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REVIEW

Caspases as Regulators of Apoptosis and Other Cell Functions

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Abstract—This review covers current knowledge on the involvement in apoptosis of a new family of endopeptidases denoted as caspases. Their structure, specific substrates, and inhibitors are considered. The recent classification of cysteine proteases of the caspase family based on their structural and functional features is presented. The biological significance of caspases not related to their proapoptotic effect is discussed.

Key words: caspases, apoptosis, caspase inhibitors, adaptor proteins, cytokines, cell proliferation, cell differentiation, cytoskeleton integrity

Apoptosis is a physiological mechanism to eliminate excess cells and/or to get rid of malfunctional cells. Such cell clearance is necessary for both the normal development of a multicellular organism during embryogenesis and the maintenance of tissue homeostasis in adults. The disorders in the induction of apoptosis resulting in its inhibition or in excess activation may represent significant factors in pathogenesis of various diseases, such as atherosclerosis, AIDS, neurodegenerative diseases, ischemic damages of tissues, and cancer [1-4].

During morphogenesis of the nematode *Caenorhabditis elegans* the programmed death of 131 cells occurs. Genetic analysis reveals genes *ced*-3, *ced*-4, *ced*-9, *egl*-1, and *mac*-1 whose products regulate this process [5, 6]. The Ced-3 and Ced-4 proteins induce the death of *C. elegans* cells, whereas Ced-9 prevents it. The Egl-1 protein inhibits the antiapoptotic effect of Ced-9, whereas the binding of the Mac-1 protein to Ced-4 prevents apoptosis (physiological cell death) in the nematode.

Genes homologous to *ced*-3, *ced*-4, and *ced*-9 were also found in mammals [5, 7, 8]. The *ced*-3-like gene encoded the cysteine protease ICE which converts the IL(interleukin)-1β precursor into the mature form of this proinflammatory cytokine. Later, other cysteine proteases were revealed in mammals. All these proteases comprise conservative sequences of sites for substrate binding and catalysis and cleave their substrates after an aspartic acid residue. Therefore, these proteases were called caspases (cysteinyl aspartate-specific proteases). By now, 14 caspases are already characterized [9, 10], and their numbering corresponds to the chronology of their identification.

According to their main biological function, caspases are subdivided into two subfamilies: CED-3 (directly involved in apoptosis) and ICE (provides the processing of proinflammatory cytokines) (the figure). It should be noted that although caspase-1 and structurally similar caspase-4, -5, and -13 are placed into the caspase group involved in the processing of cytokines, their role in apoptotic cell death also cannot be excluded. Thus, the NO-induced apoptosis of thymocytes or the Fas-mediated cell death of the T-cell Jurkat line depend on the activities of caspase-1 and caspase-5, respectively [11, 12].

The recent achievements in understanding the biochemical and molecular mechanisms of caspase activity has inspired the increased interest of biologists and clinical researchers in studying molecular biology of caspases. These proteases seem to be potential targets for therapeutic interventions in various diseases. A review concerning the involvement of proteases in development of apoptosis has been already published in *Biochemistry* (Moscow) [13]. The present article considers the recent data on the proapoptotic activity of caspases and also on their ability to regulate cell functions other than physiological cell death that significantly enlarges concepts on the biological role of these universal regulators.

CASPASE STRUCTURE

Caspases are synthesized in the cell as precursors, zymogens, consisting of four prodomains: the NH_2-terminal domain, a large subunit with molecular weight of about 20 kD, a small subunit (about 10 kD), and a linker

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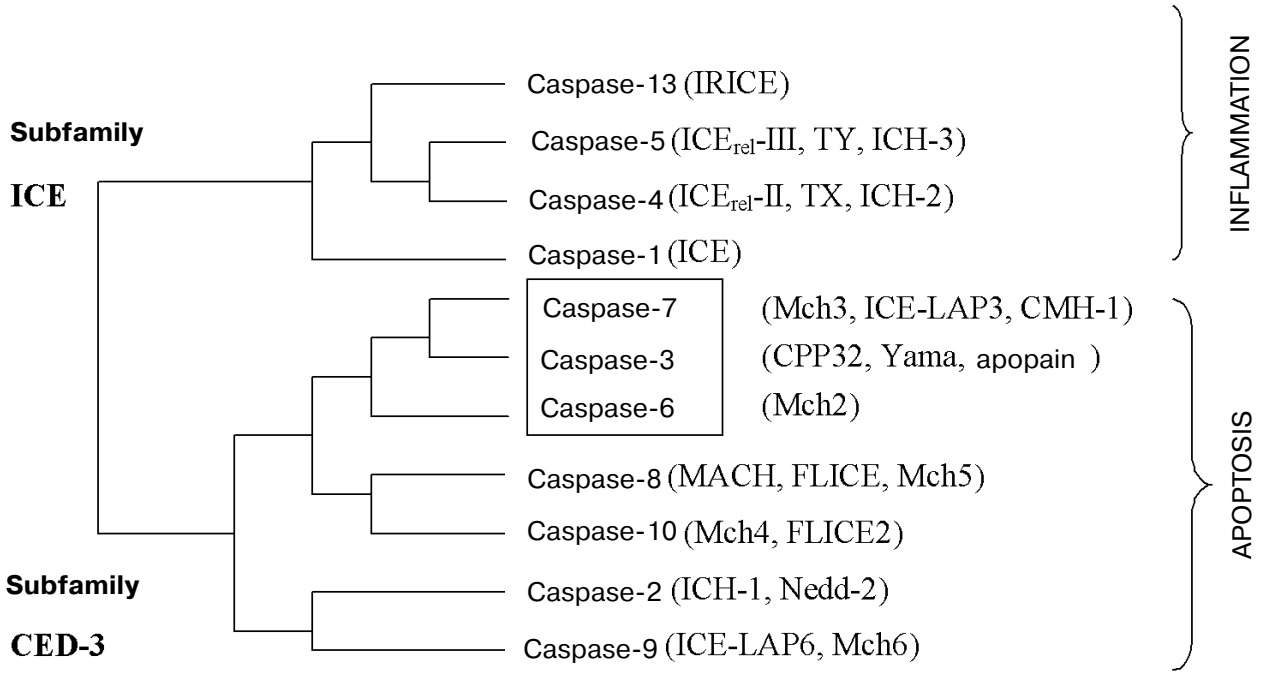

Phylogenetic relationship of human caspases (in parentheses synonyms of caspase names are presented). Caspases with the short prodomain are shown in the box (after [10])

region connecting the large and small subunits. Proteolytic cleavage of the zymogen results in separation of the large and small subunits with production of a complex (the active enzyme) consisting of two heterodimers of the large and small subunits (cited after [14]). Caspases vary in length of the amino acid sequence of the NH_2-terminal prodomain which is either short (20-30 amino acid residues) or long (Table 1). The long prodomain (more than 90 amino acid residues) contains two modular regions which mediate the interaction of caspases with adaptor proteins. These regions are the death effector domain (DED) and the caspase recruitment domain (CARD). Hydrophobic protein interactions are mainly realized via DED-DED contacts, whereas electrostatic interactions occur with involvement of CARD-CARD contacts.

In recent years significant progress has been obtained in studies on the spatial structure of caspases using X-ray crystallography and nuclear magnetic resonance techniques. Crystalline forms of caspase-1, -3, -7, -8, and -9 have been prepared and intensively studied (cited after [15]). Moreover, three-dimensional structure of the uncleaved precursor of caspase-7 (procaspase-7) has been reported [16], and this promoted further findings of the activation mechanisms of the subfamily CED-3 caspases. Structural features of cysteine proteases of the ICE subfamily (except caspase-1) involved in cytokine processing have not yet been elucidated.

CASPASE ADAPTORS

The presence of DED- and CARD-regions ensures the interaction of procaspases with special adaptor proteins which promote the caspase activation. At least nine adaptor proteins (RAIDD, FADD, Apaf-1, CARDIAK, DEFCAP, DEDAF, Nod-1, Ipaf, CARD-8) are found in vertebrata and one (Ced-4) in invertebrata [5, 17-25] (Table 1). Their interaction with caspases results in such complexes as caspase-1/CARDIAK, caspase-1/Ipaf, caspase-1/CARD-8, caspase-2/RAIDD, caspase-2/DEFCAP, caspase-8/FADD, caspase-8/DEDAF, caspase-9/Apaf-1, caspase-9/Nod1, caspase-10/FADD, caspase-10/DEDAF, and Ced-3/Ced-4, respectively. Some caspase adaptor proteins contain death domains (DD) which provide the interaction of the caspase long prodomain with "death receptors", such as Fas or TNFR1 [26], whereas the others display a protein kinase activity [20].

Dimerization of individual caspases resulting in their autoprocessing and activation (see below) occurs with the involvement of the long prodomain of the precursor molecule [27]. One of the functions of the above-considered adaptor proteins is likely to be associated with providing the maximally close interaction between procaspase molecules required for their further dimerization. Thus, the Apaf-1 protein promotes the changes in the conformation of caspase-9 monomers and makes their complementary surfaces approach one another that

Table 1. Structural and functional characteristics of cysteine proteases of the caspase family (after [14] with additions)

Enzyme	Molecular weight of enzyme precursor, kD	Prodomain type	Active subunits, kD	Activating adaptor	Recognized substrate sequence
		Apoptosis-initiating caspases			
Caspase-2	51	Long, with CARD region	20/12	RAIDD, DEFCAP	DXXD[a]
Caspase-8	55	Long, with two DED-regions	18/11	FADD, DEDAF	(L/V/D)EXD[b]
Caspase-9	45	Long, with CARD region	17/10	Apaf-1, Nod1, Bcl-10	(I/V/L)EHD
Caspase-10	55	Long, with two DED-regions	17/12	FADD, DEDAF	Unknown
		Effector caspases			
Caspase-3	32	Short	17/12	Absent	DEXD
Caspase-6	34	Short	18/11	Absent	(V/T/I)EXD
Caspase-7	35	Short	20/12	Absent	DEXD
		Caspases involved in cytokine processing			
Caspase-1	45	Long, with CARD region	20/10	CARDIAK, Ipaf, CARD-8	(W/Y/F)EHD
Caspase-4	43	Long, with CARD region	20/10	Unknown	(W/L/F)EHD
Caspase-5	48	Long, with CARD region	20/10	Unknown	(W/L/F)EHD
Caspase-11[c]	42	Long, with CARD region	20/10	Unknown	Unknown
Caspase-12[c]	50	Long, with CARD region	20/10	Unknown	Unknown
Caspase-13	43	Long, with CARD region	20/10	Unknown	Unknown
Caspase-14[c]	30	Short	20/10	Absent	Unknown
		Caspase homologs in invertebrates			
Ced-3	56	Long, with CARD region	17/14	Ced-4	DEXD
Dcp-1[d]	36	Short	22/13	Absent	Unknown

[a] The sequence of amino acid residues is presented in P_4-P_1 order; the proteolysis occurs after the aspartic acid residue in the P_1 position; X denotes the possible presence of different amino acid residues.
[b] In parentheses the number of amino acid residues is shown corresponding to the order of their preferential location.
[c] Detected in mice.
[d] Caspase-1 in fruit fly.

stimulates the production of enzymatically active dimers of this caspase [28].

MECHANISMS OF CASPASE ACTIVATION AND FUNCTIONING

Protein precursors of caspases are usually activated by proteolysis or by formation of dimer/oligomer complexes. In the presence of ATP and cytochrome C, procaspase-9 interacts with the Apaf-1 adaptor (an analog of the nematode's Ced-4 protein) that activates it and initiates a subsequent cascade of biochemical reactions with involvement of caspase-3, -6, and -7 [17]. Oligomerization of the receptor Fas activates caspase-8 and also caspase-10 which are bound to the FADD through the DED-region [18]. And the oligomerization of caspase-8 or -9 results in their autoactivation and development of apoptosis [17, 18]. Caspase-2 is activated by the RAIDD adaptor [19]. Similarly to Apaf-1, this protein includes the CARD-region required for the protein–protein interactions. The CARD-containing kinase CARDIAK also activates procaspase-1 through CARD-CARD contacts [20]. The mechanisms of activation and specific action of caspases are described more in detail in the review [15].

Because some caspases can activate others, two groups of caspases are distinguished: initiators and effectors. Caspases of the first group (caspase-2, -8, -9, and possibly, -10) transactivate those of the second group (caspase-3, -6, -7, and -14) which in their turn hydrolyze intracellular substrates. Initiator caspases can also play the role of effector caspases that seems necessary for amplification of the activity of the caspase cascade. Thus, under certain conditions, caspase-8 can be activated only after the transactivation of caspase-6 by caspase-9 [29]. Caspases are also activated with involvement of other enzymes, including granzyme B, cathepsins B and C, calpain, etc. [30-32].

As it has been noted, during apoptosis a number of caspases are activated successively. Thus, the removal of caspase-9 from cell extracts prevents the cytochrome C-dependent activation of caspase-2, -3, -6, -7, -8, and -10 [29]. And caspase-3 is required for activation of caspase-2, -6, -7, -8, and -10 under conditions of the cytochrome C-dependent activation of caspase-9. In addition to procaspase-8, caspase-8 *in vitro* activates seven zymogens of other caspases (procaspase-1, -2, -3, -6, -7, -9, and -11) [33]. Caspase-1 and -11 promote the activation of effector caspase-3 and -7 and significantly less the activation of caspase-6. Because caspase-11 activates caspase-1 and -3 [34], it may be assigned to initiator caspases. Although caspase-2 is unable to directly initiate the processing of initiator caspases, by degrading the Bid protein it stimulates the release of cytochrome C (and other proapoptotic mediators) from mitochondria that promotes the activation of caspase-9 [35]. However, caspase-2 can be activated only by caspase-3, -7, and -8 [33]. It is unclear how specific the proapoptotic effect is of any of the above-listed caspases and whether they duplicate each other by degrading the same substrates. But it should be noted that pathways of caspase activation and the succession of their interactions depend on both the cell type and the type of apoptosis inducer.

CASPASE SUBSTRATES

More than 70 pro- and antiapoptotic protein substrates of caspases are known which are located in the cell nucleus, cytoplasm, and cytoskeleton [10]. Caspases specifically recognize the sequence consisting of four amino acid residues and denoted as P4-P3-P2-P1. The most conservative in this sequence of the caspase substrate are the aspartic acid residue in the P1 position and the glutamine residue in the P3 position [36]. Hydrolysis by caspases of their substrates can either activate or inhibit biological functions of these substrates. The degradation of the nuclear matrix proteins results in disorders of the structural organization of the nucleus and in condensation of chromatin; the degradation of the cytoskeleton proteins (actin and actin-binding proteins gelsolin and fodrin) is responsible for protrusions from the plasma membrane followed by the cell lysis associated with formation of apoptotic bodies [37]. The hydrolysis by caspases inactivates Akt-1 and Raf-1 protein kinases which provide for cell division and survival [38].

Lamins, NuMa, and *acinus* which are caspase substrates are among the proteins responsible for maintenance of the structural integrity of the nucleus. The caspase-6-induced degradation of lamins A and C resulted in protein products with molecular weights of 47 and 37 kD, respectively, and the introduction into the cells of mutant lamins depleted of the aspartic acid residue delayed the DNA fragmentation and completely inhibited the nucleus condensation [39]. The condensation and fragmentation of the nucleus observed during Fas-mediated apoptosis is usually preceded by hydrolysis of the peptide bond in the nuclear protein NuMa realized by caspase-6 [40]. Unlike lamins and the NuMa which are substrates of several caspases, the protein *acinus* is affected only by caspase-3 [41]. Proteolytic degradation of this protein is required for chromatin condensation induced by various inducers of apoptosis.

Another caspase substrate is poly(ADP-ribose)polymerase (PARP) which plays an important role in the reparation of DNA because it can regulate the activity of the Ca^{2+}/Mg^{2+}-dependent endonuclease which provides the internucleosomal DNA degradation. Another mechanism of the caspase-activated DNA degradation has been recently elucidated [42]. A complex of DNase responsible for the DNA fragmentation with the protein

ICAD inhibiting this DNase was detected in the cell cytoplasm. Usually this complex was inactive, but the degradation of the ICAD protein under the influence of caspase-3 activated DNase with subsequent DNA degradation.

CASPASE INHIBITORS

The antiapoptotic effect of endogenous caspase inhibitors is thought to be associated with their ability to inhibit either the activation of caspases or the proteolytic effect of caspases activated. The first caspase inhibitors were detected among viral proteins responsible for survival of virus-infected cells. Preventing the death of the host's cells, these proteins promote the accumulation of viral particles during a lytic infection and the virus persistence in the body during a chronic infection. The poxvirus CrmA (cytokine response modifier A) protein of the serpin family inhibits apoptosis via inhibition of caspase-8 (and caspase-1) activity [43]. This protein forms with these caspases a stable complex that results in inhibition of the Fas-mediated apoptosis. CrmA inhibits not only cysteine but also serine proteases (e.g., granzyme B) [44]. Transfection of mouse hepatocytes with the *crmA* expressing adenovirus vector makes them resistant to the Fas-mediated apoptosis [45], and this also shows that the induction of apoptosis depends on the caspase activity. In addition to preventing apoptosis, the serpin CrmA also inhibits inflammatory reactions in the body, more likely due to inhibition of the caspase-1 activity and the subsequent processing of proinflammatory cytokines.

Mutants of the baculovirus *Autographa californica* with a significantly decreased virulence were prepared by directed mutagenesis [46]. These mutants had changes in the gene encoding the p35 protein. This protein can inhibit apoptosis not only in insect cells but also in mammals. In mammalian cells p35 can inhibit the *in vitro* activity of caspase-1, -3, -6, -8, and -10 [47], and this effect of the p35 protein is specific with relation to caspases and not to other serine or cysteine proteases. No homolog of p35 has yet been detected in mammals.

Baculoviruses were found to have another group of proteins called IAP (inhibitors of apoptosis) which inhibited the death of the virus-infected cells (cited after [48]). The mechanism of the anti-apoptotic effect of these viral proteins remained unknown until the discovery of cellular IAP-homologs. So far, eight proteins similar to IAP of baculoviruses have been found in mammals [48, 49]. In the structure of the IAP family proteins a specific BIR (baculoviral IAP repeat) region of about 70 amino acid residues in length has been detected. Human XIAP, c-IAP1, c-IAP2, and NAIP have three such sequences. And at least one of the BIR regions is required to provide the antiapoptotic effect of these proteins. At the C-terminus of the IAP molecule a RING domain is found which was not obligatory for inhibition of the apoptotic signal in the majority of cell types. Thus, the c-IAP1, c-IAP2, and XIAP proteins retained their antiapoptotic function in the absence of the RING domain [50, 51]. The structure of the c-IAP1 and c-IAP2 proteins is also characterized by the presence of the above-described CARD domain located between the BIR and RING regions. The significance of this domain for IAP functions is not quite clear, although it is known to be required for the c-IAP1 binding to the CARD-containing kinase CARDIAK/RIP2 which is involved in the activation of caspase-1 [52].

It is interesting that the XIAP protein which inhibits apoptosis most efficiently (as compared to other IAP proteins) can bind only to active forms of caspase-3 and -7 but not to their zymogenic precursors. And the binding to the effector caspase-3 and -7 is due to the BIR2 domain of XIAP, whereas for inhibition of the initiator caspase-9 the XIAP protein has to contain the BIR3 domain [53].

The family of IAP proteins also includes a survivin discovered in 1999 which contained only one BIR sequence [54]. Survivin which is expressed in the cells of many passaged tumor cell lines binds to caspase-3 and -7 similarly to other proteins of the IAP family and inhibits the development of apoptosis induced by various inducers [55]. The location of survivin in microtubules seems to promote its anticaspase activity during G_2/M of the cell cycle [56].

Similarly to survivin, two recently found proteins of the IAP family, ILP-2 and livin (also called ML-IAP) contain only one BIR sequence [49, 57]. However, unlike survivin, both livin and ILP-2 are specified by the presence of the RING domain. Livin can inhibit the apoptosis mediated by "death receptors" and also initiated by hyperexpression of the proteins FADD, Bax, RIP, and RIP3 [57]. Although ILP-2 fails to inhibit the Fas- or TNF-dependent apoptosis, it displays a pronounced antiapoptotic effect in cases of hyperexpression of the Bax protein or coexpression of caspase-9 and its adaptor Apaf-1 [49]. Both ILP-2 and livin can inhibit the initiator caspase-9. Moreover, similarly to other IAP proteins, livin can bind to the activated forms of caspase-3 and -7 [57].

The antiapoptotic effect of the IAP family proteins is prevented by an interesting mechanism. After the initiation of apoptosis, Smac (second mitochondria-derived activator of caspase)/DIABLO and Omi/HtrA2 (high temperature requirement A2) proteins are jointly with others released from mitochondria into the cytoplasm. Their N-terminus contains a conservative sequence AVPS which can bind IAP [58, 59]. The binding of Smac/DIABLO or Omi/HtrA2 to the XIAP protein prevents the effect of the latter that promotes activation of caspases. The hyperexpression of Smac/DIABLO or Omi/HtrA2 in the cells increases their sensitivity to induction of apoptosis by ultraviolet radiation [59, 60]. These findings confirm the ability of these mitochondrial proteins to function as endogenous activators of apoptosis.

Another endogenous inhibitor of caspases, FLIP (FLICE-inhibitory protein), has been described [61]. This protein exists as a short ($FLIP_S$) and long ($FLIP_L$) form. $FLIP_S$ contains two effector DD regions, whereas $FLIP_L$ also has a caspase-like domain free of proteolytic activity. The FLIP protein binds with high affinity to the protein complex DISC (death-induced signaling complex) that prevents activation of caspase-8 (and possibly of caspase-10) and also transduction of the proapoptotic signal from "death receptors" of the TNFR family [62]. Some viruses (γ-herpes viruses and molluscum contagiosum virus) can produce antiapoptotic viral proteins vFLIP which promote the survival of the infected cells [63].

The recently found BAR (bifunctional apoptosis regulator) protein the DED region can compete with FADD containing for binding to precursors of caspase-8 and -10 [64]. The binding of the BAR protein to these procaspases prevents their processing and activation. Due to the presence of the transmembrane domain and SAM region, the BAR protein interacts with apoptotic proteins Bcl-2 and Bcl-x_L and thus prevents the Bax-induced cell death [64]. These features of BAR determine its unique ability to inhibit cell death induced exogenously ("death receptors") or endogenously (Bax).

Similarly to the caspase inhibitor BAR, another inhibitor of caspase activation called ARC (apoptosis repressor with CARD) [65] interacts with caspase-8 (but not with caspase-9, -3, or -1). Unlike BAR, the ARC protein contains the CARD region at its N-terminus. Note, that expression of ARC is tissue-specific and occurs in skeletal and cardiac muscle [65] that suggests a selectivity of the antiapoptotic effect of ARC, especially in the case of cell death mediated by "death receptors".

Although proteins of the Bcl-2 family fail to directly interact with caspases, they are the best-studied regulators of caspase activation. The majority of Bcl-2-like proteins have a transmembrane domain which provides their location inside intracellular membranes, mainly in the mitochondria. Recombinant Bcl-x_L, Bcl-2, Bax, or Bid can generate various forms of ion channels in artificial membranes [66]. Bcl-2-induced generation of channel-like structures seems to be associated with their ability for dimerization in cell membranes. Such mechanisms have not yet been studied in detail. Bax or Bid proteins are supposed to form the channel-like structures allowing for proapoptotic factors (cytochrome C activating caspase-9, procaspase-2, -3, -9, the apoptosis-inducing factor AIF, endonuclease G, etc.) to release from mitochondrial intermembrane space. This hypothesis is supported by the finding of Saito et al. [67] that Bax can produce in liposomes pores sufficient for releasing cytochrome C from them.

Beyond the stimulation of releasing mitochondrial proapoptotic proteins into cytoplasm through the generated *de novo* channels, Bcl-2-like proteins regulate this process affecting pre-formed membrane pores. Interaction of Bax with voltage-dependent anionic channels (VDAC) results in the release of proapoptotic mediators from isolated mitochondria [68]. In the presence of ATP, cytochrome C, and Apaf-1 caspase-9 is activated that initiates a subsequent cascade of events with the involvement of caspase-3, -6, and -7 [17]. And the antiapoptotic protein Bcl-2 can close VDAC and inhibit apoptosis [69]. Note that Bcl-2 also inhibits the caspase-independent apoptosis [70] that suggests the universality of its antiapoptotic effect.

Small peptides have been recently synthesized which are pseudosubstrates of caspases and inhibitors of their activity. They have the aldehyde (-cho) or carbonyl (-fmk, -cmk) group and reversibly or irreversibly inhibit the activity of caspases by competing for their cellular substrates. And not only specific inhibitors of certain caspases have been synthesized (e.g., aldehydes of tetrapeptides Ac-WEDH-CHO and Ac-YVAD-CHO inhibit caspase-1 and -4 and Ac-VEID-CHO inhibits caspase-6) but also pancaspase inhibitors, such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, z-VAD-FMK (cited after [71]). Fluoromethyl ketones are especially interesting because they are highly efficient inhibitors of activity not only of caspases but also of serine proteases, in particular, cathepsin B [72]. Moreover, cysteine proteases are sensitive to Zn^{2+}. Thus, in the presence of $ZnCl_2$ the cleavage of the caspase-3 substrate PARP was prevented and the viability of promyelocytic leukemia HL-60 cells treated with inhibitor of topoisomerase II was retained [73].

In conclusion of the section concerning the inhibition of caspase activity it should be also noted that such a mechanism as "physiological sequestration" of caspases exists in certain cellular organelles that prevents their access to substrates located in the cytoplasm or other cell compartments. The intracellular location of caspases and their re-distribution between cell compartments are important for realization of the apoptotic cell death [74]. Table 2 summarizes the data on the subcellular location of zymogenic and activated forms of nine mammalian caspases which are best studied. The translocation of activated caspases from one cell compartment into another is important for transduction of the proapoptotic signal along the caspase cascade. Let us illustrate this with a demonstrative example. To activate caspase-12, translocation of cytosolic caspase-7 to the endoplasmic reticulum is required, whereas the subsequent activation of caspase-9 and -3 depends on the movement of active caspase-12 into the cytoplasm [81, 82]. After activation with caspase-9, caspase-3 can in its turn degrade both cytoplasmic and nuclear substrates (see above), therefore, it is easy to calculate that in the proteolytic cascade of caspase-7—caspase-12—caspase-9—caspase-3—specific caspase substrates the subcellular localization of caspases is changed at least three times.

Table 2. Subcellular location of active and zymogenic forms of various caspases in mammals

Active or zymogenic caspase form	Cytoplasm	Nucleus	Mitochondria	Golgi apparatus	Endoplasmic reticulum	Reference
Caspase-1	+	+				[75]
Caspase-2		+		+		[75, 76]
Caspase-3	+	+	+			[74, 75]
Caspase-6	+	+				[75]
Caspase-7	+	+	+		+	[75, 77]
Caspase-8	+					[74, 75]
Caspase-9	+	+	+			[74, 75]
Caspase-10	+					[75]
Caspase-12					+	[78]
Procaspase-1	+					[79]
Procaspase-2	+	+	+			[74]
Procaspase-3	+		+			[74]
Procaspase-6	+*					[80]
Procaspase-7	+					[74]
Procaspase-8	+					[74]
Procaspase-9	+		+			[74]
Procaspase-10	+					[25]
Procaspase-12					+	[78]

* On the model of rat kidney ischemia/reperfusion a translocation of activated caspase-6 from the cytoplasm into the nucleus was shown.

BIOLOGICAL SIGNIFICANCE OF CASPASES

As mentioned above, induction of apoptosis is associated with activation of various caspase cascades in dying cells. However, in experiments on caspase-1, -2, -3, or -11 knockout mice apoptosis was not inhibited completely, although cell death in several tissues was accompanied with specific disorders [83]. These data suggest that biological effects of individual caspases can be compensated by other caspases (and this has been already shown experimentally [84]) or that their functions are not limited only to activation of apoptosis.

One such "alternative" function of caspases is associated with the production of cytokines by cells. Precursors of interleukin-1β (IL-1β) and of another proinflammatory cytokine IL-18 which induces the production of γ-interferon by cytotoxic T-lymphocytes and natural killers are known to be specific substrates of caspase-1 [85]. Therefore, in mice lacking caspase-1 gene the processing of pro-IL-1β and pro-IL-18 is inhibited and also the production of IL-1α, IL-6, and γ-interferon is disturbed [86]. Proinflammatory effects of these cytokines are associated with their ability to stimulate the synthesis of proteins of the acute phase of inflammation, prostaglandins, and other mediators. *Caspase-1* deficient mice are resistant to induction of endotoxic shock and survive the treatment with the lipopolysaccharide at a dose that causes the death of intact mice within 30 h [87]. Similar features were found in mice that lacked *caspase-11* [88].

The precursor of another proinflammatory cytokine IL-16 is also a caspase substrate. Under the influence of caspase-3 the mature form of IL-16 with molecular weight of 13 kD is cleaved off the molecule of pro-IL-16 [89]. Note that the α-interferon-stimulated production of IL-16 by peripheral blood lymphocytes also depends on activation of caspase-3 whose inhibitors prevent the production of IL-16 by these cells [90]. Lymphoblast T-cells of the Jurkat cell line respond to stimulation with phytohemagglutinin or to combined treatment with CD-3 and CD-28 antibodies by secreting IL-2 into the culture medium [91]. In the stimulated cells caspase-3 is concurrently activated that is accompanied by degradation of its substrates PARP and α-spectrin [91]. And caspase inhibitors z-D-DCB and DEVD-CHO dose-dependently inhibit not only the degradation of these caspase substrates but also the production of IL-2 by activated Jurkat cells. It is unclear whether caspase-3 (or

other proteases of the caspase family) is involved in processing of the IL-2 precursor as in the case of pro-IL-16, or its effect is mediated. Thus, in the human body the activation of caspases is required not only for regulation of homeostasis of the immune system cells [92] but also for production of cytokines with oppositely directed effects; therefore, it ensures the functioning of different links of immunity.

The finding of the antiapoptotic effect of caspases seems rather unexpected. The pancaspase inhibitor z-VAD-FMK prevents the death of Jurkat cells induced by TNF-α, sodium nitroprusside, or etoposide [93]. But, on the contrary, this caspase inhibitor stimulates apoptosis in the RAW246.7 line macrophages activated with lipopolysaccharide, and this suggests the role of caspases in prevention of cell death. Temperature-sensitive cells of the RetsAF line with a mutant form of the large T-antigen of the SV-40 virus die at the restrictive temperature (39.5°C) by apoptosis which is inhibited by Bcl-2 [94]. The pancaspase inhibitor z-VAD-FMK can inhibit the Bcl-2-dependent survival of the RetsAF cells at 39.5°C. In other words, in this model caspases are also responsible for maintenance of the cell viability.

Forms of caspase-2, -3, and -9 are found which are produced as a result of alternative splicing [95-97]. Such "truncated" caspases are characterized by their effects as endogenous inhibitors of caspases. And what can be the mechanisms of the antiapoptotic activity of caspases? For the short form of caspase-9 the following possibilities have been considered [97]: 1) binding to apoptosome (the protein complex activating caspase-9), the short form of caspase-9 competes with caspase-9 that inhibits activation of the latter; 2) the short form of caspase-9 is dimerized with caspase-9 that prevents oligomerization of caspase-9; 3) the short form of caspase-9 complexes with caspase-3 that prevents the activation of caspase-3 by caspase-9.

And the ratio of the short form of caspase-9 to caspase-9 is high in such organs as ovary, brain, and heart [97]. In ovary the short form of caspase-9 is supposed to display an antiapoptotic effect during menstrual cycle phases unassociated with the pronounced cell loss, whereas in the brain and heart it provides the viability of cells which are not replaced by new ones.

We have shown on the model of the TNF-α-induced apoptosis of the U-937 cells (human histiocytic lymphoma) that the death of non-synchronized cells in the constant presence of the cytokine is not terminated within 3-4 h but continues for at least 96 h [98]. Such a "persisting" apoptosis occurs wavelike that seems to be due to recruitment of new cell subpopulations into this process. But changes in morphology specific for initial stages of apoptosis (the nucleus and cytoplasm condensation) are observed only at some moments that are likely to depend on the cell cycle phase. These changes usually precede an increase in the number of cells with fragmented nucleus. Along with these changes, the cell subpopulation is retained which has survived in the presence of TNF-α. Activation of caspase-3, -8, -and -9 [99] was shown in a similar model of apoptosis, thus, changes in ratios of pro- and antiapoptotic caspases at different phases of the cell cycle were suggested to be a factor responsible for persistence of the TNF-α-induced apoptosis of U-937 cells.

Nevertheless, truncated caspases can be produced not only as a result of the alternative splicing but also by splitting the usual form of this enzyme. Thus, Stephanou et al. [100] have found that caspase-3 activated by caspase-9 can cleave caspase-9 at Asp130. This results in the release of the CARD-domain of caspase-9 which promotes activation of the transcription factor NF-κB and subsequent expression of *bcl-x* and *iap* antiapoptotic genes [100]. Thus, different domains of the same caspase can differently affect cell viability.

Unlike caspase-1 or -11 knockout mice which had no developmental defects, mice with the caspase-3 deficiency usually die 1-3 weeks after birth [88]. These mutant mice have hyperplasia and disorganization of the brain structures because of disturbed induction of apoptosis in neurons. And mice deprived of the *caspase-3* gene are at birth smaller in size and weight than wild-type animals. This specific feature suggests that caspase-3 in addition to its involvement in neuronal apoptosis during ontogenesis can be an important regulator of processes associated with cell proliferation and/or differentiation.

In fact, the activation of caspase-3 observed in the phytohemagglutinin-stimulated lymphocytes was not accompanied by apoptotic changes in these cells [101]. Caspase-3 was also activated in T-lymphocytes stimulated with mitogens and IL-2 [102]. And the level of caspase-3 activation in proliferating cells was even higher than in apoptotic tumor cells. Moreover, on stimulation of T- and B-lymphocytes not only the activity of caspase-3 increases but also the degradation of its specific substrates (PARP, lamin B, kinase Wee1 but not factor DFF45 of DNA fragmentation) [103]. The CD3-induced proliferation of human T-cells is inhibited on their treatment with synthetic caspase inhibitors, although in this case the cell activation is accompanied by rapid processing of caspase-8 and not of caspase-3 [104]. By the way, hypotrophy of the cardiac muscle which is often observed in mice laking *caspase-8* (cited after [86]) also suggests that this caspase plays the role of endogenous regulator of *in vivo* cell survival and proliferation.

In chronic pancreatitis expression of caspase-1 was detected in atrophic acinar cells of the pancreas, proliferating cells of biliary ducts, and in acinar cells which were redifferentiating with production of channel-like structures [105]. This distribution of caspase-1 in the pancreas tissues suggests at least two *in vivo* functions of this enzyme: it induces the death of atrophic acinar cells and stimulates cell proliferation and/or differentiation in bil-

iary ducts. Moreover, on stimulation of T-lymphocytes, caspase-6 and -7 are activated (but not caspase-1, -2, or -4) [103] which suggests involvement of a wide spectrum of caspases in the regulation of cell proliferation.

And what is the mechanism of regulation of cell division by caspases? Frost et al. [106] have shown that the *in vivo* inhibition of caspase activity results in accumulation of the uncleaved form of the cyclin-dependent inhibitor $p27^{KIP1}$ (the substrate of caspase-8) and prevents proliferation of transformed lymphoid cells. Similarly, caspase-3 can degrade another inhibitor of cyclin-dependent kinases, $p21^{Cip1/Waf1}$ that promotes a dramatic increase in the activity of kinase cdk2, which is a key regulator of transition from G_1 to the S-phase of the cell cycle [107]. Thus, a partial proteolysis by caspases of proteins responsible for arrest of the cell cycle can stimulate growth of the pool of proliferating cells.

Studies on the caspase regulation of cell differentiation are only at the beginning. The differentiation of U-937 cells induced by the phorbol ester TPA is associated with the release of cytochrome C from mitochondria and with activation of caspase-3 [108]. Because the inhibitor z-VAD-FMK of caspase-3 inhibits the differentiation of U-937 cells, it is concluded that caspase activation is required to initiate the differentiation of monocytic cells [108]. Differentiation of human promyelocytic leukemia cells (HL-60 line) and of osteosarcoma cells induced by retinoic acid and triterpenoid CDDO, respectively, is also associated with the increase in expression and functional activity of caspase-3, -8, and -9 [109, 110]. The mechanism of the caspase-mediated regulation of differentiation of osteoblasts and monocytes remains unclear.

The differentiation of erythroleukemia cells K562 induced by a mutant p53 protein ($p53^{Val1335}$) also depends on caspase activity [111]. This is associated with the cleavage by caspases of c-Abl and Bcr-Abl tyrosine kinases which seem to be responsible for inhibition of the erythroid lineage differentiation. The activation of caspase-3 in immature erythroblasts succeeded with the cleavage of its substrates responsible for the integrity of the nucleus and chromatin condensation (lamin B and *acinus*, respectively) fails to cause the cell death but induces their differentiation [112]. Note that the concurrent activation of caspase-3 and -6 has no proteolytic effect on their other substrates (DFF44 and GATA1) although their cleavage is usually associated with induction of apoptosis. The differentiation of epithelial cells of the eye lens [113] and of skin keratinocytes [114] is also associated with activation of caspases required for elimination of nuclei from these cells. Although effector caspase-3, -6, and -7 are not activated concurrently, the differentiation of epidermal cells is accompanied by synthesis and processing of procaspase-14 [115]. Note that unlike caspase-3, caspase-14 is not involved in the death of differentiated keratinocytes initiated by traditional inducers of apoptosis [115].

Attention should also be paid to another feasible biological function of caspases associated with regulation of cell attachment to a substrate and of their substrate-dependent migration. Thus, cells treated with a caspase inhibitor z-Asp could not grow on a collagen-coated surface but retained their viability [116]. And caspase-3 was activated in the cells attached to collagen or to poly(L-lysine) but not in the cells in suspension. The authors of this work [117] think the activation of caspases to be associated with maintenance of cytoskeletal integrity provided by tyrosine kinase FAK which is, by the way, a substrate of caspase-3.

It is well known that dendritic cells of bone marrow origin (often called Langerhans cells) which express high affinity receptors for IgE comprise the specialized antigen-presenting cells in epidermis. Migration of these cells into lymph nodes determines activity of the cell immunity in the skin. The caspase-1 inhibitor Ac-YVAD.cmk inhibits the migration of Langerhans epidermal cells under conditions of organ culture [118]. Moreover, a skin reaction to the obligate allergen 1-fluoro-2,4-dinitrobenzene significantly decreased in mice deprived of the caspase-1 gene and when the Ac-YVAD.cmk inhibitor is applied prior to allergen on the skin of the animals. These data suggest an important role of caspase-1 in the regulation of the Langerhans cell *in vitro* and *in vivo* migration, and inhibition of its activity results in suppression of the contact hypersensitivity. However, the locomotor activity of caspase-1 can be mediated by IL-18 whose precursor is cleaved in the Langerhans cells under the influence of caspase-1 [119].

Thus, it can be concluded that our knowledge about the structure of caspases and molecular mechanisms of their activation and inhibition is markedly ahead of our concepts on their physiological functions. In eukaryotes 48 cysteine proteases of this family have been already characterized (one in yeasts, three in the nematode *C. elegans*, seven in insects, eight in amphibians, four in fishes, four in birds, ten in rodents, and eleven in man) [120, 121][1]. It seems unlikely that such a great number of endopeptidases represented by caspases are required for realization of apoptotic cell death only. It is clear that the caspase-catalyzed limited proteolysis of substrate targets is also extremely important for regulation of the immune response and processes associated with cell proliferation, survival, differentiation, and migration. Mechanisms of caspase involvement in these cell functions seem to be similar and associated with activation (as a result of processing) of endogenous stimulators or with inactivation of inhibitors of cell proliferation, differentiation, and migration. Obviously, on development of therapeutic approaches for recovery or inhibition of caspase activity [123-126] their multiple functions must be taken into account.

[1] Not considering metacaspases detected in plants, bacteria, and some protozoa [122].

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