nuclei were stored at -70^0C in 20 µl aliquots until required.

4.7.2 Induction and analysis of nuclear apoptosis

To better understand the mechanism of apoptotic execution that occurs *in vivo* we have used an apoptotic cell free system assay (studies I, II, IV, V). Cells were lysed in 1.5-2.0 volumes of ice-cold hypotonic buffer, and cytosolic extracts were prepared as described in "Analysis of protease activity" (see above). Cytosolic extracts were used at a final concentration of 5.0 mg/ml protein.

Nuclei (2 µl from the stock $2.0x10^8$ nuclei/ml) isolated from normal cells were incubated with 20 µl of cytosolic extract for 2 h at 37^0C. Then, 2 µl of incubation mixture were transferred to the 50 µl of protease assay buffer for analysis of protease activity. Rest of the mixture was embedded into low-melting point agarose, lysed and fractionated by gel electrophoresis for analysis of DNA integrity as described in the section "Analysis of DNA integrity".

5. RESULTS

5.1 Neuronal apoptosis induced by genotoxic and nongenotoxic agents is associated with distinct patterns of DNA fragmentation (I)

The results presented here demonstrate that serum deprivation and etoposide both induced apoptosis in NB 2A cells, accompanied by caspase activation and changes in nuclear morphology typical of apoptosis. However, apoptosis induced by two these insults was associated with a distinct pattern of DNA fragmentation. The treatment of the cells with etoposide led to the formation of HMW-DNA fragments of about 50-100 kb and 300 kb without the formation of an oligonucleosomal DNA ladder. Serum deprivation-induced apoptosis was associated with disintegration of nuclear DNA into 50-100 kb DNA fragments and a low molecular weight oligonucleosomal DNA ladder (Fig. 5 A, study I). Incubation of serum-deprived NB 2A cells in the presence of etoposide resulted in an additive effect on the pattern of DNA fragmentation: disintegration of nuclear DNA started earlier, was more intense and involved both 50-100 and 300 kb DNA fragments as seen in cells treated with etoposide, as well as the oligonucleosomal DNA ladder, observed in serum-deprived cells (Fig. 5 B, study I). The differences in the pattern of DNA disintegration were reproducible in a cell free apoptotic system after incubation of isolated non-apoptotic nuclei with cytosolic extracts prepared from either serum-deprived cells or from cells treated with etoposide. Incubation of nuclei with cytosol prepared from serum-deprived cells resulted in the formation of HMW fragments and a low molecular weight oligonucleosomal DNA ladder. However, cytosol prepared from etoposide-treated cells was able to induce only HMW-DNA fragmentation in substrate nuclei without formation of an oligonucleosomal DNA ladder (Fig. 6, study I). The results suggest that the lack of internucleosomal cleavage represents an intrinsic property of etoposide-induced apoptosis.

Etoposide is known to target actively proliferating cells. The high proliferative status of the cultured neuroblastoma cells could be one reason for the differences in the pattern of DNA disintegration in serum-deprived and etoposide-treated cells. To clarify this, we analysed DNA fragmentation in nonproliferating CGCs after treatment with etoposide or incubation in serum/potassium deprived medium. Both etoposide and serum and potassium deprivation induced apoptosis in CGCs accompanied by caspase-3 activation, DNA fragmentation and the appearance of nuclear apoptotic morphology (Fig. 9, study I). The results thus indicate, that the topoisomerase II inhibitor, etoposide, can induce apoptosis both in proliferating cells and nonproliferating post-mitotic neurons. In contrast to serum/potassium deprivation, etoposide induced DNA fragmentation in CGCs without DNA laddering (Fig. 9 A, study I). The results suggest, that different apoptotic stimuli can induce apoptosis with distinct patterns of nuclear DNA disintegration both in proliferating and nonproliferating cells, and that the distinct pathways are involved in apoptotic internucleosomal- and HMW-DNA cleavage at least in NB 2A cells.

5.2 Apoptotic disintegration of nuclear DNA into HMW-DNA fragments proceeds independently of internucleosomal DNA cleavage in NB 2A cells (I, II)

We extended our observations and demonstrated that genotoxic and nongenotoxic stimuli induced apoptotic DNA fragmentation that possessed a differential susceptibility to the ATA and Zn^{2+},

inhibitors of apoptotic nucleases, to the protein synthesis inhibitor, cycloheximide, and to the suramin, a multi-targeted antiproliferative drug. ATA, cycloheximide and suramin effectively prevented internucleosomal and HMW-DNA cleavage in serum-deprived or OKA treated cells, but had only a slight or no obvious inhibitory effect on HMW- DNA disintegration in etoposide or Ara-C treated cells (Fig. 7 and 8, study I; Fig. 6, study II), thus indicating that distinct pathway underlay DNA disintegration during apoptosis that proceeds with distinct pattern of DNA cleavage. Furthermore, we demonstrated that incubation of serum-deprived cells in the presence of Zn^{2+}-ions led to the abrogation of internucleosomal DNA fragmentation, but to the accumulation of HMW-DNA fragments with the pattern being similar to that observed in etoposide treated cells (Fig. 3, study II).

The cell free system experiments showed that a brief heating of the cytosolic extract prepared from the serum-deprived cells had no effect on the oligonucleosomal DNA ladder, but inhibited the cytosol-dependent formation of HMW-DNA fragments in substrate nuclei. Addition of Zn^{2+}- ions to cytosolic extract abrogated the appearance of oligonucleosomal DNA ladder, but had no effect on HMW-DNA fragments (Fig. 5, study II). These data indicate that the apoptotic formation of HMW-DNA fragments may proceed separately of the internucleosomal DNA cleavage.

5.3 HMW-DNA cleavage represents a common step in caspase-dependent and caspase - independent types of neuronal cell death (III, IV)

The treatment of CGCs with phosphatase inhibitor, OKA, or brief incubation of cells with the excitatory neurotransmitter, L-glutamate, both resulted in extensive changes in the cell and nuclear morphology, indicative of cell death. Both L-glutamate and OKA provoke similar changes in nuclear morphology associated with chromatin condensation and DNA fragmentation. At the same time the cytotoxicity induced by OKA was associated with activation of caspase-3 like proteases in contrast to the L-glutamate induced cell death (Fig. 1, study III). No activation of caspase -1, -2, -5 or -9 was observed during L-glutamate induced cytotoxicity. These results suggest that glutamate induces a caspase-independent type of cell death.

The analysis of DNA integrity showed that cell death induced by OKA was accompanied both by oligonucleosomal DNA ladder and HMW-DNA fragmentation. At the same time, L-glutamate induced only HMW-DNA cleavage without internucleosomal DNA fragmentation (Fig. 2, study III). In both cases the similar patterns of HMW-DNA cleavage proceeded with similar kinetics and intensity and involved the formation of 300 kb DNA fragments with their subsequent degradation into 50 kb DNA fragments. Despite this similarity, HMW-DNA cleavage in L-glutamate-treated cells was effectively prevented by MK-801, a L-glutamate receptor blocker, ATA, a general inhibitor of apoptotic nucleases, as well as by Zn^{2+} and Cd^{2+}-ions. At the same time the HMW-DNA cleavage in OKA-treated cells was inhibited only by Cd^{2+}-ions with no apparent inhibitory effect being observed for MK-801, ATA or Zn^{2+} (Fig. 3, study III).

In OKA-treated cells, both internucleosomal- and HMW-DNA fragmentation were effectively inhibited by Z-DEVD-fmk, a specific caspase-3 inhibitor, as well as Z-VAD-fmk, a general caspase inhibitor. In contrast, neither z-DEVD-fmk, nor Z-VAD-fmk had any apparent inhibitory effect on the formation of HMW-DNA fragments induced by L-glutamate (Fig. 4, study III).

These results suggest that different mechanisms are involved in the formation of HMW-DNA fragments during OKA and L-glutamate induced cytotoxicity.

We further investigated in the cell free system experiments the role of caspases in the disintegration of nuclear DNA into the HMW-DNA fragments. We demonstrated that incubation of a non-apoptotic cytosolic extract with recombinant caspase-3 could initiate the disintegration of DNA in substrate nuclei into the HMW- but not oligonucleosomal DNA fragments, and this HMW-DNA cleavage was almost completely suppressed by Z-VAD-fmk (Fig. 3 A, study IV). The incubation of the cytosolic extract with proteinase K resulted in similar disintegration of nuclear DNA into the HMW fragments and in activation of endogenous caspase-3. However, in contrast to the recombinant caspase-3, proteinase K-dependent induction of the HMW-DNA fragmentation was not inhibitable by Z-VAD-fmk (Fig. 3 B, study IV). Finally, the incubation of cytosolic extract with staurosporine induced HMW-DNA cleavage in substrate nuclei without activation of endogenous caspase-3. Z-VAD-fmk had no inhibiting effect on this HMW-DNA fragmentation (Fig. 3 C, study IV). Collectively, these findings suggest that there are multiple pathways of activation of HMW-DNA cleavage, and the formation of HMW-DNA fragments can be induced in both caspase-dependent and -independent manners.

5.4 Caspase/CAD pathway is not essential for HMW-DNA cleavage during apoptosis (IV,V)

Data presented so far demonstrated that formation of HMW-DNA fragments was invariably associated with both caspase-dependent and -independent cell death induced by a variety of insults in NB 2A cells. To further elucidate the role of caspases in the formation of HMW-DNA fragments during apoptosis, we have developed an experimental model in which apoptosis was accompanied by the HMW-DNA cleavage without the formation of oligonucleosomal DNA fragments. We demonstrated that incubation of NB 2A cells with staurosporine in a serum-deficient medium induced rapid cell death accompanied by changes in nuclear morphology typical of apoptosis, activation of a caspase 3-like protease and disintegration of nuclear DNA into the HMW-, but not oligonucleosomal DNA fragments (Fig. 1 A-C, study IV). The same pattern of DNA fragmentation was obtained in a reconstituted cell free apoptotic system. Cytosolic extract prepared from staurosporine-treated cells induced the formation of the HMW-, but not oligonucleosomal DNA fragments in nuclei isolated from normal (non-apoptotic) NB 2A cells (Fig. 1 D, study IV). Disintegration of nuclear DNA preferentially into HMW-DNA fragments both in staurosporine-treated NB 2A cells and in the cell free apoptotic system suggests that the absence of internucleosomal DNA cleavage is an intrinsic feature of staurosporine-induced apoptosis in NB 2A cells.

We further demonstrated that Z-DEVD-fmk, a specific caspase-3 inhibitor, as well as a general caspase inhibitor, Z-VAD-fmk, both effectively prevented activation of caspase 3-like proteases in staurosporine-treated neuroblastoma cells without inhibition of the HMW-DNA fragmentation (Fig. 4 A, study IV). The apoptotic HMW-DNA cleavage induced by staurosporine and serum deprivation was effectively inhibited by suramin (Fig. 4 B, study IV), a drug that prevented neuronal cell death provoked by several cytotoxic insults (Fig. 2, 3, study V). Interestingly, suramin, at low concentration, almost completely inhibited the formation of HMW-DNA fragments without altering the level of endogenous caspase 3-like activity, whereas a high concentration of the drug suppressed both HMW-DNA cleavage and caspase-3 activity in NB 2A cells (Fig. 4 B, study IV).

These results suggest that caspase activation is not essential for HMW-DNA fragmentation in cells induced to undergo apoptosis by staurosporine and serum deprivation.

The discovery of caspase-activated DNase (DFF40/CAD) (Enari et al., 1998; Liu et al., 1997) provided a direct link between caspases and internucleosomal DNA fragmentation during apoptosis. Our results demonstrated that activation of caspase-3 during apoptosis induced by staurosporine in serum deprived NB 2A cells was not associated with the formation of oligonucleosomal DNA fragments. Moreover, the level of both CAD-mRNA and the CAD protein was down-regulated in staurosporine-treated NB 2A cells (Fig. 2, study IV), thus suggesting that DFF40/CAD is not involved in the excision of DNA loop domains during the apoptosis induced by staurosporine in NB 2A cells.

5.5 Suramin rescues the neuronal cells against different types of cell death (III,V)

As mentioned above, treatment of CGCs with L-glutamate induced a cell death which was associated with chromatin condensation, apoptotic-like changes in nuclear morphology and fragmentation of DNA into HMW-DNA fragments. Neither activation of caspase 3-like proteases nor the formation of oligonucleosomal DNA ladder were observed during L-glutamate-induced cell death (Fig. 1 and 2, study III).

HMW-DNA fragmentation in L-glutamate-treated CGCs was effectively inhibited by suramin, whereas the general caspase inhibitor, Z-VAD-fmk, only slightly prevented DNA disintegration. The protective effect of suramin was equally apparent whether added before or after exposure of the cells to L-glutamate. The nuclear morphology of the glutamate treated cells exposed to suramin was indistinguishable from the control (nontreated) CGCs. Cell viability measured by the MTT assay was remarkably increased after suramin treatment (Fig. 2, study V). These results are evidence that suramin has a protective effect in CGCs against the excitotoxicity induced by L-glutamate.

Because the neuroprotective effect of suramin may be due to its ability to antagonise glutamate receptors, we further investigated the effect of suramin on apoptosis induced by staurosporine in NB 2A neuroblastoma cells, which are known to have no glutamate receptors. Staurosporine in standard culture medium induced apoptosis in NB 2A cells that was associated with activation of caspase 3-like proteases and the disintegration of nuclear DNA into HMW- and oligonucleosomal DNA ladder. All these parameters of apoptosis were significantly inhibited by suramin, leading to an increase in cell viability (Fig. 3, study V).

To clarify a possible mechanism whereby suramin could achieve its anti-apoptotic effect, we investigated the influence of suramin on the activity of recombinant caspase-3, the enzyme which is known to be the main executioner of apoptosis (Fig. 4, study V). The results showed that suramin inhibited the activity of recombinant caspase-3 in a concentration-dependent manner in an *in vitro* assay. Thus, the results indicate that suramin exerts its neuroprotective effect on different targets and represents an effective survival agent with multiple mechanisms of action.

6. DISCUSSION

6.1 Apoptosis in neuronal cells induced by different cytotoxic insults: distinct patterns of DNA fragmentation

Internucleosomal DNA fragmentation is one of the typical features of apoptosis in most of the cellular models. An increasing number of evidence indicate, however, that in addition to the internucleosomal DNA ladder, there is another periodicity of DNA fragmentation that yields the set of HMW-DNA fragments. HMW-DNA fragmentation proceeds in many cell types, and in some of them including neuronal ones it can occur without internucleosomal DNA cleavage (Cohen et al., 1992; Oberhammer et al., 1993; Ankarcrona et al., 1995; Saura et al., 1997). Our observations supplement these studies and further demonstrate that apoptosis even within the same cell type can proceed with distinct pattern of DNA fragmentation. We demonstrated that treatment of mouse neuroblastoma NB 2A cells with genotoxic agents etoposide and Ara-C as well as nongenotoxic agents OKA and serum deprivation induced cell death associated with biochemical and morphological changes typical of apoptosis, i.e. activation of caspase-3, cleavage of PARP, DNA disintegration, chromatin condensation and nuclear fragmentation into apoptotic bodies. At the same time, apoptosis induced by genotoxic and nongenotoxic agents was associated with a distinct pattern of DNA fragmentation. While OKA and serum deprivation induced apoptosis was associated both with HMW-DNA fragmentation and internucleosomal DNA cleavage, etoposide and Ara-C initiated apoptosis accompanied only by HMW-DNA fragmentation (**I**). Both types of apoptosis differing with DNA fragmentation patterns were associated with differential changes in a subset of DNA transcription factors, e.g. AP-I and NF-κB implying that different apoptotic stimuli can induce distinct apoptotic pathways within the same cells.

The absence of the DNA ladder at least in etoposide-treated cells was not dependent on the duration of exposure or the dose of the drug. Exposure of the cells to different concentrations of etoposide or extension of the time of treatment up to three days (when a major loss of cells and DNA occur) resulted in the same pattern of DNA fragmentation without the formation of an oligonucleosomal DNA ladder. Disintegration of DNA during apoptosis induced by etoposide appears earlier and proceeds more rapidly than DNA fragmentation induced by serum deprivation, suggesting that the lack of a ladder is a feature of etoposide-induced apoptosis. This conclusion is further confirmed by the results of our cell-free system experiments. Cell free system assay demonstrated that incubation of normal isolated nuclei with cytosol prepared from serum-deprived cells resulted in the formation of both HMW- and oligonucleosomal DNA fragments. In contrast, etoposide-treated cytosol induced HMW-, but not internucleosomal DNA fragmentation. To summarise these data we can conclude that even within the same cell line, apoptosis may be either associated with or proceed without the formation of an oligonucleosomal DNA ladder, depending on the type of apoptotic stimuli, and that the HMW-DNA cleavage, in contrast to the internucleosomal DNA fragmentation, is an obligatory event in apoptosis induced by various agents. The formation of HMW-DNA fragments may represent the initial committed step of apoptotic DNA disintegration that is accomplished by internucleosomal DNA ladder. Alternatively, HMW-DNA fragmentation may proceed independently on internucleosomal DNA cleavage and represent separate program of DNA disintegration.

6.2 Internucleosomal- and HMW- DNA cleavage: separate pathways of DNA disintegration

We demonstrated that ATA, suramin and cycloheximide inhibited both HMW-DNA fragmentation and oligonucleosomal DNA ladder during apoptosis induced by serum deprivation, whereas none of these agents were able to suppress HMW-DNA cleavage during etoposide induced apoptosis.

While ATA, suramin and cycloheximide inhibited both HMW- and internucleosomal DNA fragmentation induced by serum deprivation, Zn^{2+}-ions abrogated only the DNA ladder but not HMW-DNA fragmentation in serum deprived cells (**I, II**). These results suggest that HMW- and internucleosomal DNA fragmentation can proceed independently from each other in apoptotic cells. This was further confirmed in our *in vitro* experiments, which showed that HMW- and oligonucleosomal DNA fragmentation can be separated from each other in reconstituted cell free apoptotic system. Thus, a brief heating of cytosolic extract prepared from serum-deprived cells had no effect on the oligonucleosomal DNA ladder, but resulted in inhibition of HMW fragmentation in substrate nuclei. At the same time, Zn^{2+}-ions abrogated the appearance of oligonucleosomal, but not HMW-DNA fragments (similarly to the *in vivo* experiments), thus emphasising that different nucleases may be responsible for HMW-DNA fragmentation may proceed independently of the internucleosomal DNA cleavage (**II**).

To summarise these data, we conclude that there are at least two different mechanisms of DNA disintegration during apoptosis. The first mechanism involves the formation of oligonucleosomal DNA ladder, while the second type results in the HMW-DNA cleavage without any internucleosomal DNA fragmentation (Fig. 4). Our cell free system experiments suggest that these mechanisms can act independently from each other. The latter is consistent with recent results demonstrating that separate pathways are responsible for the induction of internucleosomal and HMW-DNA cleavage during apoptosis (Susin et al., 2000).

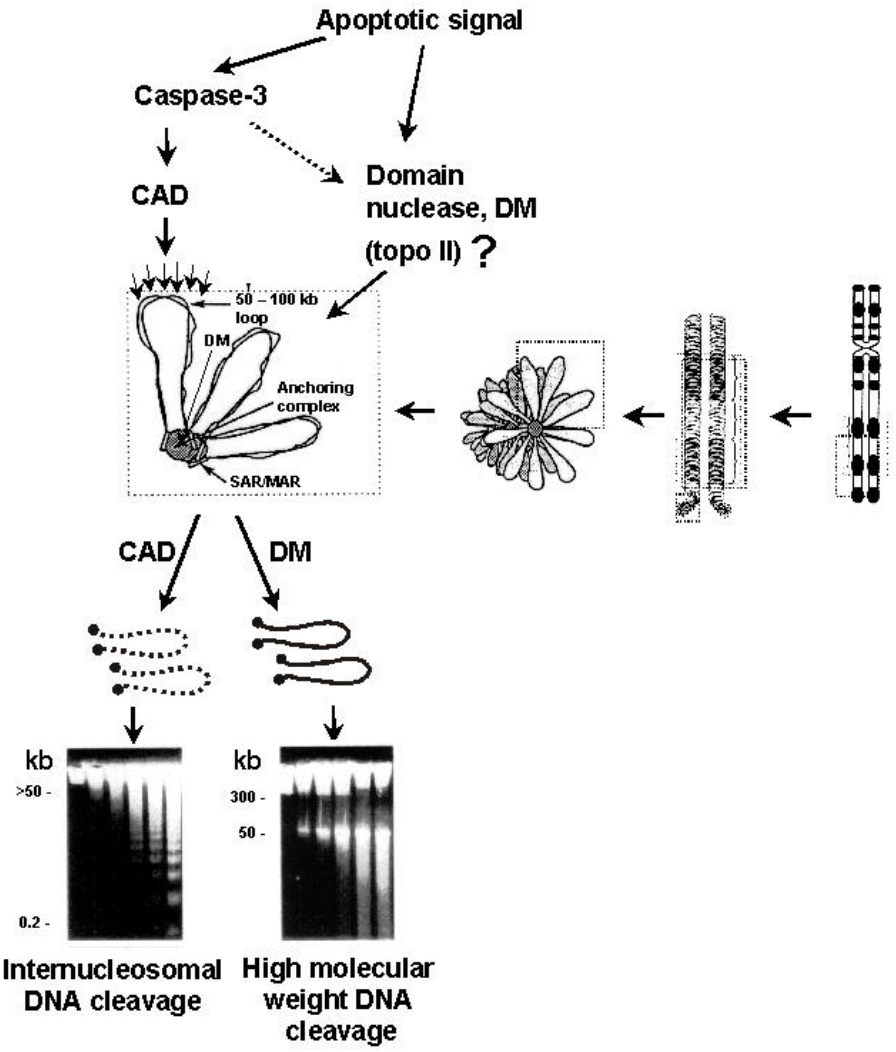

Fig. 4. Possible mechanism of DNA disintegration during apoptosis

6.3 HMW-DNA cleavage: the role of DFF/CAD

Recently, a heterodimeric DNA fragmentation factor (DFF40/DFF45) has been described responsible for the internucleosomal DNA cleavage in human cells (Liu et al., 1997) and its mouse analogue, caspase-3 activated DNase, CAD, and its inhibitor, ICAD, (Enari et al., 1998). DFF40/CAD cleaves DNA at internucleosomal spaces via an interaction with histone H1 and high-mobility group 2 protein (Liu et al., 1999). Both these factors have been shown to be activated by the caspase-3 dependent proteolytic cleavage, providing a direct link between the caspases and the CAD/DFF40-drived internucleosomal DNA fragmentation in apoptotic cells.

As shown in our experiments, apoptosis can proceed either with or without oligonucleosomal DNA ladder. HMW-DNA fragmentation induced by serum deprivation shares many common features with internucleosomal DNA cleavage and exhibits marked differences from HMW-DNA cleavage induced by etoposide. Thus, both HMW- and oligonucleosomal DNA fragmentation in serum deprived cells were effectively inhibited by ATA and cycloheximide in contrast to etoposide induced HMW-DNA fragmentation. These data allow us to propose that similar if not identical mechanisms underlie HMW- and oligonucleosomal DNA fragmentation in serum-deprived cells but quite different mechanisms are responsible for the formation of HMW-DNA fragments in etoposide treated cells.

Recent observations that DFF/CAD is able to induce simultaneously both HMW- and internucleosomal DNA cleavage (Nagata, 2000), and that both activities are absent in Jurkat cell transformants that express a mutant form of mouse ICAD (Sakahira et al., 1999 b) confirms this assumption. At the same time, our data indicate that DFF/CAD is down-regulated during apoptosis, which proceeds without formation of oligonucleosomal DNA fragments (**IV**) suggesting that nuclease activity distinct from DFF/CAD is responsible for HMW-DNA cleavage. Our observation that cycloheximide, a protein synthesis inhibitor, prevents oligonucleosomal DNA cleavage during serum deprivation suggests that ladder-forming activity belongs to the inducible component of DNA fragmentation machinery. At the same time cycloheximide had no inhibitory effect on etoposide induced HMW-DNA fragmentation activity supposing that this activity is constitutively present in NB 2A cells. The absence of an oligonucleosomal DNA cleavage in NB 2A cells induced to undergo apoptosis either by staurosporine or by genotoxic agents, etoposide and Ara-C, suggests that in this case apoptosis is associated with activation of a specific nuclease capable of inducing the HMW-DNA cleavage without performing the internucleosomal DNA fragmentation
(**IV**). Because the formation of HMW-DNA fragments is widely considered as an excision of DNA looped domains, the term " a domain nuclease" has been proposed to distinguish between internucleosomal DNA ladder and excision of DNA looped domains (Earnshaw, 1995). Our and other results suggest that domain nuclease is distinct from CAD (Susin et al., 2000; Robertson et al., 2000), and the recent data imply that it could be topoisomerase IIalpha (Li et al., 1999; Durrieu et al., 2000).

6.4 Internucleosomal- and HMW-DNA cleavage: a differential requirement for caspases

Disintegration of nuclear DNA into oligonucleosomal DNA fragments represents a classical manifestation of apoptosis (Wyllie et al., 1980) and is obligatory associated with the activation of

caspase 3-like proteases (Enari et al., 1998). Here we describe apoptosis, which is associated with caspase-3 activation and HMW-DNA fragmentation without formation of internucleosomal DNA cleavage. Our data demonstrate that HMW-DNA cleavage during apoptosis induced by staurosporine in serum deprived NB 2A cells was insensitive to caspase inhibitors Z-DEVD-fmk and Z-VAD-fmk (**IV**).

These results are consistent with our recent observation showing that caspase inhibitors completely suppressed the internucleosomal-, but not HMW-DNA cleavage during apoptosis induced by okadaic acid in CGCs (**III**), thus suggesting that caspases are not essential for the excision of DNA looped domains in apoptotic cells. Moreover, we demonstrated that in CGCs OKA and L-glutamate induce distinct types of cell death, associated either with or without caspase activation, and irrespective of the caspase activation status, both types of cell death were associated with the disintegration of nuclear DNA into similar set of HMW-DNA fragments (**III**), thus suggesting caspase-independent control of HMW fragmentation. Using cell free system we demonstrated that HMW-DNA cleavage can be induced both in caspase-dependent and -independent manner, thus suggesting the multiple mechanisms of activation of the HMW-DNA cleavage.

The recently described AIF has been shown to induce the disintegration of nuclear DNA into the HMW-DNA fragments in a cell free apoptotic system via caspase-independent mechanism (Lorenzo et al., 1999). It has also been shown, at least in a cell free apoptotic system, that caspase-independent HMW-DNA cleavage and caspase-dependent DFF40/CAD-driven internucleosomal DNA fragmentation represent two parallel pathways of apoptotic DNA disintegration (Daugas et al., 2000).

Our observations further demonstrate that disintegration of nuclear DNA into the HMW-, but not oligonucleosomal DNA fragments, represents a common event in caspase dependent and -independent types of neuronal cell death (**III-V**) and that the caspases seem to be redundant in the activation of the HMW-DNA cleavage during apoptotic cell death.

6.5 Suramin as a common protective drug against different types of cell death: a possible therapeutic implication

Suramin, a polyanionic compound, which has been used as an antihelminthic for more than 50 years in humans, is currently used in clinical trials as the antineoplastic agent for the treatment of breast and prostate cancer. One of the possible mechanisms of its anticancer activity may be the ability of suramin to inhibit protein kinase C (Funayama et al., 1993) and topoisomerase II (Bojanowski et al., 1992), which are essential in the control of cell growth and proliferation. A second mechanism could be associated with the ability of the drug to induce apoptosis in the tumour cells (Lauricella et al., 1998; Lokshin and Levitt, 1996). Induction of apoptosis has also been proposed as the reason for the cytotoxic effect of suramin in dorsal root ganglion neurons (Gill and Windebank, 1998). There is also some evidence for a protective effect of the drug against apoptosis induced by serum deprivation in cultured Chinese hamster ovary cells (Zanghi et al., 2000) or by lowering of the extracellular ATP level in murine thymocytes (Nagy et al., 2000). Suramin also has been shown to be effective in blocking naturally occurring apoptosis in the developing chick neural retina (Yokoyama et al., 1997).

To summarise these data, one can conclude that suramin alone may induce apoptosis; but, on the other hand, the drug is capable of protecting against apoptosis induced by other challenges, as is the case for a number of drugs, including the "classical" apoptosis inhibitor, cycloheximide. The different effect of suramin on cell death is hardly surprising and can be explained by the diverse mechanisms of the drug's action, which target different intracellular compartments (see above).

Our data demonstrate that suramin markedly inhibits apoptosis induced by staurosporine in cultured NB 2A neuroblastoma cells (**V**). The ability of suramin to suppress caspase 3-like activity in staurosporine-treated cells, as well as its ability to inhibit directly the activity of recombinant caspase 3, suggest that suramin exerts its antiapoptotic effect at the early stages of apoptotic execution, i.e. at least at the stage of caspase-3 activation. Whether suramin interferes with the executioner caspases, i. e. caspases -3, -6, -7, or blocks the activation of the proximal initiating caspases, i.e. caspases -8, -9 or -10, should be the focus of additional investigation. Here suramin was also shown to effectively protect cerebellar granule cells against excitotoxicity induced by L-glutamate (**V**). Glutamate-induced excitotoxicity in our experiments was not associated with caspase activation suggesting that the protective effect of suramin in this situation is not linked to the caspase pathway. The possible explanation of the protective effect of suramin against glutamate-induced excitotoxicity could be the ability of the drug to antagonise the membrane receptor channels. Nevertheless, suramin rescues the cells against caspase-mediated apoptosis and caspase-independent excitotoxicity and this implies that it possesses multiple ways of protecting against neuronal cell death.

7. CONCLUSIONS

The aim of present study was to investigate the mechanisms of DNA disintegration during neuronal apoptosis and to clarify whether HMW- and internucleosomal DNA cleavages represent separate types of apoptotic DNA disintegration.

The following conclusions can be done:

1. Apoptosis induced in neuronal cells by different apoptotic stimuli is associated with distinct pattern of DNA fragmentation.

2. HMW- and internucleosomal DNA fragmentation represent separate programmes of apoptotic DNA disintegration.

3. Caspase activation is not essential for HMW-DNA cleavage during neuronal apoptosis

4. Suramin rescues neuronal cells against different types of cell death

In conclusion, the present study provides new information about mechanisms of DNA disintegration during neuronal apoptosis and possible application of suramin as neuroprotective drug.

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