Neutrophil cell death: mechanisms and regulation
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Chapter I

Introduction

Apoptosis: general introduction

The term “apoptosis” was introduced in the early nineteen-seventies to determine a distinct type of cell death, different from necrosis, which was discovered and rediscovered several times by various developmental biologists and cytologists. Initially, it was forgotten as an irrelevant phenomenon, but the “reincarnation” of the process has come when it was realized that apoptosis is a genetically regulated (programmed) mechanism and not just an accidental event. Since then, the terms “apoptosis” and “programmed cell death” are used interchangeably as synonyms. This regulated form of cell death is as important and physiological as cell proliferation and differentiation, and is as tightly regulated. The basic knowledge of genetic regulation of apoptosis has come from outstanding work in the primitive organism *Caenorrhabditis elegans*. The genes that were found to regulate apoptosis in this nematode have later been found to have homologues in mammals, including humans. This proved apoptosis to be an important process conserved throughout evolution.

In the last 15-20 years, the number of studies devoted to apoptosis has grown exponentially. It has been revealed that apoptosis is involved in multiple physiological and pathophysiological processes. This polyfunctionality is reflected in a row of evocative names. For instance, the role of apoptosis in the sculpting of tissues during ontogenesis deserves it to be called ‘an architect’. ‘Altruistic suicide’, when cells infected with a virus “victimise” themselves, promoting a built-in apoptotic program to prevent spreading of infection, is another name of apoptosis. The balance between cell proliferation and cell death must be tuned and controlled properly, since a misbalance may contribute to disease. Examples are numerous and very relevant. Accelerated apoptosis of neurones contributes to pathogenesis of neurodegenerative disorders (such as Alzheimer and Parkinson disease). Binding of HIV or its gp120 protein to CD4+ T-cells without simultaneous engagement of the MHC-II complex triggers apoptosis of these helper cells, leading to AIDS. Delayed apoptosis is associated with cancer.

The physiological importance of apoptosis, besides its role in development, is illustrated by its crucial contribution to the regulation of the immune system. Apoptosis is involved in the control of T- and B-cell maturation and elimination of autoreactive lymphocytes. Proliferation and expansion of lymphocytes challenged by an antigen, survival of memory cells and death of activated T-cells during the decline of an immune response, all these adaptive immune
reactions are associated with apoptosis or its inhibition. Also, immune tolerance at immunoprivileged places, such as the eye and the testis, is provided by apoptosis-related mechanisms.

The present work is devoted to apoptosis of neutrophils, which serve as a first line of defense against invading pathogens. Apoptosis of neutrophils is a core element of the innate immunity. Regulation of the neutrophil life-span by apoptosis provides a fine balance between the function of neutrophils as effector cells of host defense and a safe turn-over of these potentially harmful cells. Alterations of neutrophil apoptosis are associated with a number of diseases. As do other cell types, neutrophils possess components of both extrinsic and intrinsic apoptotic routes. The intrinsic pathway of apoptosis appears to be of major importance in neutrophils, since these cells are programmed for a rapid spontaneous cell death. However, in neutrophils this mechanism of apoptosis has special features, probably due to peculiarities of neutrophil mitochondria, which are believed to be core regulators of intrinsic cell death. A better understanding of mechanisms underlying neutrophil cell death will help to understand neutrophil physiology. It will contribute to the search for new approaches for handling pathology related to disturbances in neutrophil apoptosis and also increase our knowledge of inflammation in general.
Apoptosis of Neutrophils

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Introduction

Neutrophils constitute the most abundant population of leukocytes. In humans, the daily turnover of neutrophils is about $1.6 \times 10^9$ cells/kg body weight \cite{1}, which keeps the number of mature neutrophils within defined limits despite the tremendous proliferative potential of the bone marrow precursor cells. This large turnover is mediated by the continuous egress of neutrophils from the circulation. Neutrophils do not return to the circulation but are eliminated by secretion in mucosa or die in the tissues within 1–2 days \cite{1}. Under normal conditions (without inflammation) neutrophil turnover takes place without harmful effects, despite the large bioaggressive and destructive potential of these cells displayed under various inflammatory conditions \cite{2}. A special mechanism of harmless neutrophil destruction is provided by apoptosis, genetically programmed cell suicide.

Phenotype of Apoptotic Neutrophils

Morphological changes of neutrophils undergoing apoptosis are typical for the general apoptotic scenario. Characteristic features are condensation of cytoplasm and intracellular organelles, aggregation and subsequent
cleavage of nuclear chromatin, and formation of apoptotic bodies with or without nuclear remnants. All these events are detectable in neutrophil cultures, i.e. during aging in vitro. Importantly, apoptotic changes are accompanied by downregulation of cellular functions, especially receptor-mediated reactions. Neutrophils that have entered the apoptotic process lose the ability of chemotaxis, chemotaxis, phagocytosis, oxidative burst and degranulation [3, 4]. This could be due to the loss of receptor expression and disturbed post-receptor signaling, because receptor-independent stimulation for example with PMA, is less altered in apoptotic cells [3]. Despite hyperexpression of $\beta_2$ integrins, apoptotic neutrophils cannot adhere [4, 5], due to degradation of actin filaments and increased rigidity of the plasma membrane, which prevents cellular flattening on the substrate [6]. Together, these data indicate that apoptosis of neutrophils indeed leads to deterioration of their functions.

**Induction and Inhibition of Neutrophil Apoptosis**

Neutrophils have the shortest life span among leukocytes. After egress from the bone marrow, neutrophils leave the circulation within 6–10 h and migrate into the tissues, where they undergo constitutive (spontaneous) apoptosis in 1–2 days. The duration of the neutrophil life span can be either prolonged or further shortened by signals from the microenvironment. In vitro, apoptosis of neutrophils can be modulated by a wide variety of molecules. These data are summarized in a number of recent reviews [7–11]. In general, the myelopoietic growth factors G-CSF and GM-CSF, and cytokines such as INF-γ, IL-8 and IL-1β have anti-apoptotic potential. The effect of TNF-α on neutrophil life span seems to be equivocal, i.e. the results presented by different groups of investigators are sometimes contradictory. Although the pro-apoptotic effect of TNF-α on neutrophils has been well documented [12–18], opposite results have also been published [19, 20]. Probably, this controversy can be explained by the finding that the effect of TNF-α on neutrophil survival depends on the concentration of the cytokine [21] as well as on the duration of stimulation and on the initial functional capacity of the neutrophils before exposition to TNF-α [14, 22]. The importance of the ‘input’ state of the cells in the realization of the apoptotic program was also demonstrated for IL-10, which blocks the anti-apoptotic action of LPS, whereas IL-10 alone has no effect on neutrophil apoptosis [23]. Apparently, some stimuli can influence only pre-primed cells, having no effect on resting cells.

Neutrophils are an exclusive cell type in that they do not die in response to glucocorticoids. Instead, these hormones induce neutrophil survival by suppressing apoptosis [24–27]. In this respect, neutrophils are different even from eosinophils, i.e. another subset of granulocytes, because eosinophils rapidly undergo apoptosis in glucocorticoid-containing media [27]. Apparently, depending on cell type, the same receptors can induce different signals within the apoptotic machinery, which gives additional specific features to cellular behavior. On the other hand, this peculiarity coexists with a common pro-survival pathway for neutrophils and eosinophils mediated by GM-CSF [27]. In more detail, similarities and differences in the apoptotic program of neutrophils and eosinophils are nicely summarized by Simon [28].

Delay in apoptosis coincides with preservation of neutrophil functions [24, 29]. Obviously, this is not only an effect of extrinsic mediators, but also an action of the intrinsic neutrophil resources of autocrine/paracrine regulation. IL-1β may have such a role, since the secretion of this cytokine is increased when neutrophils are treated by the anti-apoptotic agents LPS or GM-CSF [30]. Also, the pro-survival effect of IL-6 may be mediated through platelet-activating factor secreted by neutrophils [31].

The fate of apoptotic neutrophils is not unique. They are eliminated by phagocytes, both professional (macrophages) and non-professional (e.g., fibroblasts). Macrophages with engulfed apoptotic neutrophils can be observed in inflammatory loci and in vitro cultures [23, 32]. The recognition of apoptotic neutrophils is determined by their unusual surface structures, which appear on the outer layer of the plasma membrane during apoptosis. Among these are phosphatidylserine (PS) residues, normally located in the inner leaflet of cellular membrane, which exteriorize upon induction of apoptosis and are recognized by specific PS receptors on disposing cells.

Changes in surface sugars on apoptotic cells are recognized by phagocyte lectins, and modified lipids are ligands for scavenger receptors [33, 34]. Importantly, phagocytosis of apoptotic neutrophils (as well as other apoptotic cells) suppresses the production of inflammatory cytokines due to autocrine inhibition [33]. Thus, all steps of the apoptotic process are orchestrated for safe and silent turnover of a huge mass of the everyday exchanging neutrophils.
Extrinsic pathway
(death-receptor dependent)

- TNF-α/FasL
- Activation of caspase-8
- Amplification
- Activation of caspase-9
- Release of pro-apoptotic proteins
- Cytochrome c
- Smac/Diablo
- Deactivation of IAPs
- Apoptosis

Intrinsic pathway
(mitochondrial, stress-induced)

- Mitochondria
- Pro-apoptotic Bel-2 homologues
- Anti-apoptotic Bel-2 homologues
- Release of pro-apoptotic proteins
- Actvation of caspase-9
- Deactivation of IAPs
- Apoptosis

Fig. 1. Extrinsic and intrinsic pathways of apoptotic cell death and their crosspoints. The extrinsic pathway (left) is initiated upon ligation of death receptors, which results in caspase-8 activation. This is followed by activation of downstream effector caspases, such as caspase-3, and cell death. The intrinsic pathway (right) involves mitochondria, which release their pro-apoptotic constituents into the cytosol. These proteins promote activation of the initiator caspase-9 (through cytochrome c) or deactivate IAPs (through Smac/Diablo), yielding further progression of apoptosis at the level of caspase-3. The Bel-2 homologues manipulate the permeability of mitochondrial outer membrane, preventing (anti-apoptotic) or inducing (pro-apoptotic) cell death. Caspase-8 amplifies death signaling, for instance through activation of the pro-apoptotic protein Bid, which facilitates Bax insertion into mitochondria. Active caspase-3 provides a back-loop to activation of upstream caspases, thus enhancing the whole cascade.

**Routes of Neutrophil Cell Death**

It is now obvious that cell death is as important as cell life. That is why the regulation of cell death and apoptosis is equally complex and tight. To date, dozens of molecules are known to be involved in apoptosis regulation. These molecules are active in different stages of the apoptotic process, accelerate or inhibit it, relate to each other in complex cascades and networks, demonstrate redundancy or unique features, participate in other physiological reactions or act predominantly for apoptosis. Within each cell, there are two main pathways of apoptosis: an extrinsic or death-receptor-mediated pathway and an intrinsic (mitochondrial or stress) route of cell death [for a recent review, see 35]. The extrinsic pathway is initiated upon binding of so-called 'death receptors', which belong to the TNF receptor family, to their ligands (fig. 1, left). This interaction leads to the assembly of the intracellular death-inducing signaling complex (DISC). This complex then starts the activation cascade of a number of apoptosis-related proteinases, the so-called caspases, i.e. cysteine (C) proteinases that cleave target proteins after an aspartate (Asp) residue. DISC recruits and activates by autocleavage the upstream initiator caspase-8, which then starts the cleavage and activation of downstream effector caspases and the final steps of the apoptotic program [36]. In neutrophils, the death-receptor pathway seems to be inact, because two main death ligands, TNF-α and Fas ligand (FasL), are among powerful accelerators of neutrophil apoptosis. These ligands can induce activation of caspase-8, followed by processing of executioner caspases [16, 18].

The intrinsic apoptotic pathway has been less well studied in neutrophils, although it must be of major importance because neutrophils are tuned to rapid death also without external influence (spontaneously). This route of cell death involves mitochondria, which connect Bel-2 proteins and caspases (fig. 1, right). Bel-2 proteins constitute a family of proteins with either pro- or anti-apoptotic properties. The anti-apoptotic members (i.e. Bel-2, Bel-XL, Mcl-1) contain four domains of sequence similarity, designated BH1 through BH4. The pro-apoptotic members, on the other hand, are classified according to whether they contain one such homology region (the 'BH3-only' proteins, e.g. Bid, Bad, Bim, Noxa and Puma) or three of these regions (the 'multi-domain' or 'BH1-3')
proteins, including Bax, Bak and Bok). Like in other cell types, the caspases and the Bcl-2 proteins occupy a central position in neutrophil apoptosis [10]. Mitochondria may release certain proteins into the cytosol that facilitate the activation of the caspase cascade, and Bcl-2 homologues are thought to manipulate the integrity of mitochondria, thus providing regulation of caspase activation [37]. Neutrophils, although having both Bcl-2 proteins and caspases, are an exceptional cell type in that they were considered to possess no or only few mitochondria. This idea was based on the observation that mitochondrial poisons such as cyanides do not influence cellular functions in neutrophils, because these cells mainly use glycolysis for energy supply [38]. Moreover, electron microscopy studies have failed to identify the usual mitochondria in neutrophils [see references in 39], and mitochondrial respiration is very low in these cells [40]. For decades, mitochondria were out of the scope of phagocytologists as irrelevant rudiments without a role in neutrophil life. However, recent recognition of mitochondria not only as an energy plant, but also as a cellular 'plant of death' [37], has rekindled the interest in these organelles. In this respect, it was logical to suppose that if mitochondria had lost their importance in the active life of a neutrophil, perhaps they had preserved a role in cell death. That is why studies devoted to neutrophil apoptosis have also shed some light on the general physiology of neutrophil mitochondria.

Thus, mitochondria in neutrophils were visualized as a tubular network by means of specific fluorescent dyes (fig. 2a) [39, 41]. These tubular structures have been shown to possess a transmembrane potential ($\Delta \Psi_m$) that is sensitive to uncouplers and potassium ionophores [39, 42], a further indication that they are real mitochondria. In neutrophils undergoing spontaneous or accelerated apoptosis, the mitochondria change shape, forming perinuclear clusters (fig. 2b) [18, 41]. Moreover, as in other cell types, Bax protein targeting to mitochondria was evident in neutrophils [41, 43]. Such a subcellular redistribution of Bax by fusion with mitochondria upon apoptosis results in the permeabilization of the outer mitochondrial membrane, with subsequent release of pro-apoptotic mitochondrial proteins [37]. Hence, the finding that the Bax relocation occurs also in neutrophils gave a circumstantial evidence of mitochondrial participation in neutrophil apoptosis.

However, besides this fact, direct evidence that mitochondria are involved in apoptosis of neutrophils was still illusive. Neutrophil mitochondria appear to express surprisingly low amounts of the principal apoptotic player – cytochrome c. Cytochrome c itself and its release from mitochondria were only detectable after concentration procedures such as subcellular fractionation and/or immunoprecipitation [43-45]. Moreover, several other mitochondrial proteins, including the pro-apoptotic effector Smac/Diablo and the respiratory chain enzyme cyto-
chrome-c oxidase as well as the mitochondrial matrix protein HSP-60 were undetectable in neutrophil cell lysates by Western blotting [45]. However, in neutrophils, such results should be interpreted with caution, because the absence of protein detection does not necessarily mean that a protein is not there. This is due to the tremendous proteolytic potential of neutrophils, which has been reported to cause non-specific degradation of proteins, including for instance STAT [46] and procaspase-3 [43], leading to loss of their detection.

On the other hand, Murphy et al. [45] have shown that in neutrophils the cytochrome-c-dependent apoptotic pathway displays a dramatic reduction in requirements for cytochrome c. This pathway is initiated by release of cytochrome c from mitochondria, which promotes oligomerization of Apaf-1 and recruitment of the caspase-9 zymogen. Such a multimolecular complex consisting of cytochrome c, Apaf-1 and caspase-9 is called an apoptosome and contains enzymatically active caspase-9. Actually, caspase-9 is active exclusively within the apoptosome, allowing to call the latter a holozyme [47, 48]. Although dramatically reduced, the amount of cytochrome c present in neutrophils was sufficient to induce caspase-9 activation in these cells [45]. Therefore, it has been suggested that neutrophils have a lowered threshold requirement for cytochrome c, which could be partially compensated by the increased expression of Apaf-1. Indeed, upregulation of Apaf-1 is thought to increase the sensitivity of apoptosome activation to cytochrome c [49]. These results indicate that neutrophil mitochondria, although deficient in respiration, still preserve the potential to support apoptotic caspase activation. Similar findings have been reported for the neutrophil's 'relatives', eosinophils, in which mitochondria seem to play a role in apoptosis, but not in oxidative phosphorylation [40].

To date, together with the caspase-dependent apoptosis, the experimental data on a caspase-independent cell death are accumulating [50-52], although its physiological role remains to be determined. Neutrophils too, can undergo a caspase-independent cell death. This non-classical pathway of neutrophil turnover is revealed under simultaneous stimulation with TNF-α and caspase inhibition [18, 44]. The caspase-independent death of neutrophils lacks several features of apoptosis, but discloses an additional role that mitochondria could have in these cells. Several lines of evidence have demonstrated that a caspase-independent route of neutrophil cell death is mediated by mitochondria-derived reactive oxygen species (ROS) [18]. In a sense, this is not surprising, since ROS produced in the respiratory chain of mitochondria are known to be responsible for TNF-α-induced cytotoxicity in several cell lines, in which no doubts exist about the 'quality' of the mitochondrial respiration [53, 54]. However, this finding provides evidence, although indirect, that neutrophils may have some respiratory chain activity in the mitochondria. The relative cytochrome c deficiency of neutrophil mitochondria (discussed above) may cause questions about the activity of complex IV of the mitochondrial electron transport chain, but there are still complexes I, II and III that do not need cytochrome c and that can generate ROS [37]. Probably, these complexes are involved in ROS production responsible for the caspase-independent neutrophil cell death. One can also ask whether those mitochondrial ROS can participate in other signaling pathways within a cell. For instance, integrin-induced alterations in mitochondrial function lead to generation of ROS, which participate in a signal transduction cascade that leads to NF-κB activation in response to integrin-mediated cell shape changes [55]. These speculations are in line with a general reevaluation of the role of ROS in cellular physiology. During the last decade, these agents moved from a category of merely unwanted side products of oxidative metabolism to a cohort of important messenger molecules [56, 57]. Neutrophils possess perhaps the most powerful system of ROS generation among all cell types, i.e. the NADPH oxidase, which they use to kill ingested microorganisms [58]. But the extent and the rate of ROS production by this oxidase are so dramatically high that it is hard to imagine that the ROS generated by this system do anything besides killing. With respect to cell death, NADPH oxidase does not have an important role, because neutrophils from patients with chronic granulomatous disease, which have an impaired NADPH oxidase system, display the same level of spontaneous and TNF-α-accelerated apoptosis as do normal neutrophils, at least in short-term cultures [18, 21, 22]. A more finely tuned system of mitochondrial ROS synthesis or alternative NADPH oxidases [59, 60] is more likely to have a role in the signal transduction, although experimental evidence is still lacking.

Despite the obvious importance of mitochondria in the apoptotic program, it seems that these organelles are not absolutely necessary for apoptosis, at least in neutrophils. Neutrophil-derived cytoplasts, which are plasma membrane vesicles filled with cytoplasm but devoid of nuclei, granules and mitochondria [61, 62], nevertheless undergo apoptotic changes reminiscent of these reactions in intact neutrophils. During culturing, cytoplasts express PS residues on the outer leaflet of the plasma membrane with coincident caspase-3 activation [18, 41]. This observation...
indicates that neutrophil cytosol possesses an adequate machinery to proceed to cell death. It is not yet clear which pathway is operative in neutrophil cytoplasts. In Jurkat cell cytoplasts, CD95/Fas ligation leads to PS externalization and caspase-3 activation [63], suggesting that the extrinsic (receptor-dependent) pathway of apoptosis involving DISC formation and caspase-8 processing is likely to be intact in this cell preparation. However, it has not yet been studied whether the apoptosome pathway of caspase-9 activation is operative in cytoplasts. It would be intriguing to investigate the state of this mitochondria-dependent route of apoptosis in the mitochondria-free cytoplasm system.

The apoptosis studies in cytoplasts highlighted also another issue, namely the relationship between apoptosis and protein synthesis. Apparently, the apoptotic machinery is preformed in the cytosol during neutrophil development and does not require de-novo produced proteins, because the absence of protein synthesis does not prevent apoptosis in cytoplasts. This is also true for the pre-mentioned Jurkat cell cytoplasts. Moreover, shutting off the protein synthesis by inhibitors such as cycloheximide or actinomycin D markedly increases the rate of neutrophil apoptosis [64]. Obviously, the whole apoptosis machinery is inactive or inhibited before apoptosis is triggered, otherwise a cell would not survive. Such a situation invites speculation that if apoptosis does not require protein synthesis, perhaps its inhibition does so. Indeed, neutrophils, having a limited protein synthetic capacity, nevertheless need new proteins to suppress apoptosis. Thus, the pro-survival effects of G-CSF and GM-CSF depend on as yet unidentified de-novo synthesized mediators, because their anti-apoptotic activity is prevented by protein synthesis inhibitors and is absent in cytoplasts [18, 65]. Corticosteroid-induced neutrophil survival is abrogated by the inhibition of protein synthesis as well [24].

Interestingly, the pro-apoptotic action of TNF-α is dramatically enhanced by a small dose of protein inhibitors, which in itself is not apoptogenic. This powerful combination is able to induce apoptosis in 70–90% of neutrophils already within the first hours of incubation [16, 44, 66]. These observations may indicate that, under normal conditions, the existing apoptotic program is paralyzed by a permanent production of short-living inhibitors, and that blockade of protein synthesis suppresses this mechanism, thus unleashing the apoptotic process [67]. One of the candidate proteins that is likely to keep apoptosis silent and to mediate effects of anti-apoptotic agents in neutrophils is a Bcl-2 anti-apoptotic homologue called Mcl-1. This protein has a very short half-life, both at mRNA and protein level, and its expression correlates with neutrophil survival, being preserved at a certain level by pro-survival cytokines [64, 68]. In contrast to Mcl-1, pro-apoptotic Bcl-2 proteins such as Bax have a relatively long half-life, which may contribute to the short neutrophil life span. The relative expression of pro- and anti-apoptotic Bcl-2 proteins also seems to be of importance. Upon induction or prevention of apoptosis, alterations of the Bax/Bcl-XL ratio have been observed [17]. Bcl-XL was noticed to be downregulated by TNF-α stimulation, whereas Bax expression remained constant, which leads to a shift in the balance towards Bax, i.e. towards apoptosis. In contrast, GM-CSF reduced Bax levels and preserved Bcl-XL expression, thus preventing apoptosis. However, the presence of Bcl-XL proteins in neutrophils is still debated [10], although the idea that interactions between pro- and anti-apoptotic Bcl-2 proteins compose a ‘rheostat’ that can determine sensitivity to apoptosis is attractive [69].

Besides quantitative characteristics, qualitative changes of Bcl-2 proteins such as localization also represent an important mechanism of the regulation of neutrophil apoptosis. As mentioned above, upon apoptosis Bax undergoes intracellular redistribution and moves from the cytosol to the mitochondria, where this protein is likely to induce the release of pro-apoptotic mitochondrial constituents [18, 41, 43]. Prevention of Bax relocation has been suggested to contribute to the anti-apoptotic action of G-CSF. This prevention could be achieved by an association between Mcl-1 and Bax, which has been shown to occur in neutrophils during GM-CSF stimulation and to be absent in untreated cells undergoing spontaneous apoptosis [70]. This altogether indicates that the relative ratio as well as the localization and partnership interactions of anti-apoptotic and pro-apoptotic Bcl-2-family proteins are significant components of the neutrophil life and death regulation.

**Apoptosis of Neutrophils and Pathology**

Dysregulation of the apoptotic program in neutrophils and their precursors is involved in the development of several pathologic conditions. Enhanced apoptosis contributes to pathogenesis of various neutropenias, in which several mechanisms of apoptosis acceleration have been implied. Thus, a defective expression of Bcl-XL in neutrophil precursors leading to accelerated apoptosis has been shown to play a role in myelokathexis, a congenital disorder that causes severe chronic leukopenia and neutropenia [71]. Both cyclic neutropenia and severe congenital
Neutropenia (Kostmann syndrome) have been linked to an ineffective production of neutrophils due to accelerated apoptosis of bone marrow myeloid progenitor cells [72, 73]. The estimated half-life of neutrophil precursors in patients with myelokathexis, cyclic neutropenia or Kostmann syndrome is about 10 times shorter than normal [74]. Also, mutations in neutrophil elastase have been thought to predetermine the disease phenotype in some patients with cyclic and severe congenital neutropenia [73, 75], but how elastase mutations may predispose neutrophil precursors to apoptosis is as yet unclear. Another type of neutropenia, chronic idiopathic neutropenia, is also associated with impaired granulocytopoiesis because of increased apoptosis of granulocyte precursors that overexpress Fas and are induced to die within the bone marrow microenvironment [76]. The involvement of the Fas/FasL system was found in chronic neutropenia accompanying large granular lymphocyte leukemia [77]. In this disease, the high levels of soluble FasL are a pathogenetic mechanism of neutropenia, and resolution of neutropenia is associated with marked reduction in FasL levels.

Neutropenia may also be related to metabolic defects in neutrophils and their precursors, as was demonstrated in glycogen storage disease type 1b (GSD1b) [78]. Most likely, the defect in transport of glucose-6-phosphate into endoplasmic reticulum, which is a hallmark of GSD1b, leads to changes in intracellular redox state, thus driving induction of apoptosis. This hypothesis is supported by the observation that specific pharmacological inhibition of glucose-6-phosphate transport promotes neutrophil apoptosis, which can be prevented by ROS inhibition [79]. General metabolic defects such as, for instance, those occurring in liver cirrhosis, are frequently associated with a predisposition to bacterial infections and neutropenia, which can be ascribed in part to the enhanced neutrophil apoptosis as well [80]. 'Local' neutrophil insufficiency due to increased apoptosis has been reported to play a role in persistence of cardiovascular device infections [81]. The neutrophils experiencing increasing shear stress at the sites of cardiovascular device implantation display functional alterations and apoptotic signs, which was suggested to facilitate the local development of bacterial infections.

Besides the contribution of accelerated apoptosis of neutrophils and their precursors in neutropenias, there is another side of the coin, namely the pathophysiological role of delayed neutrophil apoptosis in disease. Neutrophils are sensitive indicators of homeostasis and readily react to any changes by realizing their effector potential. Reactive 'remodeling' of neutrophils may influence the apoptotic program as well. For example, spontaneous neutrophil apoptosis is delayed in septic patients with pneumonia, burns and traumatic injuries [20, 82–84] as well as after major surgery [84, 85]. A longer survival of primed circulating neutrophils due to the inhibition of apoptosis together with mobilization of the bone-marrow pool of young neutrophils tuned 'not to die' provide the accumulation of these cells in inflammatory sites. This results in excessive release of toxic metabolites, causing tissue injury and life-threatening complications (like multiple organ dysfunction), typical for systemic inflammatory response syndrome and sepsis.

In vivo, cytokines and growth factors are major regulators of neutrophil survival. Various stress insults, including stimuli originating from inflammatory lesions, may cause a dysbalance in the cytokine network. In patients with burns as well as with post-traumatic and post-operative septic complications, inhibitors of apoptosis have been detected in the blood [82–84, 86]. These inhibitors are mainly G- and GM-CSF [82, 83], although also the presence of bacteria-derived products such as endotoxin, which are assumed to promote neutrophil survival, cannot be excluded in septic patients. Taken together, these results indicate that conditions that modify neutrophil apoptotic tuning may occur in the circulation, and cytokines seem to play a crucial role in coupling neutrophils with local and systemic inflammation.

The influence of pathological conditions on neutrophil apoptosis may also be exerted via stress-dependent modifications in the hormonal status, and first of all through glucocorticoids, which depress apoptosis of neutrophils (see above). An interesting (but somewhat provocative) concept has been suggested by Sendo et al. [87]. These investigators found that spontaneous and TNF-accelerated neutrophil apoptosis was delayed in volunteers exposed to stress conditions, including lack of sleep, starvation and strenuous sport exercises. This was considered as a 'compensation' of the glucocorticoid-induced suppression of adaptive immunity. On the other hand, during longer stress (students preparing for a board examination were used as a model) neutrophil apoptosis was enhanced. As proposed, probably the latter plays a role in the weakening of the resistance to infections in chronic stress.

Delay of neutrophil apoptosis can lead to the pathological neutrophil accumulation in chronic neutrophilic leukemia, a rare syndrome characterized by excess of mature neutrophils. In this disease, neutrophils overexpress X-linked inhibitor of apoptosis (XIAP) protein due to its impaired degradation, which coincides with a delay in
spontaneous, Fas- and TNF-α-accelerated apoptosis [88, 89]. Also, neutrophils from patients with chronic myelogenous leukemia demonstrate longer survival than cells from healthy donors [90].

Disturbed elimination of apoptotic neutrophils is known to participate in the pathogenesis of inflammatory diseases. Late recognition and removal of apoptotic neutrophils, which still contain numerous aggressive mediators, from inflammatory lesions are—along with other factors—responsible for persistent inflammation during rheumatoid arthritis [91]. Clearance of apoptotic neutrophils is also impaired in systemic lupus erythematosus (SLE). In this pathology, the number of circulating apoptotic neutrophils is increased, they demonstrate increased extent of damaged DNA, and they are considered as candidate autoantigens, which may promote the production of autoantibodies to native DNA, a hallmark of SLE [92, 93]. Delayed apoptosis and clearance of apoptotic neutrophils has also been suggested as a factor of importance in the formation of autoantibodies against proteins in the granules (ANCA). More recently, it has been suggested that defective clearance of apoptotic neutrophils from the airways may contribute to ongoing airway inflammation in cystic fibrosis and bronchiectasis. This may be due to elastase-mediated cleavage of the PS receptor on resident lung macrophages, which leads to impairment of the recognition of apoptotic material [94].

**Neutrophil Apoptosis and Regulation of Inflammation**

Neutrophils form the first cellular guard to face invading pathogens. Interaction between the two parties starts to the inflammatory cascade, with various local and systemic effects such as recruitment of the additional cellular forces, increased cytokine production, etc. All these events are beneficial for the struggle with the microorganisms, but are also potentially dangerous for the host, and, obviously, to keep inflammation in a certain safe frame is as important as to be able to start it. As noticed above, apoptosis of inflammatory cells, including neutrophils, is one of the existing mechanisms known to limit phlogistic reactions. Probably, a stimulus that can initiate inflammation provides signals for its limitation as well. This suggestion is supported by a number of observations describing the ability of different pathogen to induce neutrophil apoptosis. For instance, phagocytosis of *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Candida albicans* is known to promote apoptosis of neutrophils [13, 95–99]. The mechanisms of apoptosis involved under those conditions are not clear, although a role for ROS, both NADPH-oxidase-dependent and independent, has been proposed [97, 98]. But whatever the mechanisms are, such a divergent outcome of the signals induced by a single agent supposes an initial convergence (i.e. a common signaling) somewhere upstream in the transduction cascade. In other words, activation and death signals may have to proceed through the same channel up to a certain stage, and diverge only afterwards. This common link could be represented by a caspase protease. Indeed, in the caspase family of proteases there are not only apoptotic members, but also inflammatory ones, such as caspase-1, which participates in IL-1β and IL-18 maturation. The growing body of evidence indicates that caspase-1 may also have the capacity to mediate apoptosis [100], which is, in fact, not surprising because the caspase-1 homologue gene product in *Caenorhabditis elegans*, CED-3, is known to be pro-apoptotic [101]. Neutrophils from caspase-1-deficient mice have delayed constitutive apoptosis, and LPS cannot inhibit apoptosis in these cells, but caspase-1-deficient neutrophils are still susceptible to Fas-mediated apoptosis [102]. Caspase-1 can be considered as a very upstream initiator caspase, like caspase-2 [48]. In this light, keeping in mind that mature neutrophils do not express caspase-2 [45, 103], caspase-1 gains impetus in neutrophil survival, likely combining functions related to inflammation and apoptosis. However, caspase-1 is not the only player in the field, because caspase-1-independent release of IL-1β has also been reported [104].

Hence, although the understanding of precise mechanisms of cross-talk between inflammation and apoptosis are far from complete, this concept attracts more attention in the line with the neutrophil’s prominent function in innate immunity as a first-line protection against various pathogens.


Scope of this thesis

As reviewed above, a substantial amount of literature has accumulated over the last decade on the phenomenology and mechanisms of neutrophil apoptosis as well as on the association between disturbed neutrophil apoptosis and pathology. The work described in the present thesis is focused on the clarification of the neutrophil cell death pathways and their regulation. In Chapter II, pro-survival effects of G-CSF have been studied and linked to inhibition of the mitochondrial-dependent activation of caspase-3. Chapter III describes an alternative TNF-α-induced, caspase-independent pathway of neutrophil cell death, which possesses specific features different from apoptosis and which is dependent on mitochondria-derived reactive oxygen species. As shown in Chapter IV, this “non-classical” cell death can be abrogated by inhibition of serine protease activity of the pro-apoptotic mitochondrial protein Omi/HtrA2. Chapter V provides a detailed characterization of different functional aspects of neutrophil mitochondria, which (in other cell types) combine life-supporting functions and death-promoting activity. In neutrophils, these organelles preserve mainly their pro-death potential, releasing a number of pro-apoptotic mitochondrial constituents into the cytosol during apoptosis and underlining the importance of the mitochondrial (intrinsic) pathway of neutrophil apoptosis. The experiments presented in Chapter VI reveal that the G-CSF-induced delay in neutrophil apoptosis is mediated through inhibition of Bid/Bax translocation to the mitochondria and subsequent prevention of mitochondrial dysfunction, which clarifies some details of the mechanism suggested in Chapter II. Chapter VII points out enhanced neutrophil apoptosis as a possible cause of neutropenia in a rare hereditary disorder – glycogen storage disease type 1b (GSD1b). Chapter VIII reports about another rare hereditary disease with accompanying neutropenia – Barth syndrome, in which neutrophils bind Annexin-V in the absence of apoptosis. These neutrophils are not recognized by macrophages, arguing against enhanced clearance as a cause for neutropenia.