

## REVIEW

**Apoptosis in molluscan immune defense****IM Sokolova***Department of Biology, University of North Carolina at Charlotte, Charlotte NC, USA**Accepted April 20, 2009***Abstract**

Apoptosis, or Type I programmed cell death, is a fundamental biological process involved in cellular homeostasis in metazoans. Apoptosis plays a key role in immune system homeostasis and function, defense against parasite and pathogens and self/non-self recognition. In this review, I present our current knowledge of the mechanisms and signaling pathways underlying apoptosis in mollusks and its roles in host-pathogen interactions and immune defense. Both signaling and execution pathways of apoptosis appear to be highly conserved in mollusks, although there is evidence that some apoptotic mechanisms (e.g., caspase-independent cell death) may differ from model invertebrates such as *Caenorhabditis* and *Drosophila* and more closely resemble those seen in vertebrates. Apoptosis is important for the functioning of the molluscan immune system as indicated by the high baseline apoptosis rates observed in circulating and resident hemocytes. Apoptosis also plays a role in host protection against parasites by limiting the spread of the pathogen while preventing inflammatory damage of surrounding tissues. In molluscs, interaction between immune cells and parasites or pathogens usually triggers apoptosis; however, some pathogens (especially obligatory intracellular parasites that depend on the host cell for their survival and proliferation) can inhibit this response and prevent host cell death. Currently, the mechanisms underlying pathogen-induced modulation of apoptosis in molluscs are not well understood. Summarizing our current knowledge of the immune functions of apoptosis in molluscs, and comparing them with the more widely studied vertebrate systems, this review will delineate the gaps in our understanding of this critical cellular process in molluscs and assist in the ongoing search for evolutionary novel mechanisms of apoptosis in immune defense and self/non-self-recognition.

**Key Words:** Immune response; apoptosis; parasites; pathogens; Mollusca**Introduction**

Molluscs, the second most diverse group of animals (next only to arthropods) with about 93,000 extant species, are abundant in most marine, brackish, freshwater and terrestrial habitats. Molluscs play vital roles in these ecosystems and are often used as bioindicators of the ecosystem health. In some ecosystems (most notably in marine and estuarine habitats), molluscs can function as ecosystem engineers creating and modifying habitats for other species and affecting the habitat quality by eliminating suspended particles or participating in the top-down control of algal blooms. Molluscs have also been used by humans since the dawn of the human history as food resources or to

provide other valuable commodities such as pearls and mother-of-pearl, Tyrian purple from muricid gastropods used to dye royal robes, non-opioid pain-killers derived from cone snails' toxins and anti-cancer drugs from some gastropod peptides (McFadden *et al.*, 2003; Faircloth and Cuevas, 2006; Han *et al.*, 2008). Moreover, molluscs serve as important vectors in the transmission of parasites and pathogens to humans and/or domestic animals. Given the important ecological, economical and medicinal roles of molluscs, understanding the immune defenses of these animals is critical for improving disease resistance and increasing the survival of ecologically and economically important molluscs, and for improving their aquaculture production and/or sanitation to reduce parasite/pathogen transmission. Studies of the molluscan immunity are also important from the viewpoint of evolutionary and comparative immunology. The study of the immune defense of molluscs may reveal immunobiological novelties

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thereby providing insights into the evolution of immunity, and may offer new solutions for the treatment of infectious and parasitic diseases or immunological disorders in humans and domestic animals.

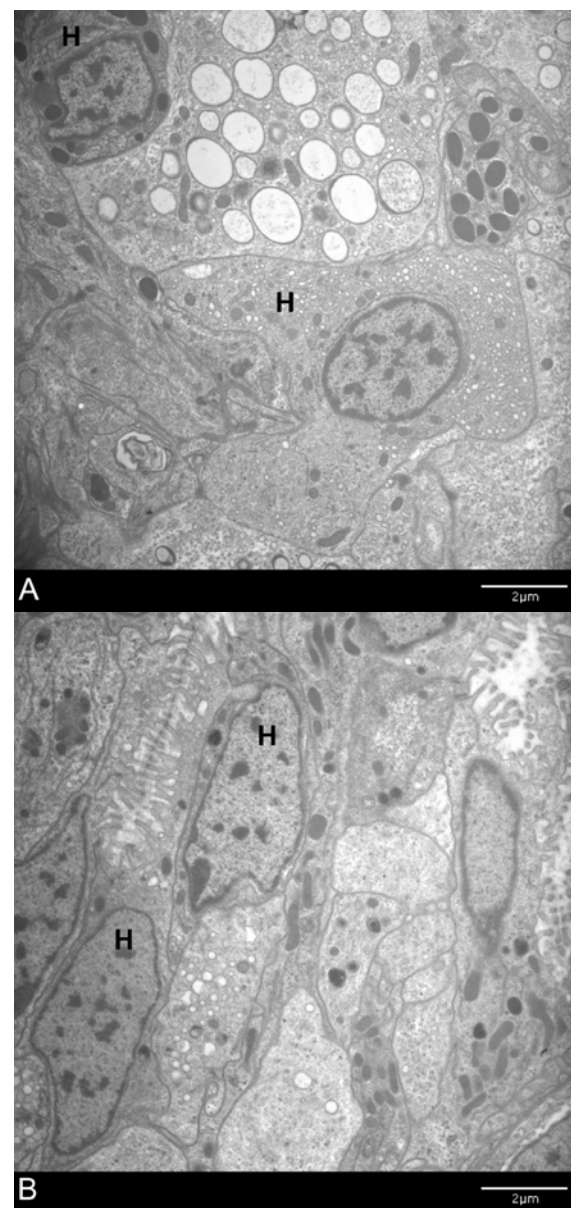
Although molluscan immunity has been actively studied in the past few decades, our knowledge about the cellular and molecular mechanisms of molluscan immune defenses and their interactions with pathogens and parasites is still far from complete. Significant progress has been made in our understanding of the phagocytic and bactericidal responses of molluscan immune cells and of the humoral factors involved in non-self recognition and pathogen killing; due to space limitations, I will only briefly mention these immune mechanisms in this review (see “Overview of the molluscan immunity” below) and further refer the reader to a number of excellent reviews focusing on these topics (see Anderson, 1996; Kennedy *et al.*, 1996; Gliński and Jarosz, 1997; Canesi *et al.*, 2002; Paul, 2003; Tiscar and Mosca, 2004; Vasta and Ahmed, 2008 and references therein). The main goal of the present review is to draw attention of invertebrate immunologists to an important but currently understudied aspect of molluscan immunity – namely, the role of apoptosis, or Type I programmed cell death (PCD I), in molluscan immune defense and host-pathogen interactions. Given that the study of molluscan apoptosis is still in its infancy, I will draw on relevant examples from vertebrate literature to illustrate molecular mechanisms and possible immunological roles of apoptosis in protection against parasites, pathogens, and tumors in mollusks. It is my hope that by providing a comprehensive overview of our current knowledge of molluscan apoptosis, this review will provide a roadmap for future studies to understand this critical cellular process in mollusks and to potentially reveal novel mechanisms and roles of apoptosis in the immune defense and self/non-self-recognition in animals.

### Overview of molluscan immunity

The immune system of molluscs, as all other invertebrates, consists only of innate immunity and is lacking the adaptive immunity components. While the innate immune system is often regarded as an evolutionary more ancient and hence more primitive form of immunity than the adaptive responses seen in vertebrates, it is actually a surprisingly complex and efficient form of protection against many parasites and pathogens encountered by molluscs. Indeed, successful adaptive radiation of molluscs and their ability to colonize a broad range of aquatic and terrestrial habitats clearly speaks to their ability to efficiently combat infections and parasitic invasions.

External barriers (such as shells, mucus and epithelia) of mollusks constitute the first line of defense against pathogens and parasites; when these barriers are breached, the second, internal line of defense involving cellular and soluble (humoral) hemolymph components come into play. Humoral components of the molluscan immune defense include lysosomal enzymes (such as  $\beta$ -

glucuronidase, acid and alkaline phosphatase, lipase, aminopeptidase and lysozyme), lectins including agglutinins, fibrinogen-related proteins (FREPS), and C-type lectins, and antimicrobial peptides that aid in recognition of pathogens and parasites by marking them for destruction via opsonizing or directly killing (review in Anderson, 1996; Kennedy *et al.*, 1996; Canesi *et al.*, 2002; Paul, 2003; Tiscar and Mosca, 2004; Vasta and Ahmed, 2008). Agglutinins, FREPS and other lectins along with pattern recognition receptors (PRRs, which in mollusks include Toll-like receptors, IMD pathway receptors and intracellular nucleotide-oligomerization domain (NOD) proteins) are responsible for the surprising degree of specificity shown by the innate immune system for pathogens and parasites (Paul, 2003; Yeretssian *et al.*, 2008).



**Fig. 1** Transmission electron micrograph (TEM) of resident hemocytes in gills (A) and hepatopancreas (B) of oysters *C. virginica*. Hemocytes, H.

While humoral immunity is very important for host defense, it is indisputable that the cellular components of the hemolymph, hemocytes, play a central role in the innate immune responses of mollusks (Fig. 1). Hemocytes are the main effector components of the molluscan immune system and are responsible for phagocytosis of parasites, pathogens, and foreign particles (which represents a complex process including recognition, adhesion, ingestion, destruction or encapsulation and final elimination of foreign cells or materials). Hemocytes are also directly engaged in pathogen and parasite killing via the oxidative burst reaction - a rapid generation of toxic reactive oxygen species (ROS) by a membrane bound enzyme NADH-oxidase. ROS (especially hydrogen peroxide) can be further converted by hemocyte myeloperoxidase to hypochloric acid (HOCl) that has strong bactericidal and viricidal properties (Tiscar and Mosca, 2004).

Molluscan hemocytes are comprised of two main types: hyalinocytes - small cells with cytoplasm containing few or no granules, and granulocytes - large phagocytic cells containing abundant numbers of cytoplasmic granules (Kennedy *et al.*, 1996; Tiscar and Mosca, 2004). These two major cell types are further classified into subtypes based on their size, morphology and (to the degree that it is known) function (e.g., acidophilic, basophilic and neutrophilic granulocytes, natural killer-like cells, etc.) (Kennedy *et al.*, 1996; Takahashi and Mori, 2000). Overall, molluscan granulocytes strongly resemble vertebrate monocytes and macrophages both in structure and function and share with the latter such key properties as avid phagocytosis, pathogen-induced oxidative burst, production and release of nitric oxide and lysosomal enzymes (Canesi *et al.*, 2002). Recent studies have revealed subpopulations of molluscan hemocytes that possess lymphocyte-like morphology and physiological properties similar to mammalian natural killer (NK) cells (Monti *et al.*, 1992).

### **Molecular mechanisms of apoptosis in molluscs**

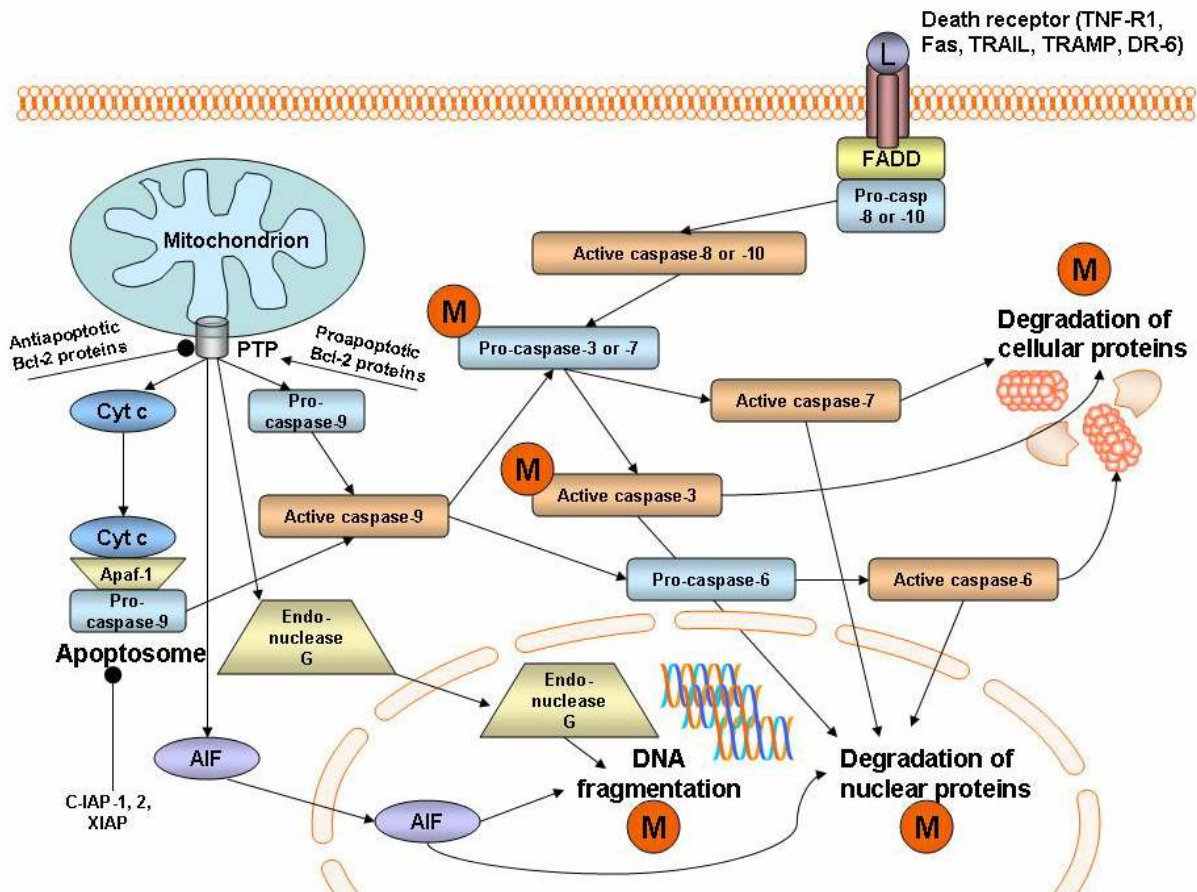
The term "apoptosis" was coined by Kerr, Wyllie and Curie in their seminal 1972 paper to describe a distinctive form of programmed cell death (PCD) defined by characteristic morphological features such as chromatin condensation, membrane blebbing and cell shrinkage (Kerr *et al.*, 1972 cited after Feig and Peter, 2007). Since then, the study of apoptosis using both vertebrate and invertebrate model systems has led to the recognition that this is a multifunctional process, highly conserved evolutionarily, and plays a critical role in cellular and tissue homeostasis, embryonic development, and immune defense in multicellular organisms. Unlike necrosis that may be considered as "accidental" cell death in response to major mechanical or chemical injuries, apoptosis is a highly orchestrated cellular process in which intracellular components are sequestered and disposed of in an orderly manner without the induction of inflammation.

Mechanisms of apoptosis have been based predominantly on the studies of vertebrates and invertebrate models such as *Caenorhabditis* and *Drosophila*. While these findings have been

extensively reviewed previously (see reviews in Hengartner, 1996, 2000; Vermeulen *et al.*, 2005; Circu and Aw, 2008; Ow *et al.*, 2008; Suen *et al.*, 2008 and references therein) and their detailed analysis is beyond the scope of the present review, a brief account of the major apoptotic pathways is in order to set the stage for a discussion of the mechanisms underlying molluscan apoptosis. Briefly, the apoptotic cell death can be triggered through two major pathways - intrinsic (or mitochondrial), in response to internal cellular damage and extrinsic, in response to death clues received from the environment (Fig. 2). Although both pathways can function independently, there is substantial cross-talk that allows for amplification of the death signal (Feig and Peter, 2007). A key characteristic of the majority of apoptotic pathways is the involvement of a family of proteases called caspases that cleave target proteins at specific sites typically containing aspartic acid residues followed by a caspase-specific three amino acid sequence (Creagh *et al.*, 2003). Caspases involved in apoptosis include initiator caspases (e.g., caspases 2, 8, 9 and 10) that cleave and activate the effector caspases (e.g., caspase 3, 6 and 7), which in turn act upon intracellular targets ranging from cytoskeleton proteins to specific cell structures such as mitochondria, nuclear lamina or chromatin (Hengartner 2000; Creagh *et al.*, 2003). Both extrinsic and intrinsic apoptotic pathways converge on the activation of caspases although it should be noted that caspase-independent apoptosis can also occur.

In vertebrates, a hallmark of the intrinsic apoptotic pathway is a release of cytochrome c from mitochondria that is usually accompanied by the formation of a large-molecular size pore in the mitochondrial membrane (the mitochondrial permeability transition (MPT) pore) and collapse of the mitochondrial membrane potential (Mignotte and Vayssiere, 1998). Factors that bring about activation of apoptosis via the mitochondrial pathway include DNA damage, oxidative damage of mitochondrial or cytosolic proteins and lipids, viral stimulation and/or removal of pro-mitogenic signals (growth factor withdrawal) (Circu and Aw, 2008). Once in the cytoplasm, cytochrome c interacts with apoptotic peptidase activating factor 1 (Apaf-1), procaspase-9 and dATP to form a complex called the apoptosome that activates the initiator caspase 9, which in turn activates the effector molecule, caspase 3. Caspase 3 activation causes degradation of proteins, DNA and other cellular components culminating in cell death (Zimmermann *et al.*, 2001). Mitochondrial permeability transition and cytochrome c release are tightly regulated by the Bcl-2 family of proteins that includes both pro- or anti-apoptotic members (Circu and Aw, 2008).

The extrinsic pathway of apoptosis is initiated via binding of specific protein ligands to so called death receptors on the cell surface. In vertebrates, six major types of death receptors have been described including Fas, TRAIL (TNF-related apoptosis inducing ligand) receptor -1 and -2, tumor necrosis factor (TNF) receptor-1, TNF receptor-related apoptosis-mediating protein (TRAMP), and death receptor-6 (DR6). Activation of death receptors



**Fig. 2** Schematic representation of major apoptotic signaling and execution pathways. Extrinsic (death receptor-activated) pathways are shown on the right, and intrinsic (mitochondrial) including caspase-dependent and caspase-independent pathways are shown on the left of the diagram. Note considerable cross-talk between the pathways. Arrows indicate activation, and lines with black dots - inhibition. Red circles with the letter "M" indicate parts of the pathways that have been demonstrated in molluscs. Abbreviations: permeability transition pore, PTP; cytochrome c, Cyt c; procaspase, Pro-casp; Fas-associated death domain, FADD; Apoptotic peptidase activating factor 1, Apaf-1; apoptosis inducing factor, AIF; inhibitor of apoptosis family of proteins, IAP.

causes rapid formation of a death-inducing signaling complex (DISC) that initiates a cascade of caspase activation (notably initiated by the activation of caspase 8) leading to induction of apoptosis (Zimmermann *et al.*, 2001; Schultz and Harrington, 2003). Homologues of death receptors and FasL have been also found in the genome of an ascidian *Ciona intestinalis* (Dehal *et al.*, 2002; Terajima *et al.*, 2003). Although previous studies have isolated several proteins containing death domains from other invertebrates, there are no unequivocal death receptor orthologs reported in *Drosophila*, *C. elegans* or *Porifera* genomes despite concerted efforts to find them (Muzio, 1998; Bridgham *et al.*, 2003). This suggests that death receptor-mediated apoptosis may have evolved within the chordate lineage and may not be functional in non-chordate invertebrates.

Despite the central role of caspases in the intrinsic and extrinsic apoptotic pathways, caspase activation is not an absolute requirement for the

induction of programmed cell death. The most prominent caspase-independent death effector molecules are apoptosis-inducing factor (AIF), a phylogenetically conserved mitochondrial flavoprotein, and endonuclease G, a mitochondrion-specific nuclease (van Loo *et al.*, 2001; Li *et al.*, 2001; Cande *et al.*, 2002; Tait and Green, 2008). Endonuclease G and AIF are released from mitochondria upon a death signal and translocate directly into the nucleus where they bind to DNA and induce caspase-independent chromatin condensation and DNA fragmentation leading to cell death (van Loo *et al.*, 2001; Li *et al.*, 2001; Cande *et al.*, 2002). Some non-caspase proteases such as cathepsins, calpains and serine proteases like granzyme A/B and Omi/Htr A can also trigger caspase-independent apoptosis (Vermeulen *et al.*, 2005). Overall, the considerable redundancy (i.e. potential triggering of the multiple apoptotic pathways by a single stimulus), cross-talk, and mutual amplification between caspase-dependent

and -independent pathways of apoptosis stresses the critical importance of this cellular process in metazoans; it appears that once the death signal has been received, apoptosis proceeds at all costs to eliminate the damaged, malignant, infected or otherwise dangerous cells.

Programmed cell death with all the characteristic hallmarks of apoptosis including cell shrinkage and blebbing, chromatin condensation, DNA fragmentation and translocation of a phospholipid phosphatidylserine into the outer leaflet of the cell membrane, have been described in a variety of mollusks (Sunila and LaBanca, 2003; Sokolova *et al.*, 2004; Buckland-Nicks and Tompkins, 2005). Moreover, many of the molecular components of apoptotic cellular machinery have also been found in mollusks and appear to be highly structurally and functionally conserved. Molluscan cells (including hemocytes) possess caspase 3-like activity (Pirger *et al.*, 2008), and similar to vertebrates, molluscan caspase 3 can be activated by cytochrome c and dATP (Sokolova *et al.*, 2004). Molluscan caspase-3 activity can also be specifically stimulated by staurosporin although, unlike in mammals, the mechanism of this activation in mollusks does not involve the activating cleavage of pro-caspase 3 (Bravarenko *et al.*, 2006).

Recent studies have demonstrated that the signaling pathways involved in the regulation of molluscan apoptosis share significant molecular and functional similarity with those seen in vertebrates. Two central players in cell death and survival, TNF- $\alpha$  and NF- $\kappa$ B transcription factors, appear to play a key role in cell signaling in mollusks. Molluscan TNF- $\alpha$  shares considerable molecular and functional similarity with vertebrate homologs (Terahara and Takahashi, 2008). For example, its expression increases in response to bacterial infections and results in the induction of apoptosis in molluscan hemocytes (Terahara and Takahashi, 2008). In contrast to TNF- $\alpha$ , NF- $\kappa$ B transcription factor typically exhibits pro-survival, anti-apoptotic effects in vertebrates (Aggarwal, 2004; Dey *et al.*, 2008). In mollusks, all the key components of NF- $\kappa$ B signaling cascade have been described and bear considerable similarity to the mammalian NF- $\kappa$ B signaling pathway (Ouwe-Missi-Oukem-Boyer *et al.*, 1994; Montagnani *et al.*, 2004; Zhu and Wu, 2008). However, the exact role of NF- $\kappa$ B in molluscan apoptosis has not been studied and requires further investigation.

Many downstream elements of apoptotic signaling cascades show significant similarity between molluscan and vertebrate cell death. Thus, similar to mammals, mitogen-activated protein kinases and Rho, a member of the Ras GTPase family, are involved in regulation of molluscan apoptosis and exhibit anti-apoptotic effects in molluscan hemocytes (Lacoste *et al.*, 2002). Protein kinase A (but not protein kinase C) also appears to be involved in molluscan hemocyte apoptosis; in the case of noradrenaline-induced apoptosis, inhibition of PKA but not PKC activity attenuated apoptosis levels (Lacoste *et al.*, 2002). Another potential key effector of molluscan apoptosis is cyclic AMP (cAMP); however, its role may be pro- or anti-apoptotic, depending on the circumstances. For

example, in oyster (*Crassostrea gigas*) hemocytes, cAMP appears to promote apoptosis since the adenylate cyclase inhibitor 2', 5'-dideoxyadenosine (DDA) reduces levels of noradrenaline-induced PCD I (Lacoste *et al.*, 2002). In contrast, elevated levels of cAMP induced by pituitary adenylate cyclase activating polypeptide (PACAP) have antiapoptotic effects in terrestrial snails (*Helix pomatia*), significantly attenuating dopamine- and colchicine-induced apoptosis in the salivary gland (Pirger *et al.*, 2008). Further research is needed to determine whether these disparate effects of cAMP are species- or cell type-specific, or whether they are differentially expressed depending on the physiological condition of an organism and/or the nature of the stimulus.

A tumor suppressor p53 homolog also plays an important role in apoptosis signaling in mollusks. In normal mammalian cells, p53 suppresses the formation of tumors by arresting the cell cycle or by apoptosis in response to genotoxic stress-induced DNA damage (Böttger *et al.*, 2008). Apoptosis can be induced by p53 either via translocation into the nucleus where it upregulates transcription of pro-apoptotic genes or by translocation to the mitochondria where it binds to and inactivates Bcl-2 and other antiapoptotic proteins (Böttger *et al.*, 2008). In clams (*Mya arenaria*), p53 protein shares significant sequence and structural similarity to human p53 and localizes to the nucleus in response to apoptotic signals (Holbrook *et al.*, 2009). Overexpression of mortalins (HSP70 family proteins) that bind p53 and prevent its translocation to the nucleus has been shown to inhibit the expression of pro-apoptotic p53-dependent genes and decrease levels of apoptosis in molluscan cells in a similar manner to that seen in vertebrate species (Böttger *et al.*, 2008). There is also evidence that the mitochondrial pathway of p53-dependent apoptosis activation is functional in mollusks (Böttger *et al.*, 2008) but further studies are needed to confirm this suggestion.

Nitric oxide (NO) is another signaling molecule that is involved in the regulation of apoptosis. The effects of NO on apoptosis vary greatly depending upon the dose of NO used, the cell type, and the physiological status of the cell, and can be either pro- or anti-apoptotic (Brune *et al.*, 1999). Molluscan cells including hemocytes contain nitric oxide synthase and produce NO (Terahara and Takahashi, 2008). However, the role of NO in apoptotic regulation has not been extensively studied in mollusks. The only documented study on the effects of NO on molluscan apoptosis shows that inhibition of nitric oxide synthase (NOS) activity during *Ilyanassa obsoleta* larvae metamorphosis induces apical ganglion cell apoptosis (Gifondorwa and Leise, 2006). In molluscan hemocytes, NO production is elevated following exposure to parasites or pathogens, or following exposure to the cytokine, IL-2 (Barsia and Ramos-Martinez, 2008; Terahara and Takahashi, 2008), but it is not known whether this induction has an effect on the level of apoptosis in these cells or if yes, whether it involves a down-stream caspase activation. Overall, the exact mechanisms and roles of NO in mollusc cell apoptosis remain to be fully elucidated.



In contrast to some of the highly conserved elements of the caspase-dependent apoptotic machinery, little is known about the caspase-independent apoptotic pathway in mollusks although its existence has been proposed. This is based on the observation that cadmium (Cd)-induced apoptosis in molluscan hemocytes occurs in the absence of mitochondrial permeability transition or caspase 3 activation (Sokolova *et al.*, 2004). A recent study from our laboratory has also shown that a pan-caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), fails to prevent apoptosis of *Crassostrea virginica* hemocytes following infection with the intracellular parasite *Perkinsus marinus*, suggesting the involvement of caspase-independent pathways (Grewal and Sokolova, unpublished data). In mammalian cells, caspase-independent apoptosis induced by trace metals ( $Mn^{2+}$  and  $Cd^{2+}$ ) or a toxic plant metabolite (allicin) is associated with protein kinase A-dependent increases in the expression and nuclear translocation of AIF in the absence of caspase 3 activation or poly(ADP-ribose) polymerase (PARP) cleavage (Ouabrahim *et al.*, 2001; Shih *et al.*, 2003; Park *et al.*, 2005). The molecular mechanisms underlying caspase-independent apoptosis in molluscs are not known and their determination would be an exciting avenue for future investigations. Notably, a partial gene sequence of an AIF homolog isolated from oysters *C. virginica* has been recently published in Marine Genomics database ([www.marinegenomics.org](http://www.marinegenomics.org), accession # MGID89694) that has significant similarity at the protein level to vertebrate AIFs (55 % amino acid identity,  $E=10^{-74}$ - $10^{-72}$  by BLASTX, Altschul *et al.*, 1997). Studies of caspase-independent PCD I in molluscs hold an especially high promise of novelty because current information from model invertebrates (*Drosophila* and *Caenorhabditis*) suggest that caspase-independent apoptosis does not occur in these organisms (Tait and Green, 2008) raising a possibility that this pathway was either lost to Ecdysozoa, or evolved independently in Lophotrochozoa (including molluscs) and vertebrates ancestors.

Exposure to environmental stressors, such as high temperatures, changes in salinity, and the presence of pollutants has been shown to induce apoptosis in molluscan cells, presumably via the intrinsic pathway. For example, high salinities result in elevated levels of oyster (*C. virginica*) hemocyte apoptosis (Goedken *et al.*, 2005). Similarly, exposure to elevated temperatures (28 °C) stimulate apoptosis in oyster hemocytes whereas moderate temperature changes in the near-optimum range (between 10 and 25 °C) has no effect (Goedken *et al.*, 2005; Cherkasov *et al.*, 2007). Pollutants such as heavy metals, tributyltin (TBT), and PAH, can also induce apoptosis in molluscan immune and non-immune cells, with the degree of induction depending on the dose and time of exposure to the pollutants, and on the exposure mode (*in vitro* or *in vivo*) (Barsiene *et al.*, 2008). Given that most of the apoptosis-inducing environmental stressors described above are also known to induce elevated ROS production and oxidative stress in mollusks (Pruski and Dixon, 2002; Abele *et al.*, 2002; Lannig *et al.*, 2006), it is reasonable to suggest that this

stress-induced apoptotic cell death could be triggered by oxidative damage. However, the precise molecular mechanisms responsible for stress-induced apoptosis in mollusks are presently not known and require further study.

Apoptosis in molluscan cells can also be triggered by activation of surface receptors including hormone receptors and integrins. In oysters (*C. gigas*), activation of integrins (either by specific ligands or by integrin-binding RGD (Arg-Gly-Asp)-containing peptides) promotes apoptosis in a similar manner to that seen in mammalian neutrophils (Terahara and Takahashi, 2008). Notably, unlike mammalian cells where RGD-induced apoptosis is due to anoikis (i.e. prevention of attachment of anchorage-dependent cells to extracellular matrix), the ability of RGD-containing peptide to induce apoptosis in non-adherent, adherent, and spreading molluscan hemocytes alike, indicates that the signaling pathways engaged by integrin activation are independent of hemocyte attachment (Terahara and Takahashi, 2008). Finally, noradrenaline, a catecholamine produced by the neuroendocrine system and by the immune cells of mollusks, also can induce apoptosis of molluscan hemocytes via  $\beta$ -adrenergic signaling pathways (Lacoste *et al.*, 2002).

Overall, current data suggests that the mechanisms underlying apoptosis in mollusks (to the degree that they are known) closely resemble those seen in vertebrates. This includes similarity of the major biochemical and molecular steps of apoptotic pathways, as well as redundancy and flexibility of the apoptotic program that can switch between caspase-dependent and -independent pathways in response to specific environmental or developmental stimuli. Moreover, there is also evidence that some of the apoptotic pathways in mollusks may be sufficiently divergent from other model invertebrates (such as *Drosophila* and *Caenorhabditis*) so as to warrant further study in the hopes that the determination of the specific regulatory and execution pathways of molluscan apoptosis will discover novel mechanisms of PCD and shed light on the evolution of this crucial cellular process in metazoans.

### Apoptosis as an immunomodulatory mechanism

Apoptosis has increasingly become a focus of study for biomedical immunologists with the discovery of the immunomodulatory and defense roles of apoptotic cell death and the recognition that high levels of apoptosis are essential for the normal functioning of immune system (Hildeman *et al.*, 2007; Feig and Peter, 2007; Birge and Ucker, 2008). In molluscs and other non-model invertebrates, studies of the roles of apoptosis in immunity have been hampered by the scarcity of genetic information and available molecular tools. However, the high degree of evolutionary conservation seen in the apoptotic pathways between vertebrates and invertebrates provide both research tools and motivation for the students of molluscan immunity to venture into this exciting new field and to study the role of apoptosis in immune defense and host-pathogen relationships in mollusks.

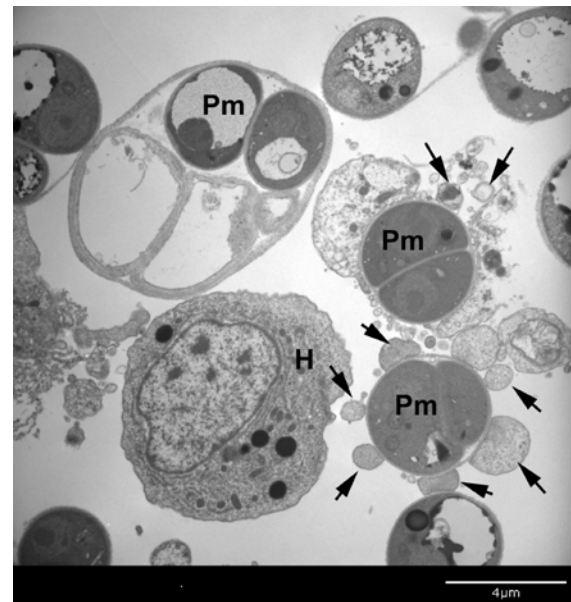
The importance of cell death in the immune defenses of molluscs has long been proposed, even before the discovery of programmed cell death. Thus, in an early paper by Michelson (1963), cell death (which at that time was considered to be exclusively due to necrosis) is cited as an important immune defense mechanism of gastropods against a variety of pathological agents including trematodes, protozoa, and bacteria (cited by Glinski and Jarosz, 1997). The importance of apoptosis in the functioning of the molluscan immune system is reflected by the detection of high baseline apoptosis rates that range from 5 to 25 % in circulating hemocytes and can reach to up to 50 % in infiltrating tissue hemocytes (Sunila and LaBanca, 2003; Sokolova *et al.*, 2004; Goedken *et al.*, 2005; Cherkasov *et al.*, 2007). Typically, granulocytes show higher levels of apoptosis than hyalinocytes possibly due to the higher phagocytic and oxidative respiratory burst activity in granulocytes (Sunila and LaBanca, 2003; Goedken *et al.*, 2005).

Apoptosis of immune cells can play an important role in protection against parasites and pathogens by the innate immune system. Apoptosis is immunologically silent and does not induce inflammation (Birge and Ucker, 2008; Yeretssian *et al.*, 2008). Thus, apoptosis of the infected cells is thought to dampen pathogen spread yet protect the integrity of surrounding tissues by limiting potentially damaging inflammation. Recent studies in vertebrate models has shown that some pathogens, especially those obligate intracellular parasites that depend on survival of the host cells for their persistence, have evolved strategies to inhibit apoptotic cell death (review in Böttger *et al.*, 2008). Inhibition of apoptosis in mammalian cells has been observed following infection with intracellular pathogens including *Leishmania donovani*, *Trypanosoma* spp. and *Theileria* spp. (Luder *et al.*, 2001; Bruchhaus *et al.*, 2007). This parasite-induced inhibition of apoptosis is associated with decreases in caspase-3 activity, inhibition of pro-apoptotic protein kinase C-mediated *c-fos* gene expression, TNF- $\alpha$  induction and/or changes in NF- $\kappa$ B expression in host cells (Nash *et al.*, 1998; Heussler *et al.*, 2001; Goebel *et al.*, 2001; Aga *et al.*, 2002). Bacteria, such as *Chlamidia*, *Pneumococci*, or *Rickettsia*, and viruses can also prevent apoptosis of the infected host cells via interference with NF- $\kappa$ B signaling, caspase activation, or balance of pro- and anti-apoptotic members of Bcl-2 family of proteins (Böttger *et al.*, 2008). Furthermore, pharmaceutical or genetic inhibition of apoptosis renders hosts more susceptible to intracellular pathogens indicating that apoptosis triggered by these pathogens is protective for the host and could play a beneficial role in eliminating the infection (Böttger *et al.*, 2008).

In molluscs, induction of apoptosis upon contact with pathogens or parasites, and parasite-induced inhibition of apoptosis have both been described. For example, hemocytes of the Pacific oyster (*Crassostrea gigas*) show an induction of apoptosis during phagocytosis of live or heat-killed marine bacteria *Planococcus citraeus* (Terahara and Takahashi, 2008). Elevated levels of apoptosis have been associated with the oxidative burst of hemocytes and are abolished by treatment with

antioxidants suggesting that apoptosis may be induced by oxidative damage to hemocytes during bacterial killing (Terahara and Takahashi, 2008). Symbiotic bacteria of some cephalopods (*Vibrio* spp.) can also induce apoptosis in host cells (McFall-Ngai, 1999). Importantly, our earlier studies have also shown an early increase in apoptosis of oyster hemocytes upon infection by the intracellular protozoan parasite, *Perkinsus marinus*, which then returns back basal levels, perhaps due to parasite-induced inhibition of the apoptotic response (Fig. 3; Sokolova, Grewal and Hughes, unpublished data). Apoptosis levels in resident tissue hemocytes of oysters has also been shown to be dramatically reduced from approximately 50 % to 10-11 % in oysters naturally infected by *P. marinus* or another obligate intracellular protozoan parasite, *Haplosporidium nelsoni* (Sunila and LaBanca, 2003). Notably, infection with *P. marinus* results in a significantly faster induction of apoptosis in hemocytes derived from *P. marinus*-resistant Pacific oysters (*C. gigas*) than those from *P. marinus*-susceptible eastern oysters (*C. virginica*) suggesting that faster induction of apoptosis may be an effective defense mechanisms against this intracellular parasite (Goedken *et al.*, 2005).

Overall, it is apparent that while apoptosis induction and the possible subversion of this response by successful parasites and pathogens may play a critical role in both disease resistance and parasite/pathogen development and transfer, our knowledge about the occurrence of, and mechanisms responsible for, apoptosis in infected mollusks is currently limited. Clearly, further studies are urgently needed to determine how specific and wide-spread the apoptotic response to infections is



**Fig. 3** Parasite-induced apoptosis in molluscan hemocytes. Hemocytes of oysters *Crassostrea virginica* were infected with an intracellular pathogen *Perkinsus marinus*, and TEM obtained 30 min post-infection. (hemocyte, H; *P. marinus* cells, Pm). Arrows show apoptotic bodies of dying hemocytes.

in molluscs, and whether it differs in response to bacterial and viral pathogens, and to uni- and multi-cellular eukaryotic parasites (especially *Schistosoma* and other trematodes).

### Summary and future directions

Recent advances in the fields of molluscan immunology and fundamental cell biology have brought about significant breakthroughs in our understanding of the mechanisms underlying apoptotic cell death in molluscan cells and, in particular, their immune cells. These studies reveal a high degree of evolutionary conservation of key signaling and execution pathways of apoptosis and indicate that programmed cell death likely plays a key role in homeostasis and functioning of the molluscan immune system. However, our knowledge about the molecular mechanisms of molluscan apoptosis and its immunomodulatory and immune defense roles is currently far from complete. Further studies are urgently needed to delineate the mechanisms underlying apoptosis in molluscs. Despite the high degree of evolutionary conservatism (attesting to the key role of this process in this organisms' survival), some molluscan apoptotic pathways and induction mechanisms appear to be sufficiently different from those defined in invertebrate models such as *Drosophila* and *Caenorhabditis*, or in vertebrates, to expect that studies of molluscan apoptosis will yield important discoveries of the novel and evolutionarily distinct ways in which "molluscs do it".

Our current studies of the abilities of parasites and pathogens to dysregulate molluscan apoptosis have also barely scratched the surface, and many exciting discoveries await researchers in the field. Molluscan genome sequencing projects (such as those proposed for *Biomphalaria glabrata* and *C. gigas*) and molluscan EST libraries may provide essential new molecular tools for probing these mechanisms and their role(s) in the molluscan immune system. More studies are needed to determine the molecular mechanisms underlying pathogen-induced modulation of apoptosis in molluscan cells and these will provide significant insights into immune avoidance and the evolution of the host-parasite arms race that is apparent between molluscs and their parasites/pathogens. Such studies will also help to identify the crucial physiological and biochemical pathways that may prove therapeutic targets to prevent proliferation and spread of the mollusc diseases.

Another interesting immunological aspect of apoptosis that has not been addressed in molluscs (or any other invertebrate model) is the suppression of the immune responses by apoptotic cells, and the role of parasite-induced apoptosis dysregulation in intra-host competition between parasites. In mammals, interaction of macrophages with apoptotic cells inhibits inflammatory cytokine and chemokine secretion thereby limiting immune responsiveness (Birge and Ucker, 2008). This immunosuppressive effect of apoptotic cell bodies appears to be conserved across metazoan evolution (Birge and Ucker, 2008) and so it would be interesting to determine whether parasite-induced

suppression of apoptosis in molluscan hemocytes (such as seen in *Perkinsus* spp. or *Haplosporidium* spp. infections) not only assists in proliferation and spread of the parasites responsible for this inhibition, but may also prevent secondary infections by subsequent invaders thus reducing intra- and interspecific competition between parasites within a host individual.

Unlike homeostatic mechanisms in mammals that closely regulate the organism's body temperature, osmolarity, and pH, these parameters are allowed much wider variations in molluscs following changes in their environment. The ramifications of extrinsic and intrinsic changes in apoptosis on the immune system of mollusks are poorly understood and require further investigation. While excessive apoptosis due to an environmental stress may suppress the immune system, moderate stimulation may modulate host-parasite relationships and alter disease outcome. In either case, understanding these interactions will be crucial to predict the proliferation and dissemination parasites and pathogens in natural populations of molluscs and potentially, their role as vectors in the diseases of domestic animals and humans.

Knowledge of the molluscan apoptotic mechanisms could also provide critical information for the development of immortalized mollusk cell lines. Currently, no such lines are available for marine mollusks, and the existing cell lines of *Biomphalaria* are notorious for their difficulty to maintain in culture. Development of new molluscan cell lines by suppressing apoptotic cell death in culture would provide critical new tools for advancement of experimental cell biology and physiology of mollusks.

Important as it is, apoptosis is not the only type of the programmed cell death in multicellular organisms. Other highly conserved forms of PCD such as autophagy and pyroptosis (a pro-inflammatory, caspase-1-dependent PCD) have been described and may play a role in immune defense. Currently, the role of the alternative forms of PCD in the immune defense has been underexplored, and their mechanisms, or even occurrence, in mollusks awaits further investigation.

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