Stroke, myocardial infarction, acute and chronic inflammatory diseases: caspases and other apoptotic molecules as targets for drug development

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Summary

Mapping of the human and other eukaryotic genomes has provided the pharmacological industry with excellent models for drug discovery. Control of cell proliferation, differentiation, activation and cell removal is crucial for the development and existence of multicellular organisms. Each cell cycle progression, with sequences of DNA replication, mitosis, and cell division, is a tightly controlled and complicated process that, when deregulated, may become dangerous not only to a single cell, but also to the whole organism. Regulation and the proper control of the cell cycle and of programmed cell death (apoptosis) is therefore essential for mammalian development and the homeostasis of the immune system. The molecular networks that regulate these processes are critical targets for drug development, gene therapy, and metabolic engineering. In addition to the primary, intracellular apoptotic suicide machinery, components of the immune system can detect and remove cells and tissue fragments that no longer serve their defined functions. In this review we will focus on apoptotic pathways converging on caspase family proteases, summarizing pharmacological attempts that target genes, proteins, and intermolecular interactions capable of modulating apoptosis and the inflammatory response. The upcoming pharmacological development for treatment of acute pathologies, such as sepsis, SIRS, stroke, traumatic brain injury, myocardial infarction, spinal cord injury, acute liver failure, as well as chronic disorders such as Huntington’s disease, Parkinson’s disease, ALS, and rheumatoid arthritis, will be discussed in details. We also suggest new potential molecular targets that may prove to be effective in controlling apoptosis and the immune response in vivo.

Key words: apoptosis • Bcl-2, inflammation • myocardial infarct • stroke • sepsis


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INTRODUCTION

Programmed cell death (apoptosis) is of central importance for the physiology of every multicellular organism; therefore the majority of malfunctions of this process will have pathologic consequences. The importance of apoptosis is underscored by the fact that most medicines that have been developed in recent years target molecules that are an integral part of or at least modulate apoptotic pathways. While this has been expected for the treatment of cancer (reviewed in 46), stroke 32, 83, acute liver failure 49, 98, and myocardial infarct 17, 50, 54, 101, drugs which target molecules involved in apoptosis also enter the treatment of rheumatoid arthritis (RA), "systemic inflammatory response syndrome" (SIRS) 20, 97, 105, Huntington's disease 19, 103, Parkinson's disease, "amyotrophic lateral sclerosis" (ALS) 143, spinal cord injury 73, and even sepsis 51, 60, 126, alcoholic hepatitis 99, and other forms of chronic liver diseases, at least in animal models. Even in cancer, in addition to the classical antiproliferatory and proapoptotic approaches, the consequences of such treatments for the integrity of the immune system are increasingly gaining attention 89. In addition to the classical clinical applications, inhibition of caspases has been shown to be very successful in improving cryopreservation of cells and tissues by counteracting cryo-induced stress 100, 129.

The apoptotic pathway was first delineated to a significant extent in the nematode (worm) model organism Caenorhabditis elegans 45, 46. The set of genes that regulate the apoptotic process in the worm have their counterparts in higher multicellular organisms, including mammals. Various cloning approaches have allowed us to identify these genes quickly and to analyze them functionally. Thus the nematode proapoptotic molecules Ced-3, Ced-4, and Egl-1 are represented in higher organisms by the family of caspase-proteases, Apaf-1, and the BH3-only family of proteins. The counterpart of the antiapoptotic Ced-9 protein is represented in higher eukaryotes by the antiapoptotic Bcl-2 family of molecules 25, 46, 75, 80, 94.

The first apoptotic pathway detected and described in higher eukaryotes was the so-called death receptor (extrinsic) pathway. Cell surface death receptors are able to activate caspases and induce apoptosis and interleukin (IL)-1β secretion upon the appropriate ligand-binding 66, 79. First, a subfamily of caspases, termed apical/initiator caspases, becomes activated upon enrolment to the death-inducing signaling complex (DISC), a multiprotein conglomerate recruited to the death receptor within seconds or minutes after its triggering 63. Once activated, the initiator caspases activate downstream effector caspases and other apoptosis-relevant molecules 66, 120. Modulation of interaction among DISC components, or triggering death receptors by naturally occurring or artificial ligands, provides another means of control of the apoptotic process in the immune system and during cancer therapy. Another caspase activation pathway exists that depends on the release of cytochrome c from mitochondria, which is strongly modulated by Bcl-2 proteins and is often used by the cell to activate the caspase cascade 114, 144. This so-called, "intrinsic", apoptosome-dependent pathway is frequently triggered by anticancer drugs and stimuli that cause cellular stress 47, and it is strongly modulated by Bcl-2 family members.

The Bcl-2 family comprises both antiapoptotic and proapoptotic proteins. The large number of molecules that belong to the family, its diversity, different intracellular localization, and sometimes opposing mode of action makes Bcl-2 family molecules among the most intriguing apoptosis modulators 2, 5, 10, 112. The antiapoptotic subfamily is best represented by Bcl-2 and Bcl-xL. All subfamily members share three or four BH domains, and they localize to the cytoplasmic sides of intracellular membranes, such as the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope 11. The proapoptotic Bcl-2 family members can be sorted into two subgroups. Members of one subgroup, with Bax and Bak as the best examples 11, 62, 102, have two or three BH domains and are structurally very similar to their prosurvival relatives, especially Bel-xL 130. The second subgroup comprises the so-called “BH3-only” proteins, including Bad, Bcl-Gs, Bid, Bik, Bim, Bmf, Hrk, Noxa, and Bbc3 (reviewed in 11, 33), which have only a short BH3 domain. Despite more than ten years of intensive research, the mechanism of apoptosis regulation by Bcl-2 family members is not fully understood 113, 128. It is widely believed that Bcl-2 functions to preserve mitochondrial membrane integrity and to prevent the release of cytochrome c and other proapoptotic molecules from the mitochondria. In addition, at least some Bcl-2 molecules have a significant impact on calcium homeostasis in the cell 6, 13, 41. A rise in cytoplasmic calcium is a powerful biological signal that can, for example, activate calpain family proteases or open the mitochondrial permeability transition pores, two events that are of significant importance for the propagation of death signals (see below). BH3-only proteins appear to sense stimuli that cause cellular stress and initiate the death cascade. Proapoptotic Bax and Bak are essential for cell killing initiated by BH3-only proteins and can functionally replace each other, and this form of cell death is antagonized by the overexpression of Bcl-2 (reviewed in 86).
CASPASE ACTIVITY – IMPLICATIONS FOR APOPTOSIS AND CYTOKINE MATURATION

In primitive multicellular organisms such as the worm *C. elegans*, caspase (Ced-3) seems to be involved only in apoptosis. In higher eucaryotes, including mammals, caspases form a large family comprising 12 members. They play an important role in the apoptotic process and also as cytokine activators in the immune system. They are the executioners of the apoptotic program. Biochemically, caspases are classified as cysteinyl-aspartases with the conserved active-center motif QACXG68. Based on their differential substrate specificity, structural differences of their zymogens, preferred subcellular localization, as well as their known role in cellular processes, they can be divided into subfamilies with distinct roles in cell (patho)physiology. Caspases assure the irreversibility of apoptosis by proteolytically chopping off selected cellular proteins. This leads to the controlled degradation and disruption of all essential cellular pathways. The semi-hierarchical and partially redundant organization of the caspase cascade guarantees strong amplification and the rapid progression of the apoptotic process even in the absence of some family members80, 127, 142. Caspases as potential targets had been in the focus of interest of the pharmacological industry for years before the discovery that these proteases had a key effector role in apoptosis. The target of interest has been the IL-1β-converting enzyme (now caspase-1) that, together with caspase-4 and -5, is a regulator of secretion of the inflammatory cytokines such as IL-1β, IL-16, IL-18 and indirectly interferon-γ in humans14, 141. Therefore, explorative programs focusing on the identification of caspase inhibitors or activators are being pursued by a number of pharmaceutical companies (see below and in Table 1). This immunologically relevant pathway is initialized by the so-called “inflammosome” (Fig. 1), a multiprotein complex.

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<th>Table 1. Apoptosis research-based drug development strategies</th>
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<td><strong>Target</strong></td>
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<td>Caspase-3</td>
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Table 1. Cont.

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<th>Target</th>
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<tr>
<td>Caspases</td>
<td>caspase inhibitor</td>
<td>M-920 (L-826,920)</td>
<td>strongly (~80%) reduces mortality in a murine and rat sepsis model by preventing sepsis-related apoptosis of B and T cells(^{51})</td>
<td>Merck Frosst Canada &amp; Co</td>
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<tr>
<td>Caspases</td>
<td>highly selective caspase-3 inhibitor</td>
<td>M-791 (L-826,791)</td>
<td>strongly (~80%) reduces mortality in a murine and rat sepsis model by preventing sepsis-related apoptosis of B and T cells(^{51})</td>
<td>Merck Frosst Canada &amp; Co</td>
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<td>Caspases</td>
<td>selective activation of caspase-3</td>
<td></td>
<td>caspase-3 zymogen is maintained in an inactive conformation by a regulatory triple-Asp-motif, so-called “safety-catch”, localized within a flexible loop near the large-subunit/small-subunit junction(^{118})</td>
<td>Merck Frosst Canada &amp; Co</td>
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<td>Caspases</td>
<td>peptide-based, irreversible inhibitor</td>
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<td>in a rat-model, a broad-spectrum caspase inhibitor, zVADfmk (dose: 3 mg/kg, i.v.) when co-injected with endotoxin completely prevented endotoxin-induced myocardial dysfunction evaluated at 4 and 14 h following endotoxin challenge</td>
<td>INSERM, France, (non-profit, gov.-sponsored org.)</td>
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<td>Caspases</td>
<td>selective inhibitor originating from specific substrate peptide motif</td>
<td>pralnacasan VX-740, HMR-3480</td>
<td>in a type II collagen-induced rat RA model, pralnacasan is effective at 50 mg/kg for over 60 days; well tolerated in animal models(^{111}); encouraging results in phase I clinical studies, currently in phase II trials for RA treatment(^{72})</td>
<td>Vertex Pharmaceuticals Inc/Aventis Pharma AG</td>
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<td>Caspases</td>
<td>recombinant caspase-3 linked to an antibody</td>
<td>MX-2060</td>
<td>“small-molecule” caspase activator, a potential anticancer agent tested in human cancer xenograft animal models</td>
<td>Maxim Pharmaceuticals Inc.</td>
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<td>Mitochondria/</td>
<td>tetracycline family member, inhibits cytochrome c release</td>
<td>minocycline</td>
<td>direct inhibition of mitochondrial cytochrome c release(^{143}); inhibits caspase-1 and caspase-3 mRNA upregulation(^{19}); blocker of the inducible NO-synthetase; beneficial effects in animal disease models including Huntington’s disease, ALS, acute brain injury, Parkinson’s disease and multiple sclerosis</td>
<td>Neuroapoptosis Laboratory, Harvard Med. School, Boston, MA, USA</td>
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<td>Antiapoptotic genes, Bcl-x, IAP, downregulation of proapoptotic genes</td>
<td>upregulation of antiapoptotic genes, Bcl-x, IAP; downregulation of proapoptotic genes</td>
<td>Xigris, (rhAPC)</td>
<td>Xigris directly modulates patterns of endothelial cell gene expression clustering into anti-inflammatory and cell survival pathways (demonstrated by broad transcriptional profiling)(^{80})</td>
<td>Lilly Research Laboratories, Indianapolis, USA</td>
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<td>Antiapoptotic genes, Jak2, NF-(\kappa)B pathways</td>
<td>erythropoietin</td>
<td>r-Hu-EPO</td>
<td>in rodent experimental models shows significant neuroprotection when given up to 6 h after an experimental stroke, reduced injury by approximately 50–75%; an acute and delayed beneficial action of r-Hu-EPO in ischemic spinal cord injury (rabbit model); EPO: 350–1,000 u/kg of body weight, administered intravenously immediately after the onset of reperfusion(^{19})</td>
<td>Kenneth Warren Labs, NY, USA; Burnham Institute, La Jolla, CA, USA; Dept. Anesthes., Dokuz Eylul Univ., Izmir, Turkey</td>
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<td>Bcl-2</td>
<td>antisense 18-mer-oligo-nucleotide, (Phosphorothioate)</td>
<td>G-3139, Genasense</td>
<td>very promising results in combination with a standard chemotherapy(^{21}); phase I/II studies of Genasense have demonstrated an excellent safety profile with toxicity observed in 20% of patients, fatigue in 10% and rash in 5%, the symptoms reverse upon withdrawal of treatment; in phase III trials for malignant melanoma(^{8})</td>
<td>Genta Inc.</td>
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<td>Antiapoptotic, mech. not fully defined</td>
<td>ant apoptotic compound, (Monoaminooxidase B inhibitor), dibenzo[b,f][o-xepin-10-ylmethylene]-methyl-prop-2-ynylamine</td>
<td>CGP 3466B</td>
<td>positive results observed in vitro(^1) and rodents(^4, 138), and in primate (Rhesus Macacus) models(^4)</td>
<td>Nervous System Research, Novartis Pharma AG, Basel, Switzerland, and Dept. Psychoneuropharmac., Univ. Nijmegen, The Netherlands</td>
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<td>Glutamate excitotoxicity</td>
<td>interferes with the glutamate excitotoxicity</td>
<td>riluzole</td>
<td>clinical trials in ALS</td>
<td>Pharmacia Upjohn/ Rhone-Poulenc Rorer, Philadelphia, PA, USA</td>
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<td>in ALS mouse model (expression of mutant human Cu/Zn superoxide dismutase), riluzole significantly preserved motor function as assessed by nightly running in a wheel</td>
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<td>a phase I trial of riluzole in spinal muscular atrophy(^1)(^9)</td>
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<td>Retinoid receptor-driven transduction, synergy with TRAIL</td>
<td>retinoid acid derivative: 6{-3-[1-adamantyl]-4-hydroxyphenyl}-2-naphthalene carboxylic acid</td>
<td>CD-437 AHPN</td>
<td>mitochondria and caspase-3-dependent apoptosis</td>
<td>Anderson Cancer Center, USA/ CIIRD Galderma</td>
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<td>increases expression of Bad and down-regulates Bcl-2 expression</td>
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<td>synergy effect between recombinant TRAIL and CD-437 observed in a number of cancer cell lines and in human tumor xenografts</td>
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<td>Survivin</td>
<td>antisense oligodeoxynucleotides</td>
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<td>following transfection of antisense oligonucleotides to mouse surviving mRNA, a time- and dose-dependent increase in polyplody of approximately 2- to 3-fold and induction of apoptosis were observed in most of the tumor cell lines(^1)(^8)</td>
<td>Isis Pharmaceuticals/ Abbott Laboratories</td>
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<td>Smac/Diablo</td>
<td>exclusive rights patented</td>
<td></td>
<td>exclusive rights to develop Smac-based therapy have been patented</td>
<td>Idun Pharmaceuticals</td>
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<td>IL-1(^\beta) and IL-1(^R)</td>
<td>recombinant IL-1Ra</td>
<td>Anakinra/Kineret</td>
<td>approved for clinical use in the treatment of RA</td>
<td>Amgen Inc. Thousand Oaks, CA, USA</td>
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<td>in vitro attempts to use cells genetically modified to constitutively express IL-1Ra(^2)</td>
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Abbreviations: i.v. – intravenous, i.p. – intraperitoneal, ALS – amyotrophic lateral sclerosis, RA – rheumatoid arthritis, IL-1Ra – interleukin 1 receptor antagonist.

Figure 1. Activation of inflammatory caspases by “inflammosome” protein complex. Analogously to the apoptosome complex that initiates activation of the apoptotic caspase cascade, inflammosome is involved in the activation of “inflammatory caspases” (caspase-1 and -5). Nalp-1 forms the backbone of this structure. It has a modular structures composed of several distinct domains. The ligand that triggers Nalp-1 association with the adaptor Pycard and caspases is unknown; however, this unknown ligand (probably lipopolysaccharide) is proposed to bind to the leucine-rich repeat (LRRs) of Nalp-1, in a way similar to which cytochrome c binds to the WD-40 repeats which are localized to the amino terminus of Apaf, the backbone of the apoptosome. The domains of Nalp-1 and Pycard have been schematically represented. The PYD and CARD, both of which are protein-protein interaction domains of Pycard, link Nalp-1 with caspase-1. The NACHT is an oligomerisation domain of Nalp-1 and probably binds to a NACHT domain within the same or an adjacent molecule of Nalp-1. Upon formation of the mature complex that includes also caspase-1 and -5, both caspases become activated and can process and activate inflammatory cytokines such as IL-1\(^\beta\) or IL-18.
that allows activation of the inflammatory caspase family members caspase-1 and -5. Pycard/Asc and Nalp-1 (a member of pyrin family), other components of the inflammosome, are responsible for the proper positioning of both caspases and they play regulatory functions. Proteolytic maturation of some key activatory cytokines, such as IL-1β, IL-16 and IL-18, allows the immediate secretion of mature, biologically active cytokines without the time-consuming process of de novo synthesis.

In this way, cells not only save time in mobilizing an immediate and adequate immunologic response, but, when under viral attack, proteolytic signaling allows them to mount a proper reaction under such circumstances by shutting off cellular transcription, and translation machinery is a powerful defense mechanism in itself. In addition, increased proteolytic activity may be protective against some cell-invading species. Moreover, at least some caspases are likely to be involved in other crucial cellular processes, including activation, differentiation, and even cell cycle progression (reviewed in76). Caspase activity, in conjunction with other molecules, may influence cellular energy (ATP) consumption, thus affecting the (apoptotic or necrotic) mode of cell death77. Although these areas of caspase action still await exact definition, they may be responsible for the unexpected effects of caspase-based pharmacological approaches. Efforts are on the way to negatively or positively modulate caspase activity to achieve physiological effects are listed in Table 1.

But caspases are not the only pharmacologically attractive targets in the pathway; naturally occurring molecules that can modulate caspase activity are also increasingly gaining interest as potential targets for drug development. In recent years a family of caspase inhibitors called IAPs that bind and inactivate already active caspases have attracted increasing attention from the pharmaceutical industry. Their attractiveness as potential targets has grown by the discovery of the inhibitor of IAPs Smac/Diablo, which allows an additional level of modulation of caspase activity and apoptosis. Depending on the part of IAP that is targeted by a designed inhibitor, the net outcome can be either caspase activation, and thus an apoptotic or immunomodulatory effect if the interaction with caspases is disrupted, or downregulation of caspase activity and apoptosis inhibition if the interaction with Smac/Diablo becomes disrupted69. Below we discuss in more detail the progress as well as the positive and negative aspects of the mentioned targets for drug development, focusing on diseases other than cancer. Apoptotic pathways as targets for cancer treatment are covered only to a limited extent since they have already been covered in detail in our recent review76.

PROSPECTIVE CLINICAL RELEVANCE OF CASPASE ACTIVITY MODULATORS

The central role of caspases in the propagation of the apoptotic process and their function as activators of certain interleukins make the individual family members or subfamilies an attractive target for pharmacological intervention. Several pathologic conditions, acute and chronic, involve the activation of members of the caspase family of proteases. Below we discuss therapeutic approaches involving caspases and other apoptosis modulatory molecules in acute pathologies such as sepsis, stroke, myocardial infarction, spinal cord injury, and acute liver failure and also chronic disorders such as Huntington’s disease, Parkinson’s disease, ALS, RA and SIRS. Evolving data indicate that apoptosis or the over-activation of components of the immune system, or even a combination of both, significantly contributes to the pathophysiology of each disorder. Caspase inhibitors, or apoptotic modulators, have demonstrated pharmacological activity in animal models for these disorders and in a number of cases drugs have entered at least clinical trials.

Stroke, traumatic brain injury, and spinal cord injury as inducers of proapoptotic conditions in the central nerve system

Stroke, the consequence of arrested blood flow in a vessel supplying the affected area of the brain, is characterized by a mixture of apoptotic and necrotic cell death. As apoptosis is an energy-dependent process, the center of the ischemic area is dominated by necrosis, whereas neurons and other cells that are located in the periphery of the affected area will likely die by apoptosis. Thus it is not surprising that in experimental models the inhibitors of apoptotic pathways have significantly decreased the size of brain infarction. The administration of peptide-based caspase inhibitors, given up to 9 h after initiation of ischemia, significantly decreases the lesion size. Mice deficient in key enzymes in the apoptotic pathway, caspase-3 and -9, demonstrated relative neuroprotection after cerebral ischemia. Moreover, the data on caspase inhibitor treatment in animal stroke models indicate a much wider time window compared with, for example, inhibitors of coagulation or fibrinolytica, for the initiation of treatment by targeting apoptosis. This is not trivial, as the physician is usually not immediately available to give the medication when the stroke occurs, and the failure of many agents in stroke clinical trials may have been
caused by too late administration of the given medication, since crucial time had been lost in, for example, transport, diagnosis, and other activities. Consistent with the notion that targeting apoptosis may also be possible at later time points after the initiation of ischemia, recombinant erythropoietin, which appears to act via the induction of specific antiapoptotic genes, also has demonstrated significant neuroprotection when given up to 6 h after focal cerebral ischemic insult. Another pharmacological problem frequently encountered by the development of brain-active antiapoptotic drugs is the relative impermeability of the brain-blood barrier (BBB) for short peptides, the most frequent form of caspase inhibitors that are currently in use. Despite the stroke-related increased permeability of the BBB in the affected area, which may allow for the penetration of larger molecules, BBB-permeable caspase inhibitors are expected to be much more effective due to their bioavailability in the marginal zones of a stroke, where apoptosis is the predominant death mode.

Another sudden condition that causes significant apoptosis is traumatic brain injury. Apoptosis is here probably a secondary event that follows the necrosis seen mainly in the most affected areas. Traumatic brain injury is accompanied by an array of pathologic processes related to hemorrhage, fluid-electrolyte and mitochondrial disturbance, excitotoxicity, other calcium-related events, and immunological processes. Caspase inhibition has been proven to be beneficial at least in some animal models. However, other authors have found no significant improvement in neurological functions following the application of caspase inhibitors despite a reduction of the affected area. The observed discrepancies between improved histology without a positive effect on the function may be due to poor intracellular bioavailability of peptide-based caspase inhibitors. The achieved concentrations may prevent cell death, but this may not be sufficient to fully counteract deterioration of axons and dendrites. “Small-molecule” caspase inhibitors, with improved bioavailability, may be better able to fulfill their function in traumatic brain injury.

Acute spinal cord injury

Because of its anatomy (long, located in a semi-flexible channel) the spinal cord is frequently damaged during car or bicycle accidents or other conditions associated with sudden and substantial deceleration. Apoptosis has been well documented in pathologic samples from patients who died after acute spinal cord injury. Local administration of caspase inhibitors provided protection in a mild injury animal model. However, systemic administration of caspase inhibitors did not show beneficial effects in a more severe model of spinal cord damage. Hope has been raised by an already clinically used drug, erythropoietin. It has recently been shown that erythropoietin is active in experimental spinal cord injury. The beneficial effect of this hormone seems to be related to the activation of expression of the antiapoptotic molecules.

Myocardial infarction, a classical clinical condition that causes apoptosis

Myocardial infarction shows significant pathophysiological similarities to a stroke. Both originate in metabolically very active tissues after a sudden stoppage of blood supply caused by arterial occlusion. In both cases the central part of the lesion is poorly supplied with oxygen, thus providing favorable conditions for necrosis to occur in the center and for apoptotic death in the penumbra. Histopathological samples taken from ventricles of patients who died within 2 months after myocardial infarction showed a correlation between the apoptotic rate and the occurrence of early-onset congestive heart failure. Furthermore, apoptotic cell death has been visualized in vivo in patients with acute myocardial infarction using radiolabeled Annexin V, which binds cells that expose phosphatidylserine (semi-specific indicator of the apoptotic process) on their surface. Caspase inhibition has been shown to reduce the size of infarction. Yet one report shows no effect of these inhibitors in experimental models. Idun Pharmaceuticals Inc. (Table 1) has successfully tested its small-molecule caspase inhibitor, IDN-6734, in rat and pig infarct models. In both studies a significant reduction of the lesion size could be documented. These findings indicate that antiapoptotic therapy for myocardial infarction has the potential to reduce the incidence of post-myocardial infarction congestive heart failure, the cause of significant morbidity and mortality in the first months and year after myocardial infarction.

Overreaction of the immune response as a cause of pathologic apoptosis in sepsis, SIRS, and acute liver failure

The dramatic battle between invading microorganisms and components of the immune system leads to “collateral damages” by the latter to other tissues, which may cause a fatal outcome. Organ damage is a prominent aspect of the clinical presentation of sepsis, and it is likely that in addition to apoptosis,
necrotic cell death is involved. Therefore, the application of cell-death-blocking drugs in these conditions can protect valuable body tissues. Antiapoptotic drugs will have no protective effect on the invading organisms due to the lack of state-of-the-art eukaryotic apoptotic machinery in their cells. In particular, in the liver and in the components of the immune system apoptosis plays a prominent role in sepsis and SIRS\textsuperscript{23, 53}. Caspase inhibitors are particularly effective in the CD95 (APO-1/Fas) antibody-mediated model of hepatic damage\textsuperscript{49}; in the model the antibody binds the CD95 receptor and acts as an agonist to send a death signal to the cell’s apoptosis machinery. Soluble FasL (the ligand for CD95) levels have been shown to be elevated in sepsis and in conditions such as alcoholic hepatitis that have a prominent SIRS component\textsuperscript{97, 105, 107}. Even in relatively mild preclinical models of sepsis (cecal ligation and puncture, murine model), with no significant damage to internal organs, caspase inhibitors showed considerable protection\textsuperscript{51}. Caspase inhibitors significantly prevent the depletion of peripheral lymphocytes, which otherwise get extensively lost as sepsis advances clinically. The observed effects compare favorably with a model involving caspase-3\textsuperscript{−/−} mice, indicating that at least the pharmacotherapy of sepsis should target multiple caspases. The above results imply that caspase inhibitors act in sepsis by preventing apoptosis of the patient’s immune cells, thus maintaining the host’s ability to fight the invading microorganisms. Furthermore, the increased lymphocyte apoptosis in the circulating blood of patients suffering from sepsis has directly been demonstrated\textsuperscript{71} and was associated with poor treatment outcome. Consistent with the above data, overexpression of the antiapoptotic protein Bcl-2 in T cells also improved survival in a murine sepsis model\textsuperscript{54}. Moreover, activated protein C (Xigris®), a drug used in the clinic to treat sepsis, not only up-regulated anti-inflammatory genes, but also antiapoptotic genes, including Bcl-x\textsubscript{L} and IAP; in addition, it strongly down-regulated the expression of proapoptotic genes, as determined by microarray gene profiling\textsuperscript{40}. Thus, septic patients treated with Xigris benefit in addition to its well-known anti-coagulant activity also from the anti-inflammatory and antiapoptotic action. Accordingly, a broad-spectrum caspase inhibitor, VX-799, recently developed by Vertex Pharmaceuticals Inc., was shown to be effective in several models of bacterially induced sepsis and an apoptosis-dependent model of organ failure. While VX-799 has not yet entered clinical trials, a broad-spectrum caspase inhibitor developed by Idun Pharmaceuticals Inc. IDN-6556 has been proven effective as a conservant that prevents from cold- and ischemia-induced damage of donor liver organ transplants and is used in the surgical treatment of acute liver disease\textsuperscript{98}. Phase I clinical trials have indicated that this compound is also beneficial in patients with chronic liver disease.

**Chronic activation of apoptotic pathways in Huntington’s disease, Parkinson’s disease, ALS, RA, and other disorders**

Cell death in chronic neurodegenerative diseases is often a consequence of a genetic mutation that either directly or indirectly affects cell viability or triggers evasion of the immune system. Environmental factors may contribute, trigger, or accelerate the chronic neurodegenerative process. Despite decades of intensive research, the cause of many of these disorders remains to be elucidated. The contribution of caspase activation to the pathophysiology of ALS and Huntington’s disease has been shown in vivo and in various experimental models (see below). Furthermore, experimental evidence indicates the role of caspases in Parkinson’s disease, Alzheimer’s disease, and dementia associated with human immunodeficiency virus infection\textsuperscript{34, 35, 64}. Thus, caspases and other components of apoptotic pathways are emerging as targets for drug development, at least in some neurodegenerative diseases. However, due to the chronic character of these pathologies, the shortage of selective antiapoptotic drugs currently “in the pipeline” that cross the BBB becomes an important problem. Patients suffering from chronic diseases have to take medications for years, so ideally their action should be free from side effects. In some cases, when the etiology is better elucidated, targeting the primary, early event, rather than protecting neurons from apoptosis by inhibitors, would be more effective. Apoptosis in these cases is a relatively late consequence of pathologic changes in the affected cell. For example, rather than targeting apoptosis, understanding the specific biochemical consequences of mutant Huntingtin protein and targeting those processes will more likely allow successful therapy development. Furthermore, some diseases may depend selectively on certain apoptotic pathways in the cell. It has already been demonstrated that specific components of the apoptotic machinery dominate in certain cell types but not in others\textsuperscript{42}. This would allow cell type-specific intervention, perhaps selectively modulating apoptosis in desired tissues while leaving other tissues unaffected. While following this approach it will be necessary to define disease-related apoptotic pathways that are both dependent on specific caspases and which are bypassed by other caspases in normal cells.

Huntington’s disease is an autosomal dominant neurodegenerative disorder affecting primarily neostria-
tum and cortex. First symptoms usually occur between the 4th–5th decade of life and, with a mean survival of 15–20 years after the occurrence of the first symptoms, prognosis is frequently fatal. Symptoms include a characteristic movement (Huntington’s chorea), cognitive dysfunction, and psychiatric signs. The disease is caused by a mutation encoding an abnormal expansion of CAG-encoded polyglutamine repeats in a protein called Huntingtin. The discovery of the mutant gene responsible for the disease made it possible to create transgenic mouse models suitable for drug tests. The mutated Huntingtin causes disturbances of the mitochondrial metabolism leading to an energy shortage that is compensated by increased glycolysis and other mechanisms. The observed death mode resembles neither apoptosis nor necrosis clearly. Nevertheless, the upregulation of caspase-1 and -3 expression, followed by activation of caspase-8, -9 and the release of cytochrome c, indicates activation of components of the apoptotic machinery. Both the toxic effects of Huntingtin fragments and the depletion of a wild-type Huntingtin in Huntington’s disease seem to be responsible for the neuronal death. Huntingtin is cleaved by caspase-1 and -3, thus, as the disease progresses, caspases increasingly generate toxic fragments of Huntingtin and deplete the wild-type protein. Neuronal dysfunction caused by the down-regulation of neurotransmitter receptors is another important feature of Huntington’s disease, and it is at least in part a caspase-mediated event, since it can be counteracted by the inhibition of caspases. Most of the findings first detected in the studies of murine models of Huntington’s disease have also been verified in human striatal brain tissue. This includes the activation of caspase-1, -3, -8, and -9 and cytochrome c release. Transgenic mice have been used as a tool for evaluating and demonstrating the efficacy of caspase inhibitors, creatine, and minocycline in an animal model of Huntington’s disease. While the protective role of caspase inhibitors was expected due to the blocking of proteolytic death cascade, the neuroprotective effect of minocycline, a member of the tetracycline antibiotic family, is more complex. Its protective action seems to be related not only to the inhibition of nitric oxide (NO) production by the inducible form of NO-synthetase, but probably more significantly to the inhibition caspase-1, and -3 expression and to the direct inhibition of mitochondrial cytochrome c release, thus to the prevention of the activation of the intrinsic (apoptosome-dependent) death pathway. Creatine’s beneficial function is most likely related to its “energy-buffering” capacity. A phosphorylated form of creatine can quickly release energy on demand, a capacity very important in neuronal and other cells with impaired mitochondrial function in the course of Huntington’s disease. The antiapoptotic activity of minocycline is probably responsible for its beneficial effects in other animal disease models, including ALS, acute brain injury, Parkinson’s disease, and multiple sclerosis.

The involvement of apoptosis in Parkinson’s disease has been suggested by some researchers, but others have questioned its key role in the pathology of the disease. Molecular evidence, such as the increased expression and/or activity of Bax, caspase-3, and p53 in the midbrain and striatum of patients that suffered form Parkinson’s disease, have been found. Several Parkinson’s disease models have been used to test antiapoptotic and other drugs. They include: 1) depletion of dopaminergic neurons by 6-hydroxydopamine (6-OHDA) or by 2) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment, and 3) partial inhibition of mitochondrial respiratory chain complex-I by rotenone (several weeks of continuous intravenous administration). The rotenone-based model seems to best reproduce the histological and neuropathological features of Parkinson’s disease. Numerous attempts to treat the disease by the prevention of apoptosis have been made. At least in the 6-OHDA- and MPTP-based models, the antiapoptotic compound CGP 3466B (dibenzo[b,f]oxepin-10-ylmethyl-methyl-prop-2-ynyl-amine), which also acts as an inhibitor of monoamine oxidase A, has been shown to cause cytoprotective and functional beneficial effects. The positive results were observed in in vitro models, as well as in primate (Rhesus Macacus) models. The antiapoptotic therapeutic approach may not always be effective, as at least in some in vitro models inhibition of caspase-8 by zIETD-fmk (N-benzoylcarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone) or the application of a broad-spectrum caspase inhibitor zVAD-fmk (N-benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone) converted apoptosis into necrotic death rather than protected the neurons.

ALS is associated with neuronal cell death in the anterior horn of the spinal cord and in the motor corex. The typical apoptotic features, such as DNA-fragmentation, caspase-3 activity, and macrophages with ingested cell fragments, have been documented in the course of ALS. The primary pathway indicated by some researchers is the p53-dependent signaling cascade that activates the mitochondrial/apoptosome death pathway. Accordingly, a decreased level of Bel-2 and an increased level of Bax and Bak have been found in post mortem samples from the spinal cords of ALS patients.
Recently, transgenic animal models used to study the pathophysiology of ALS have been created by the overexpression of various mutations of (Cu/Zn) superoxide dismutase 1, previously identified in familiar forms of ALS. Data obtained from some transgenic models, e.g. carrying the mutation G86R, support the importance of the p53 pathway in the pathology of ALS, whereas other models, e.g. transgenic mice that carry the G93A mutation, seem to develop ALS phenotype independently of the p53 pathway. Nevertheless, regardless of the pathway activated, there is a general consensus supporting the involvement of apoptosis in the ALS pathology. The mice carrying the G93A mutation show an increased caspase-1 and -3 activation in the anterior horns of the spinal cord that can be counteracted by Bcl-2 overexpression. The expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL are also diminished in the affected spinal cord areas. Accordingly, intracerebroventricular administration of zVAD-fmk caspase inhibitor delays disease onset and mortality. Moreover, zVAD-fmk inhibits caspase-1 activity as well as caspase-3 mRNA upregulation, the latter by yet an unknown mechanism. Despite several clues indicating the direct role of apoptosis in the development of ALS, these authors are unaware of any preclinical or clinical trials targeting apoptosis. However, riluzole, a drug already used in the clinic to treat ALS, interferes with the glutamate-mediated excitotoxic pathway and shows a significant beneficial effect in the murine G93A model. Glutamate excitotoxicity has been previously implicated in ALS development. Thus, riluzole may indirectly serve to prevent or at least slow down the apoptotic process in the affected neurons.

RA is a chronic inflammatory and destructive joint disease that affects 0.5–1% of the population in the industrialized world and commonly leads to significant disability and, consequently, a reduction in the quality of life. Drug therapy for RA goes in two different directions: 1) symptomatic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and 2) disease-modifying antirheumatic drugs. Whereas NSAIDs do not specifically target the underlying immuno-inflammatory events and work just by interfering with it in a rather unspecific way, the disease-modifying drugs try to interfere with specific immunologic pathways, thus altering the disease process. The role of the cytokine network in mediating inflammation and joint destruction in RA has been investigated extensively in recent years. IL-1β and tumor necrosis factor are two pivotal proinflammatory cytokines that have been shown to contribute to the clinical manifestations of RA. The ability of IL-1β to drive inflammation and joint erosion and inhibit the tissue repair processes has been clearly established in in vitro systems and in animal models. Under physiological conditions the activity of IL-1β is balanced by IL-1 receptor antagonist (IL-1Ra). The understanding of the respective roles of IL-1β and IL-1Ra in RA has led to the development of a recombinant IL-1Ra, anakinra (Kineret; Amgen Inc., Thousand Oaks, CA), and caspase-1-specific inhibitor VX-740 (pralnacasan, Vertex Pharmaceuticals Inc.) that offers a new therapeutic modality for RA. After a series of very promising results obtained in in vitro as well as an animal model, pralnacasan is in phase II clinical trials as an anti-inflammatory agent for RA. Similarly, disease mechanism-oriented approaches are being followed by other pharmaceutical companies.

CONCLUSIONS

The pace of research into the understanding of the biological processes involved in apoptosis, coupled with the interest in related pharmaceutical drug discovery, induces the expectation that major pharmaceutical products that modulate apoptosis will result. The “proof of principle” experiments, often made with broad-spectrum caspase inhibitors, are usually followed by the testing of “designed” inhibitors that target only a subfamily of, for example, caspases or other key modulators of apoptotic pathways. The remarkable efficiency of zVAD-fmk, a prominent example of broad-spectrum caspase inhibitors, in the various animal models presented above may reflect its ability to inhibit multiple enzymes not only from the caspase family, but also from other cysteine proteases with a similar mechanism of action. Accordingly, selective and/or reversible inhibitors usually show lower efficacy in multifactorial models such as ischemia, NMDA-induced excitotoxicity, or hepatitis. Importantly, caspase inhibitors may exhibit significant activity in vivo even when they are applied post insult. This is clinically very important, as it is very often the case that medication is not available immediately, but a few hours after the occurrence of the emergency. Clinicians seek drugs with significantly wider therapeutic windows than that offered, for example, by anticoagulants in stroke or heart infarction. At least for acute central nerve system pathologies the first systemically active inhibitors have emerged. Functional recovery has been demonstrated in some ischemia models, but long-term protection by caspase inhibitors is still being questioned. Recent developments in drug design have enabled the first caspase inhibitors to enter the clinic. To
assure sustained effectiveness and good pharmacokinetic characteristics, the peptide character of the current inhibitors will have to be further reduced. Small-molecule nonpeptidic caspase inhibitors, which have appeared recently, indicate that this goal can be accomplished. Before these therapeutic approaches enter the clinic, a few important issues have to be resolved. In particular, the necessary spectrum of inhibitory activity required to achieve the beneficial effect needs to be determined. Safety aspects associated with prolonged administration have to be resolved. Therefore, broader-range caspase inhibitors and other apoptosis modulators are likely to enter the treatment of acute clinical conditions first. Recent results with the synergistic effects between MK-801 and caspase inhibitors in ischemia suggest that caspase inhibitors may need to be used in conjunction with other drugs. The combination of anticoagulants and, for example, caspase inhibitors will likely be tested in stroke and heart infarction in the very near future. The application of antiapoptotic therapies for chronic disease is further away than the other approaches discussed; a deeper understanding of apoptotic pathways associated with these chronic disorders holds promise that treatments truly influencing disease progression can emerge. The research on caspases and their inhibitors as well as other modulators of apoptotic process will remain a rapidly developing area of biology and medicinal chemistry, and it will stay in the focus of interest of pharmaceutical industry CSOs and their R&D departments. With the first caspase inhibitors already in clinical trials, it can be predicted that novel drugs exploring apoptosis modulation will appear on the market within the next few years. Certainly in oncology research, the era of specific, targeted cancer therapy that explores proapoptotic drugs has already arrived and the resultant drugs are at the core of current and future cancer treatment. But some caution is advisable in this rapidly developing field, as the likely expected and unexpected side effects of these treatments will emerge. For example, despite their positive effect in sepsis, caspase inhibitors may worsen conditions associated with viral infections. In these cases, apoptosis of infected cells is a powerful protective mechanism.

REFERENCES


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