Host–Parasite Interactions

Edited by
G.F. Wiegertjes and G. Flik

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Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
APAF1	apoptotic protease activating factor 1
APC	antigen-presenting cell
ATP	adenosine triphosphate
CBH	cutaneous basophil hypersensitivity
CCM	cell-conditioned medium
CD	cell differentiation
CL	chemoluminescence assay
CRMA	cytokine response modifier A
dd	degree days
DIC	disseminated intravascular coagulation
dpi	days post infection
DTH	delayed-type hypersensitivity
EGC	eosinophilic granular cell
EHNV	epizootic haematopoietic necrosis virus
EST	expressed sequence tag
GPI	glycosylphosphatidylinositol
HBP	histamine-binding protein
HPLC	high-pressure liquid chromatography
HSP	heat shock protein
IAP	inhibitor of apoptosis proteins
IEL	intra-epithelial leukocyte
ICE	interleukin-converting enzyme
IDI	invertebrate developmental inhibitor
IL	interleukin
iNOS	inducible nitric oxide synthase
IPNV	infectious pancreatic necrosis virus
IRE	iron-responsive element
IRP	iron-regulatory protein
IVDKM	*in vitro*-derived kidney macrophage
LMP	latent membrane protein
LP	lamina propria
LPS	lipopolysaccharide
lsr	large subunit of ribosome
MAC	membrane attack complex
MAF	macrophage-activating factor
MHC	major histocompatibility complex
mMCP	murine mast cell protease
NFκB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide
NRAMP	natural resistance-associated macrophage protein

NVI	necrotic volume increase
OUC	ornithine–urea cycle
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PCD	programmed cell death
PG	prostaglandin
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
PTK	protein tyrosine kinase
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
SOD	superoxide dismutase
SRBC	sheep red blood cells
ssr	small subunit of ribosome
SVCV	spring viraemia in carp virus
TGF	transforming growth factor
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial cell growth factor
VSOR	volume-sensitive outwardly rectifying


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Preface

This book gives an up-to-date overview of host–parasite interactions, using a comparative approach and covering vertebrates from fish to mammals. Four of the chapters in this book, or at least parts of them (Chapters 6, 7, 9 and 10) were originally presented at an intersociety meeting of the American Physiological Society, '*The Power of Comparative Physiology: Evolution, Integration and Application*', held in San Diego, California, August 24–28, 2002. It was decided then to invite partners of a European Union Research Training Network called 'Parity', working on host–parasite interactions in fish, to contribute with chapters and publish the joined papers in this book format. The first few chapters deal with more fundamental questions regarding host–parasite interactions and, as such, in Chapter 1, Steinhagen, and in Chapter 2 Hoole and Williams address fundamental aspects of apoptosis and necrosis. Although, for fish structural changes following cell death are well documented, studies on the physiological responses preceding cell death remain scarce. However, even if these changes would be better documented, the roles of apoptosis in the interactions between parasites and hosts are multifaceted and highly dependent on individual associations between the two organisms involved. Still, with the information obtained from studies in mammals and invertebrates, the next decade promises to be both exciting and productive with respect to our knowledge of the relationship between apoptosis in non-mammalian animals and infection.

Chapter 3 by Nielsen, Lindenstrøm, Sigh and Buchmann considers the role of thionine-positive cells in relation to parasites. Thionine-positive cells comprise such apparently diverse types as mast cells, goblet cells, basophils, eosinophils and cells from amyloid cartilage and mucous tissues. Thionin-positive cells are unique in the sense that they contain granules in their cytosol that are metachromatically stained with the cationic dye thionine (cf. toluidine blue, methylene blue, gallocyanin and pinacyanole). These cells derive from $CD34^+$ haematopoietic progenitors and play an important role in the innate response to parasites. Anti-parasitic activity of the cells is discussed in the context of cellular pathways including the NF-κβ activation via Toll-like receptors, phagocytosis and reciprocal signals between hosts and parasites. A review is presented on the vertebrate evolution of thionine-positive cells, with an emphasis on anti-parasitic mechanisms in fish.

In their chapter (Chapter 4), Joerink, Saeij, Stafford, Belosevic and Wiegertjes look at protozoan infections in fish. Kinetoplastid parasites such as *Trypanosoma* and *Leishmania* species are well studied in mammalian models but little is as yet known about the immune response against comparable parasite species in fish. Carp are host to at least two kinetoplastid species (*T. borelli* and *T danilewskyi*) and leeches act as vectors. The roles of NRAMP (natural resistance-associated macrophage protein), transferrin and the omnipresent, but still only partly understood, messenger NO is discussed as well as the role of cytokine profiles in macrophage polarization and function. In-depth analysis of the immune response to infection(s) by protozoan kinetoplastid parasites in fish may shed light on the evolution of host–parasite relationships.

In Chapter 5, Woo also addresses kinetoplastid haemoflagellates, i.e. two groups that cause disease in fish or in cattle, with an emphasis on the former. *Cryptobia* species affect

freshwater and seawater fish species, transmission occurs by leeches. *Trypanosoma* species are a nuisance for cattle, with blood-feeding flies their best-known vector. Susceptibility, tolerance and resistance to *Cryptobia* are addressed, as well as trypanosomiasis-related anaemia. Several aspects of immunosuppression are dealt with as well as the interaction of the parasites with the endocrine system of the host.

In Chapter 6, Walker, Wendelaar Bonga and Flik review the biology of the freshwater louse genus *Argulus* and its interactions with its hosts. They describe this obligate ectoparasite from an ecological point of view with a focus on the host–parasite interaction. The life cycle, morphology of developmental stages, locomotion, attachment strategies, feeding and sensory systems are addressed as well as geographic distribution and seasonality. The host–parasite interaction section focuses on host choice and specificity, with an emphasis on fish as host and their stress and immune responses to this parasite. Suggestions for parasite control and prevention are given.

In Chapter 7, Johnson and Fast describe the new developments in our understanding of host–parasite interactions between a seawater louse: the salmon louse *Lepeophtheirus salmonis* and its hosts. The salmon louse is a major problem in intensive marine salmon aquaculture, the costs of losses due to this parasite exceed over $40 million annually. The ultimate goal is development of a vaccine to this parasite, but classical (identification of protective antigens) approaches are unsuccessful so far. Biochemical, genomic, proteomic and immunological approaches are now used to unravel the interaction between this parasite and its host. Salivary components of the parasite are tested for their effects on the immune response of the host. New techniques such as the screening of subtractive cDNA libraries are predicted to provide the required resolution.

In Chapter 8, Buchmann, Lindenstrøm and Bresciani focus on fish and monogeneans. Helminths are parasites with a particular appetite for fish. There are an estimated 30 000 species of these parasites, including nematodes, acanthocephalans, trematodes, cestodes and monogeneans. They are almost exclusively ectoparasites, although some are found in cloaca, body cavity and heart. The chapter gives an overview of diversity, anatomy, gut and feeding, nervous system, osmoregulation, life cycle and reproduction of these parasites. Both the parasite and the host produce molecules for mutual communication, albeit that this communication is only poorly understood. However, the immune system of the host seems fully activated when infected by these parasites: both cellular (innate) and humoral (adaptive) responses with their particular machinery and signalling pathways are seen. At the end of the chapter measures to control these parasites are presented.

Alarcon-Chaidez and Wikel address, in Chapter 9, the topic of the tick–host relationship. Immunobiology, genomics and proteomics are the keywords for this chapter. They discuss life cycles and ecology of ticks, present the latest insights on tick-borne pathogens and host-acquired immunity to tick infestation. What is it in tick saliva that makes these parasites so nasty, how do they modulate host immunity? Ticks are vectors of diseases (Lyme), and a further in-depth analysis of host cytokine response to infection by this parasite is indicated to facilitate vaccination against this nuisance. Proteomic and genomic analysis will certainly bring more details on the parasite, possibly allowing control in the not too distant future.

In Chapter 10, Vermeulen describes avian coccidiosis. Intensive chicken rearing is a major meat industry yielding 35 billion broilers per year worldwide. *Eimeria* species

inhabit the intestinal lining of the chick, enteritis, disruption of intestinal villi and death being the classical symptoms of this parasite infection. Even when the parasite is not life-threatening, it causes poor food absorption and water resorption resulting in impeded growth. Chickens become readily immune to the parasite but the mechanism is still poorly understood. Newly discovered antigens that stimulated T-cell subpopulations and have protective capacity are targets for the development of new vaccines to improve the relationship between host and parasite.

Special thanks go to all contributors to this volume for their constructive interaction with the editors. Significant financial support was received from the SEB, London, and the Osmoregulation group of the SEB, which we gratefully acknowledge. Daisy Maurits, Nijmegen, The Netherlands, is acknowledged for excellent secretarial support. Kay McNamee, Wageningen, The Netherlands is acknowledged for excellent editing support. Special thanks go to my co-editor and co-ordinator of the EU-RTN Parity programme, Geert Wiegertjes. Without his invaluable help and persistent drive the realization of this book would have been at serious risk.

Gert Flik
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Structural and physiological aspects of cell death

Dieter Steinhagen

1 Introduction

Cells are active participants in their environment and thus constantly adjusting structure and function to extracellular demands or stressors resulting from organismal needs or from outside. Beside structural or functional adjustments, developmental processes or physiological adaptations can cause death of cells. It is of significant importance during development of organisms, and it occurs in the context of stressors such as toxicology, oxygen deprivation or infection, as a result of irreversible injury. A concept of cell death was developed in the 19th century together with cell theory; and during the 20th century, cell death was characterized morphologically (reviewed by Majno and Joris, 1995).

Associated with cell death, alterations of subcellular organelles, as well as morphological changes appear in cells or tissues subjected to injury. Subcellular alterations include loss of cellular differentiation (cell junctions, pseudopodia), swelling and disruption of mitochondria, formation of membrane blebs, and deviation in cytoplasm density.

In histology, dead cells show eosinophilia and have a more glassy appearance, the basophilia of nuclear chromatin may fade (karyolysis), or nuclear shrinkage and increased basophilia occurs (pyknosis). Pyknotic nuclei may fragment, a pattern, which is called karyorrhexis. In fishes, these post-mortem changes were noted, for instances in hepatocytes (Braunbeck *et al.*, 1992), splenocytes (Spazier *et al.*, 1992), or branchial pillar cells (Speare *et al.*, 1999) in response to infection or exposure to various chemical compounds.

Once masses of necrotic cells occur, further distinctive morphological patterns are recognized, depending on whether enzymatic digestion of the cell or protein denaturation predominates. In processes that are dominated by protein denaturation, the basic outline of the dead cell is preserved, the cells appear acidophilic, coagulated. This 'coagulative necrosis' is characteristic of hypoxic cell death in various tissues. This occurs, when blood supply to a tissue area is lost (ischaemia), for instance in the context of fish tubercles or renal infection with amoebae (Lom and Dykova, 1992, Timur *et al.*, 1977).

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When enzymatic digestion of the dead cells predominates, colliquative or liquefactive necrosis is the result. This often is seen in context of bacterial or fungal infections in fish, for instance in infections with *Vibrio anguillarum* (Ransom *et al.*, 1984). These morphological changes are results of processes, which follow cell death and have to be regarded as post-mortem changes, but do not refer to physiological processes which resulted in cell death (for review see Majno and Joris, 1995; Sastry and Rao, 2000). A synthesis about cell physiological and biochemical processes resulting in cell death, however, was achieved only very recently. In this chapter major physiological events that determine the route to and how they lead to cell death are summarized. In addition, implications of cell death for local immune responses are discussed. Structural changes, which follow cell death are well documented in fish under pathogen infection. Biochemical or physiological processes resulting in cell death and its significance for pathological conditions in the context of fish diseases still needs considerable attention.

2 Cell death: necrosis or apoptosis?

In many occasions, cell death is associated with an orderly disassembly of cell structures, orchestrated by the action of biochemical pathways and associated with several distinct biochemical, molecular and structural characteristics. This process is called 'apoptosis' (Kerr *et al.*, 1972). Morphologically apoptosis is associated with shrinkage of cell volume, chromatin condensation, DNA fragmentation, and membrane blebbing. Cells undergoing 'shrinkage necrosis', as this process was labelled by Kerr, kept a physiologically intact cell membrane. As this process is mediated by proteins with no other known functions, apoptosis was regarded as active. It was postulated, that cells have the ability to self-destruct by the activation of an intrinsic, genetically encoded cellular suicide program, which becomes active when the cell is no longer needed or seriously damaged. This idea is now accepted and the term programmed cell death (PCD) has been coined (McConkey and Orrenius, 1994) for a cell death, that is mediated by a built-in suicide program. It is activated by a wide variety of stimuli (reviewed by Sastry and Rao, 2000) and involves a cascade of cysteine proteases (cleaving after particular aspartate residues) and proteins of the Bcl family (reviewed by Fiers *et al.*, 1999; McConkey, 1998). Although observed under some pathological conditions, apoptosis is now recognized as a normal feature in development and ageing and thus is regarded as the physiological form of cell death (Nicotera, 2002; Sastry and Rao, 2000).

In contrast to this, accidental cell injury is considered to induce cell death by a passive, loss-of-function phenomenon, for which the term necrosis is retained (Leist and Nicotera, 1997). It is considered to result from primary energy depletion (Leist and Nicotera, 1997), which leads to failure of ion pumps and other ATP-driven processes, loss of ion homeostasis, membrane blebbing, and finally cell lysis occurs (Okada *et al.*, 2001). Proteins known to be involved in necrosis also participate in other physiological functions such as volume regulation or energy metabolism. Therefore, necrosis is regarded as accidental and non-specific (Leist and Nicotera, 1997).

There is increasing evidence that it is not always possible to distinguish between apoptosis and necrosis. In neuronal cells for instance, oxidative stress induced by a

treatment with glutamate initiates events which lead to a form of cell death distinct from either necrosis or apoptosis (Tan *et al.*, 1998). The inhibition of oxidative phosphorylation induces processes, which abruptly increase the permeability of mitochondrial inner membrane to solutes of high molecular mass, induced necrotic killing of cells, when intracellular ATP levels fell profoundly. When ATP levels were partially maintained, apoptosis followed mitochondrial permeability transition, and in many cells, features of both apoptosis and necrosis occurred together after toxic stresses or death signals (Lemasters *et al.*, 1999). This supports the assumption that classical apoptosis and necrosis represent the extreme ends of a wide range of possible forms of death with morphological and biochemical different parameters (Nicotera *et al.*, 1998). In addition, the primary cause of cell death, such as exposure to reactive oxygen species, nitric oxide, or binding of tumour necrosis factor alpha, not always determines whether apoptosis or necrosis results from the insult. This has been shown in cell death induced, for instance, by oxidative stress (Bonfoco *et al.*, 1995; Dypbukt *et al.*, 1994; Kim *et al.*, 2000), or by activation of specific death receptors (Denecker *et al.*, 2001; Vercammen *et al.*, 1998). Important for the final outcome was the severity of the insult, and among others, the type of the cell and its metabolic status rather than the nature of the insult. Thus apoptosis or necrosis are considered as 'different execution of the same death' (Nicotera *et al.*, 1999). Can some events be identified, which determine that cell death is executed as necrosis?

3 Intracellular ion and energy levels

Necrotic cells death is paralleled by cell swelling (Kroemer *et al.*, 1998), termed necrotic volume increase (NVI). Living cells need to regulate their volume, because the presence of membrane-impermeable polyvalent anion macromolecules within the cytosol causes colloid-osmotic pressure which leads to a cationic leak influx. The volume regulation is coupled to Na^+ pump-mediated mechanisms (Okada *et al.*, 2001). Accidental cell damage or injury induces Na^+ uptake and ATP release due to membrane leakage as well as dissipation of ATP by constrained overworking of the Na^+ pump. The resulting ATP depletion then leads to a reduction of Na^+ pumping and cell swelling. Cell swelling by this mechanism can be induced, for instance by hypoxia or ischaemia (Hoffmann and Simonsen, 1989; Lipton, 1999). In addition, reactive oxygen species (ROS) including superoxide anion, hydroxyl radical or hydrogen peroxide may activate non-selective cation channels (Barros *et al.*, 2001b; Herson and Ashford, 1997) and by this induce Na^+ overload. The significance of Na^+ overload for the induction of necrosis was shown in experiments on hepatocytes exposed to menadione or KCN (Carini *et al.*, 1999). Both substances induced a drop of intracellular ATP concentration. In the presence of sodium in the bathing medium, the cell volume increased and the cells suffered necrosis, while cells in sodium-depleted medium did not swell or lose viability. Sodium was not toxic itself, as cells exposed to ouabian wherein intracellular Na^+ can reach extracellular levels did not lose viability (Orlov *et al.*, 1999). Thus, a combination of Na^+ overload and ATP depletion might be required for the induction of cell necrosis.

Intracellular ATP-deficient conditions, induced by the disruption of mitochondrial respiration, provoked neuronal cell swelling (Patel *et al.*, 1998). Under low intracellular ATP conditions, this cell swelling must persist, because volume-regulating,

volume-sensitive outwardly rectifying (VSOR) anion channels depend on the binding of non-hydrolytic intracellular ATP (Okada, 1997; Okada *et al.*, 2001). In neuronal cells under hypoxia or mitochondrial inhibition, a marked inhibition of Cl^- handling VSOR channels was observed (Patel *et al.*, 1998). It is assumed that ATP depletion or metabolic inhibition induces a persistent cell swelling and finally cell rupture (Barros *et al.*, 2001b; Okada *et al.*, 2001).

In principle, any changes which lead to ATP depletion may be detrimental for the cell and may direct the execution of cell death towards necrosis. Pre-emptying human T-cells of ATP switches demise by apoptotic triggers such as staurosporine or CD95 stimulation from apoptosis to necrosis (Nicotera *et al.*, 1998). In these experiments, the death program was driven towards necrosis when mitochondrial or glycolytic generation of ATP was blocked and re-directed towards apoptosis with repletion of the cytosolic ATP pool with glucose supplementation (Nicotera *et al.*, 1998).

In free-radical-induced necrosis, the mechanism of intracellular energy depletion is not always obvious. It can be explained by metabolic exhaustion by the activity of ion pumps in addition to an inhibition of gycolysis or mitochondrial functions (Leist *et al.*, 1999; Redegeld *et al.*, 1990). Furthermore, excessive DNA damage causes poly (ADP-ribose) polymerase 1 (PARP-1) hyperactivation (reviewed by Chiarugi, 2002), an abundant nuclear enzyme involved in DNA-repair, DNA stability and transcriptional regulation. PARP-1 is activated by DNA strand breaks, and overactivation of PARP after cellular insults can lead to cell death caused by depletion of the enzyme's substrate β-nicotinamide adenine dinucleotide (NAD^+) and ATP. Caspases, in particular caspase-3 and -7, cleave PARP-1 and thus prevent the recruitment of the enzyme to sites of DNA damage (Cohen, 1997), a hallmark of apoptosis. Fibroblasts from PARP-deficient ($PARP^{-/-}$) mice were protected from necrotic cell death and ATP depletion, which was induced in fibroblasts from PARP ($^{+/+}$) mice upon hydrogen peroxide exposition (Ha and Snyder, 1999). In fibroblasts expressing caspase-resistant PARP, enhanced necrosis coupled with depletion of NAD^+ and ATP was induced by treatment with tumour necrosis factor alpha. The PARP inhibitor 3-aminobenzamide prevented the NAD^+ drop and concomitantly inhibited necrosis and elevated apoptosis (Herceg and Wang, 1999). Activation or cleavage of PARP is suggested to function as a molecular switch between apoptotic and necrotic modes of death receptor-induced cell death (Los *et al.*, 2002).

A current concept of cell death induced by free radicals or activation of death receptors assumes that ion homeostasis of cells is challenged by an activation of non-selective cation H channels, and cells experience a sodium influx (see *Figure 1*). In addition, the cell experiences a K^+ efflux. Rising cytosolic concentrations of sodium are met by, increased Na^+/K^+ pumping consuming extra energy, and ATP depletion together with other events such as calpain activation (Wang, 2000) can induce a swelling of the cell until it bursts (Barros *et al.*, 2001a). If the ATP depletion is less severe, Na^+/K^+ pumping maintains the cytosolic level of sodium low, the efflux of potassium would prevail and together with mitochondrial changes such as cytochrome c release (Büki *et al.*, 2000) the cell will be directed towards apoptosis. This concept of physiological events leading to cell death explains why frequently a combination of apoptosis and necrosis is found in the same tissue. Crucial for the execution of death appear to be the severity of the insult and the degree of ATP depletion associated with the insult (Leist *et al.*, 1997).

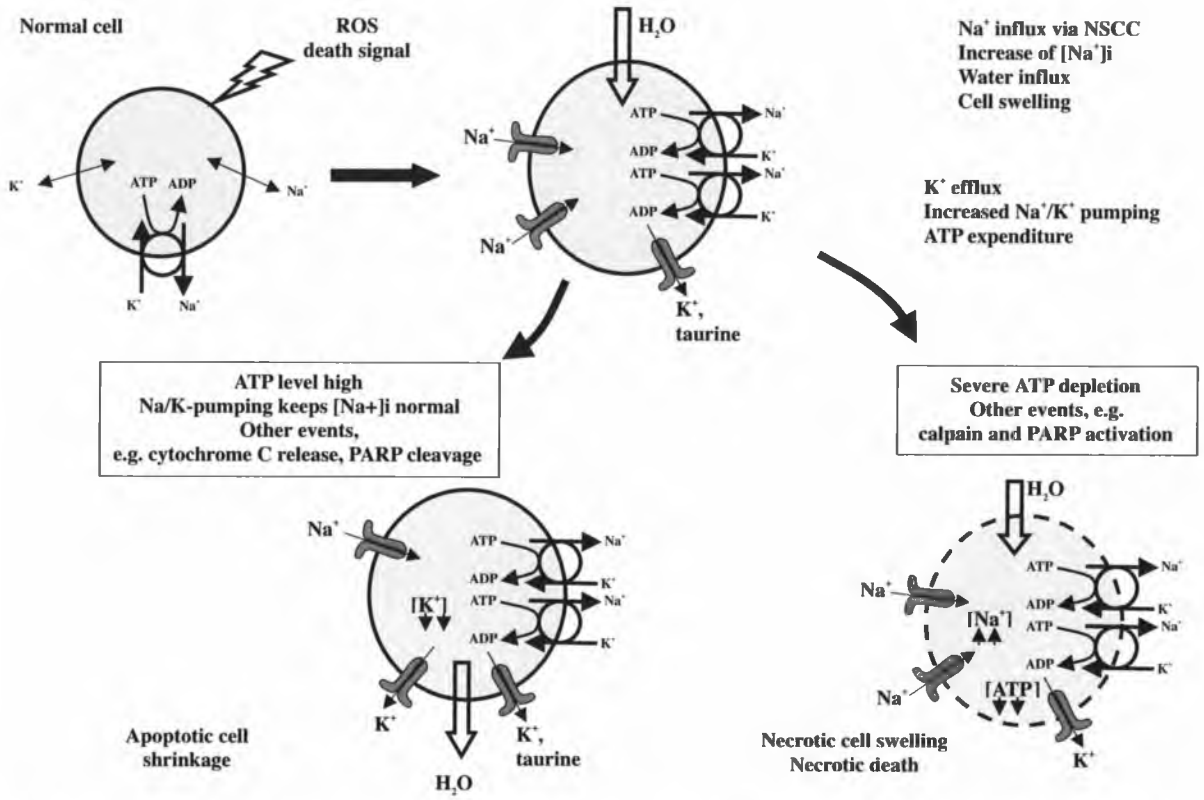

Figure 1 *Schematic summary of physiological mechanisms associated with necrotic versus apoptotic cell death. The physiological status of cells with a pump-leak balance is shown in the upper left. In response to stressors, the ionic homeostasis of cells is challenged, which is met by energy consuming ion pumping mechanisms. The severity of the insult appears to have an influence on energy demand for ion pumping and DNA repair. When energy depletion is severe, cell demise frequently is directed towards necrosis and towards apoptosis, if the depletion is less severe.*

4 Immunological implications

Whether cells die by apoptosis or by necrosis can have profound implications for local immune responses such as tissue inflammation. Apoptosis *in vivo* is followed almost inevitably by the rapid uptake of dead cells into adjacent phagocytic cells. Binding to, or uptake of, apoptotic cells by macrophages inhibited the secretion of inflammatory mediators such as interleukin (IL)-1β, IL-8, IL-10, and tumour necrosis factor alpha (TNF-α) as well as leukotriene C4 and thromboxane B2. In contrast, production of transforming growth factor (TGF)-β1, prostaglandin E2, and platelet-activating factor (PAF) was increased (Fadok *et al.*, 1998, 2001).

Macrophages exposed to necrotized tumour cells (Reiter *et al.*, 1999) or lysed neutrophils stimulated the induction of inflammatory signals such as IL-8, or IL-10, and TNF-α (Fadok *et al.*, 1998; Gough *et al.*, 2001). These pro-inflammatory effects were markedly reduced when serine protease inhibitors were added, or when separated neutrophil membranes only were used (Fadok *et al.*, 2001). Experiments with human monomyelocytes, which do not express phospatidylserine (PS) on the outer leaflet of the plasma membrane during apoptosis showed that suppression of inflammatory mediators and promotion of TGF-β1 secretion appeared to require PS on apoptotic cells (Huynh *et al.*, 2002). The presence of TGF-β1, prostaglandin E2, or PAF appeared to be involved in the suppression of pro-inflammatory cytokine production because exogenous supplementation of these substances inhibited lipopolysaccharide-stimulated cytokine production (Fadok *et al.*, 1998).

Necrosis is pro-inflammatory, while apoptosis is anti-inflammatory. The anti-inflammatory property of apoptotic cells may be instrumental to avoid tissue disruption by the high number of cell deaths, which occur in multicellular organisms during development, or in normal life. In human life, for instance, 100 000 cell deaths occur every second (Vaux and Korsmeyer, 1999). In other words, multicellular organisms with a high cell turn over and high numbers of cell deaths occurring in normal life reduce the risk of inflammation by the inflammation-resolving properties of apoptosis.

In the context of infection or tumour defence, however, inflammation plays an adaptive role. In tumour defence, for instance, inflammation-resolving properties of apoptosis have an unwanted side in the sense of reduced anti-tumoural activity of macrophages exposed to apoptotic cells (Reiter *et al.*, 1999). The ability of *Entamoeba histolytica*, a gut-dwelling and tissue-invading parasite of man, to kill and phagocytose host cells correlates with parasite virulence. The parasite induces apoptotic killing of host cells, and the subsequent phagocytosis of these cells may limit inflammation and enable amoebas to evade the host immune response (Huston *et al.*, 2003). In such contexts, necrosis would be adaptive, or even more physiological.

5 Detection of necrosis

Detection of necrotic cell death mainly relies on assays which include morphology, plasma membrane integrity, DNA content, and cell surface expression of phosphatidylserine. Morphological features of necrosis include cell swelling and development of translucent cytoplasm, as seen in human monocytes and in cells of a monocytic cell line by light microscopy and flow cytometry (see *Table 1*; Warny and Kelly, 1999). In contrast to this, apoptotic cells show shrinkage and fragmentation

(Sastry and Rao, 2000). In cell cycle analysis, necrotic cells are characterized by random DNA fragmentation compared to patterned DNA cleavage in apoptotic cells (Evans *et al.*, 2000). In addition, the plasma membrane of necrotic cells loses integrity, which can be determined by uptake of indicating dyes, or by leakage of intracellular enzymes. Uptake of chemicals frequently is detected by flow cytometry using propidium iodine (Warny and Kelly, 1999), or by light microscopy using trypane blue (Bonfoco *et al.*, 1995). Enzyme leakage frequently is traced *in vitro* by measuring lactate dehydrogenase activity in the culture medium (Bonfoco *et al.*, 1995; Kim *et al.*, 2000).

Table 1 *Some features of cell undergoing necrotic versus apoptotic cell death*

Necrosis	Apoptosis
Cell swelling	Cell shrinkage
Development of translucent cytoplasms	Fragmentation, membrane blebbing
Chromatin clumping	Chromatin condensation
Organellar swelling	
Loss of membrane integrity	Intact cell membrane
Leakage of intracellular enzymes	Cell surface expression of phosphatidyl serine
Random DNA fragmentation	Interchromosomal patterned DNA cleavage

6 Necrotic cell death in the context of fish diseases

In fish, morphological features of necrosis frequently are associated with infections with pathogens of viral, bacterial or parasitic origin. In viral diseases, affected organs are, for instance, nervous and retinal tissues in nodavirus infections of marine finfish (Munday and Nakai, 1997), liver and renal tubules in halibut (*Hippoglossus hippoglossus*) infected with an aquareovirus (Cusack *et al.*, 2001), haematopoietic tissues in the kidney in rainbow trout (*Oncorhynchus mykiss*) infected with the epizootic haematopoietic necrosis virus (EHNV; Reddacliff and Whittington, 1996), or pancreatic tissues in rainbow trout infected with the infectious pancreatic necrosis virus (IPNV; Hong *et al.*, 1998). In IPNV, cells from a fish cell line initially displayed structural and biochemical indications of apoptotic demise (Hong *et al.*, 1998), but at a later stage of the process leading to cell death, pores were formed in the outer cell membrane (Hong *et al.*, 1999). By this, the cell membrane of infected cells lost their integrity, and cellular compounds were leaking into the environment. Membrane leakage was considered to be a core event in necrotic cell death, mainly involved in the promotion of inflammation, which was found to be associated with this form of cell death.

Accumulations of necrotic cells in various tissues of fish were associated with inflammation. This was mainly noted in histology as an infiltration of granulocytes, macrophages and lymphocytes into tissue areas where cell necrosis had appeared. In

was seen for instance in the liver of medaka *Oryzias latipes* (Braunbeck *et al.*, 1992; Hoft *et al.*, 2003), or spleen of eels (Spazier *et al.*, 1992), as well as in carp infected with the blood fluke *Sanguinicola.* Here, eggs and emigrating miracidia caused necrosis of vascular cells and induced inflammatory infiltration of epithelial tissue, which encapsulated and subsequently eliminated parasite stages by degradation (Kirk and Lewis, 1998). In rainbow trout infected with the blood flagellate *Cryptobia salmositica*, a generalized inflammation with lesions in connective tissue and in the reticuloendothelial system was associated with tissue necrosis in liver and kidney. The severity of this response was related to parasitaemia, and most likely induced by occlusion of blood vessels due to parasites and mononuclear cells (Bahmanrokh and Woo, 2001). In carp infected with *Trypanoplasma borreli*, a parasite closely related to *Cryptobia salmositica*, renal tubule cells experienced mitochondrial damage and subsequent necrosis (Rudat *et al.*, 2000). In infected carp, an extensive proliferation of the lymphoid renal tissue and a congestion of blood vessels with parasites and inflammatory cells occurred (Bunnajirakul *et al.*, 2000). This might have caused changes in renal microcirculation and thus induced hypoxic conditions in renal tubule cells. In addition, this parasite induced a strong activation of phagocytic cells, which resulted in high secretion of nitric oxide and high production of ROS (Saeij *et al.*, 2000, 2002). Production of nitric oxide and ROS in excess could induce oxidative stress to cells and tissues, as suspected by Saeij *et al.* (2003), who found that peripheral blood derived leucocytes were highly susceptible to NO-induced cell death. In addition, rainbow trout phagocytes were suspected to be sensitive to high production of ROS in association with high intracellular Ca^{++} levels (Betoulle *et al.*, 2000). Chemical depletion of glutathione-S-transferase in cells of medaka significantly elevated the induction of necrotic cell death by methyl-nitro-nitrosoguanidine (Kwak *et al.*, 2000).

Taken together, a link between necrotic cell death and inflammation is well established in fish. In local responses, like in infections with *Sanguinicola inermis* (Kirk and Lewis, 1998) or with gut-dwelling coccidia (Jendrysek *et al.*, 1994 Landsberg and Paperna, 1987) inflammation induced by necrotic cells helps eliminating the parasite. Responses which are associated with ischaemic conditions in vital organs, such as liver or kidney, appear to promote oxidative stress to cells and tissues, which in consequence may result in progressive cell death by necrotic pathways. The significance of inflammation for the induction of necrotic cell death, and the significance of necrosis for pathological conditions, however, has hardly been addressed in the context of fish diseases and still needs considerable attention.

7 Summary and perspective

In the context of infectious diseases of fish, cell death is described as part of pathology on the basis of structural alterations in cells and tissues. Morphological changes, which can be observed by microscopy, however, are results of processes that follow cell death and thus have to be regarded as post-mortem changes. In mammalian cells, stressors such as oxygen deprivation (hypoxia), or oxidative damage due to the production of ROS or NO most frequently were found to induce cell injury and subsequently cell death. Oxidative damage has the capacity to trigger both main pathways of cell death, necrosis and apoptosis. While high levels of oxidative damage can cause necrosis, lesser degrees of oxidative stress induce cell death via the apoptotic cascade. Necrotic cell demise was found to be

associated with energy depletion, cell swelling, membrane rupture and release of intracellular enzymes. Immunological consequences of necrotic cell death were a pronounced induction of pro-inflammatory effects such as the release of IL-8, IL-10 or TNF-α by macrophages or neutrophils exposed to necrotized cells.

High levels of reactive oxygen species were also released from inflammatory cells such as neutrophils and macrophages in fish infected with various infections agents and induced oxidative stress to cells or tissues (Saeij *et al.*, 2003). Physiological responses of fish cells confronted with reactive oxygen species are not studied in detail. It remains unclear whether cells die or can repair the damage resulting from the insult. Can a high-level release of nitric oxide induce necrotic cell death, which then could be responsible for functional deficiencies, or does it help to clear the pathogen from the tissue? Studies on the induction of cell death and on immunological implication of pathogen-induced demise of cells are completely lacking for fish, but would be highly needed for an understanding of pathogen–host interactions on the physiological level. Here, a wide field is open for future work.

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References

Bahmanrokh, M. and Woo, P.T.K. (2001) Relations between histopathology and parasitaemias in *Oncorhynchus mykiss* infected with *Cryptobia salmositica*, a pathogenic haemoflagellate. *Dis. Aquat. Org.* **46:** 41–45.

Barros, L.F., Hermonsilla, T. and Castro, J. (2001a) Necrotic volume increase and the early physiology of necrosis. *Comp. Biochem. Physiol. A* **130:** 401–409.

Barros, L.F., Stutzin, A., Calixto, A., Catalan, M., Castro, J., Hetz, C. and Hermonsilla, T. (2001b) Non-selective cation channels as effectors of free radical-induced rat liver cell necrosis. *Hepatology* **33:** 114–122.

Betoulle, S., Duchiron, C. and Deschaux, P. (2000) Lindane increases in vitro respiratory burst and intracellular calcium levels in rainbow trout (*Oncorhychus mykiss*) head kidney phagocytes. *Aquatic Toxicol.* **48:** 211–221.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton, S.A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methly-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl Acad. Sci.* **92:** 7162–7166.

Braunbeck, T.A., The, S.J., Lester, S.M. and Hinton, D.E. (1992) Ultrastructural alterations in liver of medaka (*Oryzias latipes*) exposed to diethylnitrosamine. *Toxicol. Pathol.* 20: 179–196.

Büki, A., Okonkwo, D.O., Wang, K.K. and Povlishok, J.T. (2000) Cytochrome c release and caspase activation in traumatic axonal injury. *J. Neurosci.* 20: 2825–2834.

Bunnajirakul, S., Steinhagen, D., Hetzel, U., Körting, W. and Drommer, W. (2000) A study of sequential histopathology of *Trypanoplasma borreli* (Protozoa: Kinetoplastida) in susceptible common carp *Cyprinus carpio*. *Dis. Aquat. Org.* **39:** 221–229.

Carini, R., Autelli, R., Bellomo, G. and Albano, E. (1999) Alterations of cell volume regulation in the development of hepatocyte necrosis. *Exp. Cell Res.* **248:** 280–293.

Chirarugi, A. (2002) Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited. *Trends Pharmocol. Sci.* **23:** 122–129.

Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.* **326:** 1–16.

Cusack, R.R., Groman, D.B., MacKinnon, A.M., Kibenge, F.S., Wadowska, D. and Brown, N. (2001) Pathology associated with an aquareovirus in captive juvenile Atlantic halibut *Hippoglossus hippoglossus* and an experimental treatment strategy for a concurrent bacterial infection. *Dis. Aquat. Org.* **44:** 7–16.

Denecker, G., Vercammen, D., Steemans, M., *et al.* (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. *Cell Death Differ.* **8:** 828–840.

Dypbukt, J.M., Ankarcrona, M., Burkitt, M., Sjöholm, A., Ström, K., Orrenius, S. and Nicotera, P. (1994) Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *J. Biol. Chem.* **269:** 30553–30560.

Evans, D.L., Bishop, R. and Jaso-Friedmann, L. (2000) Methods for cell cycle analysis and detection of apoptosis of teleost cells. *Methods Cell Sci.* **22:** 225–231.

Fadok, V.A.; Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y. and Henson, P.M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE_2, and PAF. *J Clin Invest* **101:** 890-898

Fadok, V.A., Bratton, D.L., Guthrie, L. and Henson, P.M. (2001) Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role for proteases. *J Immunol* **166:** 6847-9854

Fiers, W., Beyaert, R., Declercq, W. and Vandenabeele, P. (1999) More than one way to die: apoptosis and reactive oxygen damage. *Oncogene* **18:** 7719–7730.

Gough, M.J., Melcher, A.A., Ahmed, A., Crittenden, M.R., Riddle, D.S., Linardakis, E., Ruchath, A.N., Emiliusen, L.M. and Vile, R.G. (2001) Macrophages orchestrate the immune response to tumour cell death. *Cancer Research* **61:** 7240-7247

Ha, H.C. and Snyder, S.H. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc. Natl. Acad. Sci.* **96:** 13978-13982

Hamm, J.T., Wilson, B.W. and Hinton, D.E. (1998) Organophosphate-induced acetylcholinesterase inhibition and embryonic retinal cell necrosis in vivo in the teleost (*Orzyias latipes*). *Neurotoxicology* **19:** 853–869.

Herceg, Z. and Wang, Z.Q. (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspase leads to induction of necrosis and enhanced apoptosis. *Mol Cell Biol* **19:** 5124-5133

Herson, P.S. and Ashford, M.L.J. (1997) Activation of a novel non-selective cation channel by alloxan and H_2O_2 in rat insulin-secreting cell line CRI-G1. *J. Physiol.* **501:** 59–66.

Hofmann, E.K. and Simonsen, L.O. (1989) Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69:** 315–382.

Hoft, P.T., van Dongen, W., Esmans, E.L., Blust, R. and de Coen, W.M. (2003) Evaluation of the toxicological effects of perfluorooctane sulfunic acid in the common carp (*Cyprinus carpio*). *Aquat. Toxicol.* **62:** 349–359.

Hong, J.R., Lin, T.L., Hsu, Y.L. and Wu, J.L. (1998) Apoptosis precedes necrosis of fish cell line with infectious pancreas necrosis virus infection. *Virology* **250:** 76–84.

Hong, J.R., Lin, T.L., Hsu, Y.L. and Wu, J.L. (1999) Dynamics of nontypical apoptotic morphological changes visualized by green fluorescent protein in living cells with infectious pancreatic virus infection. *J. Virol.* **73:** 5056–5063.

Huston, C.D., Boettner, D.R., Miller-Sims, V. and Petri, W.A. Jr. (2003) Apoptotic killing and phagocytosis of host cells by the parasite *Entamaeba histolytica*. *Infect. Immun.* **71:** 964–972.

Huynh, M.L., Fadok, V. and Henson, P.M. (2002) Phospatidylserine-dependent ingestion of apoptotic cells promotes TGF beta secretion and the resolution of inflammation. *J Clin Invest* **109:** 41-50

Jendrysek, S., Steinhagen, D., Drommer, W. and Körting, W. (1994) Carp coccidiosis: intestinal histo- and cytopathology under *Goussia carpelli* infection. *Dis. Aquat. Org.* **20:** 171–182.

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26:** 239–257.

Kim, Y.M., Chung, H.A.T., Simmons, R.L. and Billar, T.R. (2000) Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. *J. Biol. Chem.* **275:** 10954–10961.

Kirk, R.S. and Lewis, J.W. (1998) Histopathology of *Sanguinicola inermis* infection in carp, *Cyprinus carpio*. *J. Helminthol.* **72:** 33–38.

Kroemer, G., Dellaporta, B. and Resche-Rigon, M. (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Ann. Rev. Physiol.* **60:** 619–642.

Kwak, H.I., Lee, M.H. and Cho, M.H. (2000) Interrelationship of apoptosis, mutation, and cell proliferation in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)- induced medaka carcinogenesis model. *Aquatic Toxicol.* **50:** 317–329.

Kwak, H.I., Bae, M.O., Lee, M.H., *et al.* (2001) Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordfish (*Xiphophorus helleri*). *Environ. Toxicol. Chemist* **20:** 787–795.

Landsberg, J.H. and Paperna, I. (1987) Intestinal infections by *Eimeria* (s.l.) *vanasi* n. sp. (Eimeriidae, Apicomplexa, Protozoa) in chichlid fish. *Ann. Parasitol. Hum. Comp.* **62:** 283–293.

Leist, M., Single, B., Castoldi, A.F., Kühnle, S. and Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.* **185:** 1481–1486.

Leist, M., Single, B., Naumann, H., Fava, E., Simon, B., Kuhnle, S. and Nicotera, P. (1999) Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. *Exp. Cell Res.* **249:** 396–403.

Leist, M. and Nicotera, P. (1997) The shape of cell death. *Biochem. Biophy. Res. Commun.* **236:** 1–9.

Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, A.L. and Herman, B. (1999) Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J. Bioenerg. Biomembr* **31:** 305–319.

Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol. Rev.* **79:** 1431–1568.

Lom, J. and Dykova, I. (1992) Protozoan parasites of fish. *Develop. Aqua. Fish. Sci.* **26:** 315.

Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, Z.Q. and Schulze-Osthoff, K. (2002) Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signalling. *Mol. Biol. Cell.* **13:** 978–988.

Majno, G. and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* **146:** 3–15.

McConkey, D.J. (1998) Biochemical determinants of apoptosis and necrosis. *Toxicol. Lett.* **99:** 157–160.

McConkey, D.J. and Orrenius, S. (1994) Calcium and cyclosporin A in the regulation of apoptosis. *Curr. Top. Microbiol. Immunol.* **200:** 95–105.

Munday, B.L. and Nakai, T. (1997) Nodavirus as pathogens in larval and juvenile marine finfish. *World J. Microbiol. Biotechnol.* **13:** 375–381.

Nicotera, P. (2002) Apoptosis and age related disorders: role of caspase dependent and caspase-independent pathways. *Toxicol. Lett.* **127:** 189–195.

Nicotera, P., Leist, M. and Ferrando-May, E. (1998) Intracelluar ATP, a switch in the decision between apoptosis and necrosis. *Toxicol. Lett.* **102–103:** 139–142.

Nicotera, P., Leist, M. and Ferrando-May, E. (1999) Apoptosis and necrosis: different execution of the same death. *Biochem. Soc. Symp.* **66:** 69–73.