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REVIEW

Cell death and the immune responses of the sipunculan worm *Themiste petricola*

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Abstract

We have recently studied the role of cell death in the immune system of the sipunculan worm *Themiste petricola*. Typical biochemical and morphological changes of apoptosis were induced in celomocytes of these marine worms after in vitro exposure of cells to hydrogen peroxide. Apoptosis was time and dose dependent, and required several hours to become apparent. Surprisingly, in unexposed samples a subtype of granulocyte was observed to undergo homotypic aggregation, extensive cytoskeletal changes, and degranulation followed by cell death. This spontaneous response ending in cell death occurred in a divalent cation-dependent manner, served to entrap foreign particles, and was blocked by EDTA-containing saline solutions. Even though the mode of granulocyte cell death shares some features with apoptosis, it appears to be a different form of programmed cell death since it occurs within minutes and does not produce single cell-derived apoptotic bodies but transforms itself into one or several syncytial masses with haemostatic and immune purposes. Since numerous granulocyte types and multicellular masses involved in cellular immunity have been described in sipunculan worms, the review also discusses the potential influence of activation of granulocytes by sea water in expanding the variety of morphological types and multicellular structures identified through morphological studies among sipunculan species.

Key Words: Sipuncula; immune responses; hemostasis; cell death; celomocytes; apoptosis; coagulation

Introduction

Recent studies on celomocyte death in the sipunculan worm *Themiste petricola* have introduced some new perspectives of cellular immune responses of these worms. The finding that a specific cell type of celomic granulocytes demonstrates rapid cell death as part of a well orchestrated response with hemostatic and immune purposes (Blanco, 2007; Blanco *et al.*, 2008; Cavaliere *et al.*, 2010) has implications to the interpretation of celomic cell morphological categories and cellular immune responses described hitherto in sipunculans. The first part of this review will present a brief summary of sipunculan celomic cells and cellular immune responses, as commonly described in previous studies conducted in different species of the phylum. Then the main findings on a peculiar mode of rapid cell death of celomic granulocytes in *T. petricola* will be presented, and its implications to the immune system and hemostasis of sipunculans will be discussed.

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List of abbreviations:
LHA, large hyaline amebocyte; LGL, large granular leukocyte; SGL, small granular leukocyte; PGRP peptidoglycan recognition protein; PGRP-S, PGRP-small; FSC, forward light scatter; SSC, side light scatter; PS, phosphatidylserine; TUNEL, Terminal-Deoxynucleotidyl-Transferase-Mediated-dUTP-Nick-End-Labeling; DAPI, 4',6-Diamidino-2-Phenylindole; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

Sipunculan body plan and the absence of a true circulatory system

Sipuncula is a phylum of unsegmented vermiform celomates with a plump trunk and a slender introvert that ends in a mouth encircled by tentacular outgrowths (Stephen and Edmonds, 1972; Gibbs and Cutler, 1987; Rice, 1993). The body wall enclosing the celom cavity consists of several layers that include cuticle, epidermis, dermis, circular muscle layer, longitudinal muscle

layer, and peritoneum (Fig. 1). The dermis varies from quite thick to very thin stratum and consists of a mesh of fine fibres containing connective-tissue cells, pigment cells, multinucleate bodies and leukocytes (Hyman, 1959). A definite circulatory system is lacking in sipunculans and celomic fluid operates mechanically as a hydroskeleton. However, in some thick-walled species the dermis contains celomic canals or spaces communicating with each other and with the general celom (Hyman, 1959).In larger species of the genus *Siphonosoma* canals take the form of celomic diverticula that penetrate into the dermis as blind sacs of irregular shape, branched, and somewhat transversely arranged (Hyman, 1959). The canals are lined by peritoneum and contain the same elements as the celomic fluid. Thus celomic cells can reach the dermis and they can be found interspersed between a felt of fine fibres and connective-tissue cells (Fig. 1). The main muscles of the interior are the retractors of the introvert. The contraction of the retractors causes the invagination of the introvert, which is extruded again by the general contraction of the circular layer of the body wall acting to compress the celomic fluid. This is the main factor causing celomic fluid flow (Zuckerkandl, 1950). The tentacles consist of a separate lumen that contains the same elements as the celom, and although there are no openings into the celom the celomocytes seem able to penetrate into the system (Maiorova and Adrianov, 2005; Adrianov *et al.*, 2006). All sipunculans are dioecious and the sex cells are shed into the celom at an immature stage and complete their maturation while floating in the celomic fluid. They are voided by way of the nephridia, which act as gonoducts (Hyman, 1959; Adrianov *et al.*, 2002).

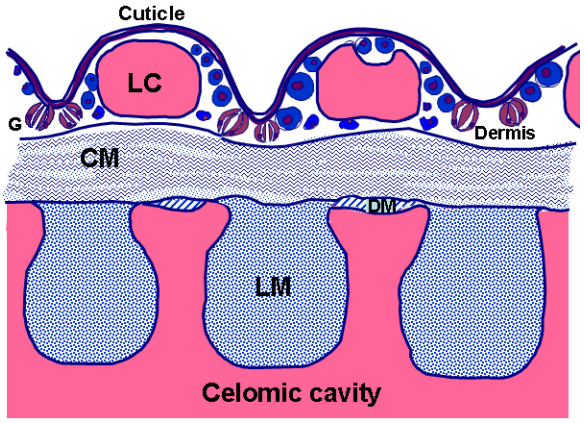

Fig. 1 The body wall of sipunculan worms consists of an external cuticle everywhere underlain by an epidermis with numerous glands (G). In some genera the dermis contains longitudinal canals (LC) communicating with the general celom. The body-wall musculature consists of outer circular (CM) and inner longitudinal layers (LM). A thin diagonal layer (DM) exists between the other two in some sipunculans but is infrequently mentioned (Modified from Hyman, 1959).

Celomic cell types and cellular immune responses in sipunculans

Hemerythrocytes

The celomic cavity contains a variety of dissimilar cell types. Unfortunately, nomenclature of cell types is far from being standardized making it difficult to compare studies from different species. Hemerythrocytes, the most abundant cells in the celomic fluid, are found in all species and confer the celomic fluid a pinkish tint due to the iron-containing substance hemerythrin (Hyman, 1959; Valembois and Boiledieu, 1980; Dybas, 1981a; Rice, 1993; Matozzo *et al.*, 2001; Lunetta, 2004; Meyer and Lieb, 2010). Most authors agree in the morphological description of hemerythrocytes as nucleated biconvex disks, varying in size among different species, and often having one or more acid vacuoles (Ochi, 1970; Ochi and Ohnishi, 1971). It has been suggested that they are of some assistance in ridding the celom of foreign particles since injected dyes are taken into these vacuoles (Towle, 1975). Other main cell types in the celom are leukocytes, multicellular structures and gametes (Ochi, 1970).

Hyaline leukocytes

Leukocytes encompass a heterogenous group of cells mostly involved in cellular immune responses. Main types of leukocytes, not necessarily all present in the same species, include hyaline cells with fine or no granules, and granulocytes with coarse granules that may be acidophilic, neutrophilic, or basophilic (Marcou and Volkonsky, 1933; Hyman, 1959; Matozzo *et al.*, 2001). The presence of granules, extension of pseudopodia or an ameboid cell shape is often used as a morphological criterion to distinguish leukocytes from the most abundant biconcave disc-shaped hemerythrocytes. Amebocytes are often referred to as phagocytic cells, with a hyaline cytoplasm having few or no granules and having one or several large lysosomic vacuoles often similar to that of hemerythrocytes (Ochi and Ohnishi, 1971).

Granular leukocytes

Most authors coincide in the occurrence of granulocytes among celomic leukocytes and their involvement in cellular immune responses (Rice, 1993; Matozzo *et al.*, 2001; Lunetta, 2004). They are often described as having numerous granules masking details of the cytoplasm and the nucleus (Dybas, 1981b; Matozzo *et al.*, 2001; Lunetta, 2004). The few studies that have quantified the relative abundance of granulocytes within celomic cells indicate a range between 5 % and 20 % (Towle, 1975; Matozzo *et al.*, 2001; Lunetta, 2004) More recently Lunetta (2004) has introduced a classification that separates two broad categories of granulocytes from *Sipunculus nudus* designated type I and Type II, and has provided differential characteristics between these two categories at the biochemical, morphological and functional level. This classification criterion may be applicable to most species of sipunculans and the categories proposed by Lunetta (2004) will be recalled while discussing results obtained in *T. petricola*. Granules

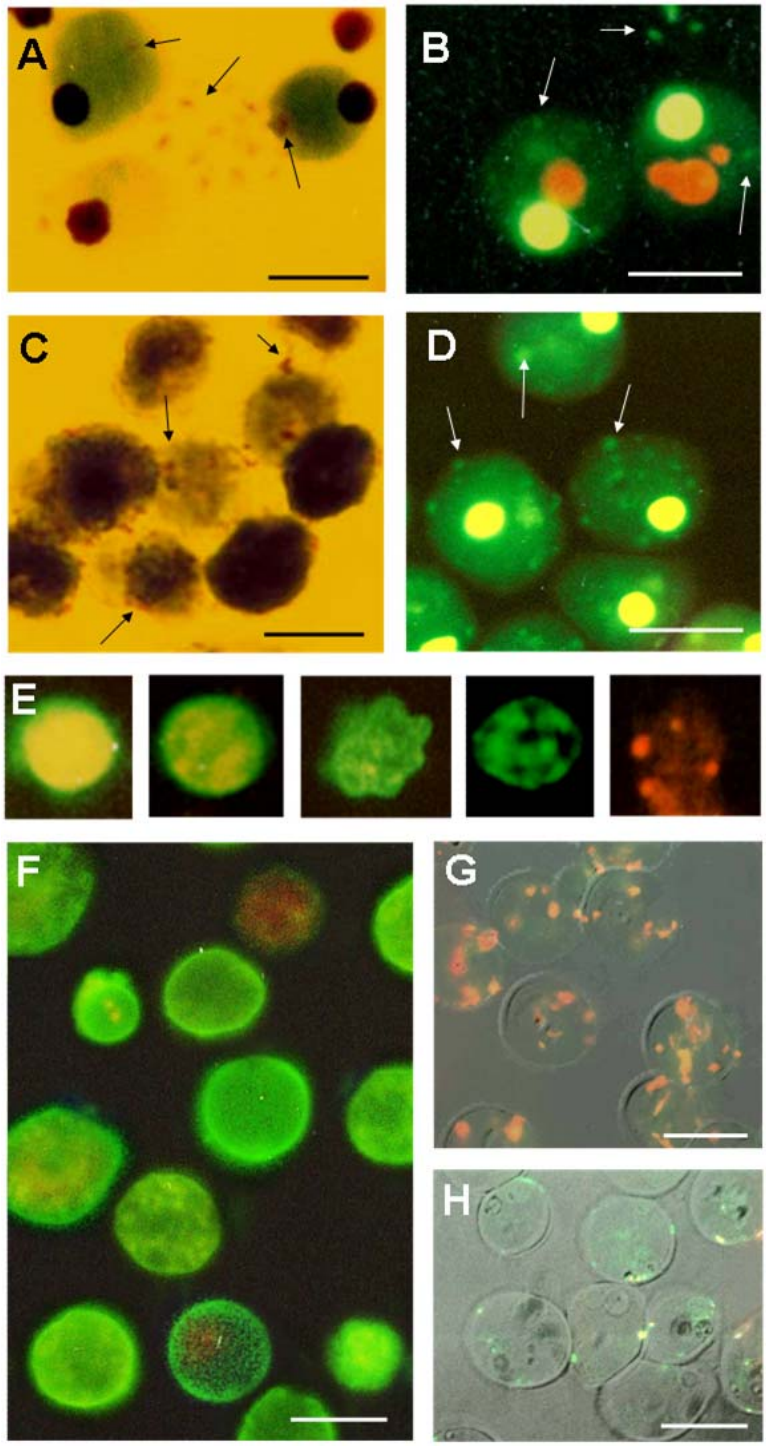

Fig. 2 Phagocytosis of zymosan particles (arrows) by large hyaline amebocyte (LHA) stained with Giemsa (A), and with acridine orange (B). In panels C and D degradation of particles was inhibited by alkalinizing the lysosomes with ammonium chloride. Zymosan is engulfed but not degraded, thus the cytoplasm becomes packed with basophilic zymosan particles (C). Note also that when lysosome vesicles are alkalinized acridine orange no longer stains them red-fluorescent (D). A sequence of nuclear apoptotic changes induced in LHAs by exposure to hydrogen peroxide during 2 to 6 hours is shown in (E). Cells were stained with acridine orange and ethidium bromide; green fluorescence indicates that cell membrane permeability is preserved (apoptotic live), while red fluorescence indicates loss of membrane permeability (apoptotic dead). Exposure of phosphatydilserine during apoptosis of LHAs and hemerythrocytes detected by Annexin V-FITC and propidium iodide is shown in (F). Red fluorescence indicates loss of membrane permeability. In panel (G) LHAs and hemerythrocytes were stained with the potentiometric dye JC-1 showing normal mitochondrial membrane potential (orange fluorescence), while in (H) mitochondria became depolarized as indicated by shift from orange to green fluorescence. Bar = 15 μm (Modified from Blanco *et al.*, 2005).

from both categories of granulocytes have lytic enzymes including peroxidase, acid esterases, alkaline and acid phosphatases, and lipase (Lunetta, 2004, 2005). Even though type I granulocytes have lytic enzymes they do not contain any foreign material and do not show any phagocytic capability (Lunetta, 2005). Degranulation of type I granulocytes and production of extracellular acidophilic material has been noted by several authors (Lunetta, 2004). In addition type I granulocytes often show thin cytoplasmatic filaments or filopodia, oriented in all directions.

A wide range of morphological descriptions that may well be included in the type I category of granulocytes has been introduced by different authors. Descriptions include elongated granulocytes, fusiform or curious shaped cells projecting pseudopodia, which often appear to join with one another (Towle, 1975). Yet Dybas (1975) has reported a paired non-phagocytic granulocyte, occurring as a cell within a cell at a frequency of less than 0.5 % of total celomic cells. A recent study in *Phascolosoma esculenta* reported granulocytes with condensed and clumped chromatin, syncytial granulocytes, cell complexes of granulocytes with podocytes, granulocytes forming pseudopodia and devoid of granules at the pseudopodia protrusion, and finally granulocytes having a large nucleus and often found in association with hemerythrocytes (Ying *et al.*, 2010). It should be noted that all of these cytological studies have been conducted in live or fixed preparations without concurrent assessment of cell viability prior to fixation. In addition it has been a common practice to use sea water as saline solution to dilute celomic fluid during cytological studies, either for supravital studies or prior to fixation of cells in suspensions. As it will be discussed further, non-phagocytic granulocytes may be activated in contact with sea water or saline solutions containing Ca^{++} and show rapid cytoskeletal changes before dying within minutes.

In contrast, type II granulocytes of the classification proposed by Lunetta (2004), are motile cells, actively phagocytic with lytic enzymes involved in intracellular digestion, and contain engulfed particulate material and lipids. Both categories of granulocytes are reported to accumulate in the vicinity of foreign particles trapped within the celom together with masses of acidophilic material (Lunetta, 2004). As will be discussed further type II granulocytes of the category proposed by Lunetta (2004) correspond to cells that do not lose viability as part of the cellular defence reactions but remain motile and actively phagocytic engulfing remnants of dying type I granulocytes and foreign particles as well.

Multicellular bodies

Various types of multicellular bodies are reported to occur in the celomic fluid of different species of sipunculans (Hyman, 1959; Rice, 1993). The main criterion to define these bodies has been the morphological identification of cells by light and electron microscopy. Several authors have noted that the multicellular bodies include adhered cells and variable degrees of amorphous material that is thought to entrap particles and aid in adhesion. These masses, often of sizes from 30 to 100 μm, have received several names such as cell plates, giant multinucleate corpuscles or more recently brown bodies (Lunetta, 2004). Experimentally they have been induced by the injection of foreign particles into the celom and it has been considered a main cellular immune response of sipunculans (Hyman, 1959; Lunetta, 2004). Brown bodies and other similar multicellular structures entrapping foreign bodies have been also referred to as capsules (Tripplet *et al.*, 1958), although there is not a histological resemblance to capsules occurring in other invertebrates such as insects and crustaceans. More recently Lunetta (2004) described the structure of brown bodies induced by mammalian red blood cell injection as made of a core of acidophil material, probably derived from degranulation of type I granulocytes. The function of acidophilic material would be to cement the various parts of the brown body and external layers containing type I and type II granulocytes (Lunetta, 2004). Granulocytes in brown bodies were described as misshaped, containing little cytoplasm, and having a large quantity of granulated material (Lunetta, 2004).

Free urns

The free urns, described only in some species of sipunculans, are cell complexes that swim freely within the celomic cavity and play an important role in the sipunculan immune defence by releasing sticky, mucoid tails that promptly trap invading pathogens (Bang and Bang, 1975; Nicosia, 1979; Bang and Bang, 1980; Nicosia and Sowinski, 1995). Urns are composed of two cells, a vesicle cell and a saucer-shaped ciliated cell, fitted together like an acorn into its cap (Dybas, 1976). A smaller and variable population of cells, often referred to as third- type cells, is frequently found in close juxtaposition to the ciliated cell. Lunetta (2004) showed that third type cells are adhering granulocytes that reacted positively with chloroacetate esterase and were enmeshed in acidophilic material.

Celomocyte cell death in *T. petricola*

Apoptosis in celomic cells

Apoptosis has been a main focus of research during the last 20 years and there is no doubt that it is a crucial process in adaptive and innate immunity of higher animals (Kerr *et al.*, 1972; Opferman and Korsmeyer, 2003; Wang *et al.*, 2003). Apoptosis occurs during normal T and B cell ontogeny, central tolerance development, and downregulation of T and B cell responses (Feig and Peter, 2007). Cell death in neutrophils during inflammation was classically thought to occur exclusively by passive necrosis due to release of lytic enzymes and damage by microbial products (Sendo *et al.*, 1996). However, recent studies have shown that neutrophils undergo apoptosis to ensure complete destruction of intracellular microbes, facilitate uptake by macrophages, and limit proinflammatory stimuli protecting bystander self cells (Everett and McFadden, 1999; Fadok and Chimini, 2001; Fadok *et al.*, 2001).

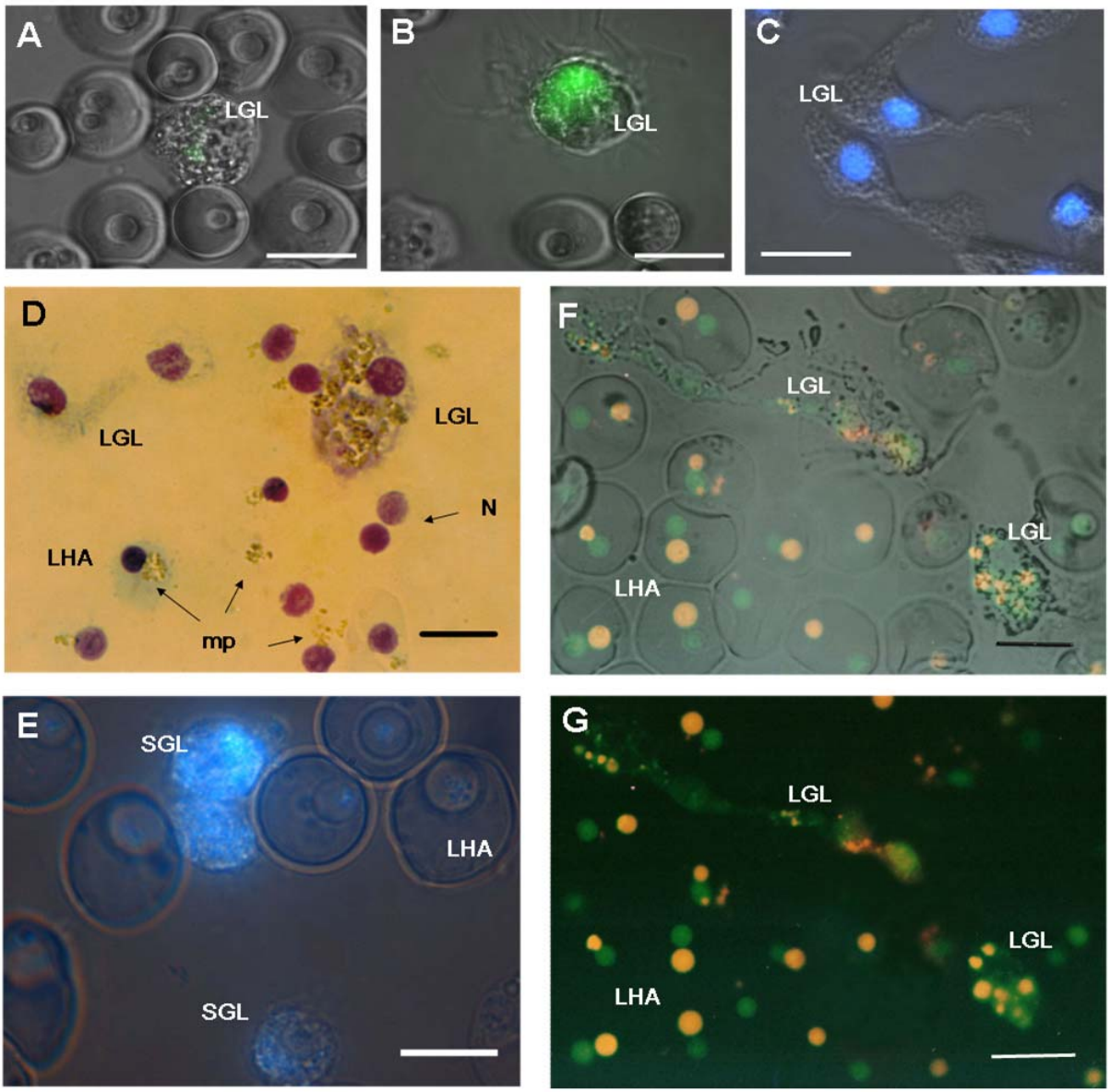

Fig. 3 Activation, shape changes and cytoplasmic disintegration in large granular leukocytes (LGL).A resting LGL is shown in (A). Green fluorescence corresponds to intracellular Ca^{++} level as indicated by Ca^{++} probe Fluo-4. Early activated LGL emitting filopodia is shown in (B). Note the increase in intracellular Ca^{++} levels as indicated by green fluorescence. Extensive shape changes observed at later time points after activation are shown in (C). Nuclei were stained with DAPI in a paraformaldehyde-fixed preparation in (C). A Giemsa stained preparation is shown in (D) where cytoplasmic disintegration yields microparticles (mp), and isolated nuclei (N). Microparticles are observed to be engulfed in a LHA. In live preparation shown in (E) DAPI stained only DNA present in nuclear remnants and dead cells because the dye is non-permeant to live cells. Note that DNA-containing remnants were engulfed in LHAs and SGLs and remained in the cytoplasm, while nuclei were not stained indicating that the phagocytic cells were viable. In panels (F) and (G) a live preparation was stained with acridine orange showing acid granules in an early activated round LGL (right-bottom), and partial loss of granules in a late activated LGL with extensive pseudopodia formation (centre). Bar = 15 μm (Modified from Blanco, 2007; Blanco *et al.*, 2008; Cavaliere *et al.*, 2010).

The role of apoptosis in innate immunity has been also recognized in invertebrate animals where programmed cell destruction can aid in degradation of intracellular pathogens preventing spread of infection beyond the cell boundary (Wang *et al.*, 2003; Russo and Madec, 2007; Wang *et al.*, 2008; Sokolova, 2009; Kiss, 2010). Yet some pathogens appear to have the ability to modulate the induction of apoptosis to their own advantage (Sunila and LaBanca, 2003; Wang *et al.*, 2003; Terahara and

Takahashi, 2008). Other studies have focused on cell death induced by ecotoxicants, since exposure to these substances may pose a threat to wildlife invertebrates by their ability to induce apoptosis in several cell types including immune cells (Sokolova *et al.*, 2004; Cima *et al.*, 2008; Matozzo *et al.*, 2008).

All these findings have fostered the interest in characterizing apoptosis mechanism of immune cells in several invertebrate groups (Podrabsky and Krumschnabel, 2010). It should be pointed out that apoptosis is no longer a synonym of programmed cell death since other forms, including autophagic cell death and programmed necrosis, have an increasing relevance in immune response, amongst many other fields of study (Sperandio *et al.*, 2000; Yorimitsu and Klionsky, 2005; Samara *et al.*, 2008; Kroemer *et al.*, 2009).

Our first study on cell death in sipunculan celomocytes was focused on identifying standard indicators of apoptosis after exposure to hydrogen peroxide and evaluating to what extent hydrogen peroxide-induced cell death was similar to what had been described in other animals (Blanco *et al.*, 2005). That study was conducted on cell suspensions from celomic fluid containing hemeryhtrocytes and large hyaline amebocytes (LHA) as their main constituents. Both cell types have large acid vacuoles and particularly LHA are actively phagocytic (Blanco *et al.*, 1995), and were shown to migrate in vitro toward gradients of endogenous or exogenous chemoattractants (Cabrera *et al.*, 2002). The LHA vacuole has an extraordinary capacity to degrade engulfed material. When exposed to zymosan particles LHA engulf the particles, route them to the vacuole and degrade it in such an efficient manner that only a scant number of particles can be observed in the cytoplasm (Figs 2A, B). When acid degrading enzymes were inhibited by elevating the pH of the vacuole with ammonium chloride the cytoplasm became densely packed with particles since they were phagocytosed but not degraded (Figs 2C, D) (Blanco *et al.*, 2005). Exposure of this suspension of hemerythrocytes and LHAs to hydrogen peroxide induced morphological and biochemical changes that are known to be present in apoptotic cells (Blanco *et al.*, 2005; Galluzzi *et al.*, 2009). Changes at the nuclear level included chromatin condensation and fragmentation, nuclear membrane ripples, and DNA degradation to oligonucleosome-sizes (Fig. 2E) (Blanco *et al.*, 2005). Extranuclear changes consisted of loss of mitochondrial membrane potential, cell volume decrease, exposure of phosphatidylserine (PS) on the outer membrane leaflet, and cell membrane blebbing (Figs 2F-H) (Blanco *et al.*, 2005).

Activation and cell death in a subtype of granulocytes

The celomic cell suspension used in our first study was particularly depleted of granular cells. The cell suspension was obtained by harvesting, washing, and incubating cells in saline solutions or culture media containing Ca^{++} (Blanco *et al.*, 2005). When celomic fluid was stained with acridine orange and propidium iodide immediately after harvesting without washing and observed by fluorescence microscopy, we noted that a celomic cell type having acid granules was present in the samples (Blanco, 2007). These cells, that we designated large granular leukocyte (LGL), were about 8 % of celomic cells, showed extensive morphological changes within minutes emitting filopodia and pseudopodia upon contact with glass surface (Figs 3A-C), aggregated to each other, loss the acid granules and became dead as indicated by red fluorescence of the nuclei stained with propidium iodide (Figs 3F, 3G, 4G-J). These granulocytes are similar in description to type I granulocyte category proposed by Lunetta (2004). When celomic fluid was harvested in EDTA-containing solutions, LGLs did not show morphological changes, preserved their round shape densely packed with granules and remained viable (Blanco, 2007; Blanco *et al.*, 2008). Moreover, when sea water was added to celomic fluid harvested in Ca^{++} free saline preparations, LGLs started to form aggregates and demonstrated shape changes, being more noticeable the larger the amount of sea water dispensed (Blanco, 2007) (Figs 4A-D). Since all changes were blocked by EDTA we concluded that sea water and Ca^{++} containing solution caused activation and aggregation of LGLs in multicellular masses.

A surprising finding was that although cell death of LGLs occurred very rapidly, several apoptotic-like features were still observed including chromatin condensation and fragmentation, nuclear membrane ripples, loss of mitochondrial membrane potential and PS exposure (Blanco, 2007; Blanco *et al.*, 2008). However, other typical apoptotic cytoplasmic changes were absent, including cell volume decrease, membrane blebbing and formation of apoptotic bodies. Instead, the cytoplasm of LGLs was often seen to disintegrate in small particles unless the cells remain in clumps, in which case the cytoplasmic remnants were kept as integral part of a syncytial mass (Cavaliere *et al.*, 2010) (Figs 3D, 4E-K).

A suspected hemostatic role: isolating the cellular clot by the pullout method

Activation and aggregation of LGLs was to some extent reminiscent of platelet aggregation and thrombus formation (George, 2000; Heemskerk *et al.*, 2002; Harrison, 2005). Both platelets and LGLs show a series of prominent cytoskeletal changes after activation and continue to form a cell-cell aggregate, with exposure of PS residues in the outer leaflet of the plasma membrane and loss of mitochondrial membrane potential (Pereira *et al.*, 1999, 2002; Li *et al.*, 2000). The end product is in both cases an insoluble mass. However activation and aggregation systematically followed by death is not an accepted concept in platelet physiology due to its non-nucleated condition (Perrotta *et al.*, 2003). Programmed cell death in platelets is still a concept that raises several concerns to most authors (Wolf *et al.*, 1999; Zhang *et al.*, 2007).

However our finding in LGLs was also reminiscent of cell death and disintegration of coagulocytes as occurs during arthropod coagulation (Theopold *et al.*, 2004). Coagulocytes have an accessory role in arthropod clot formation

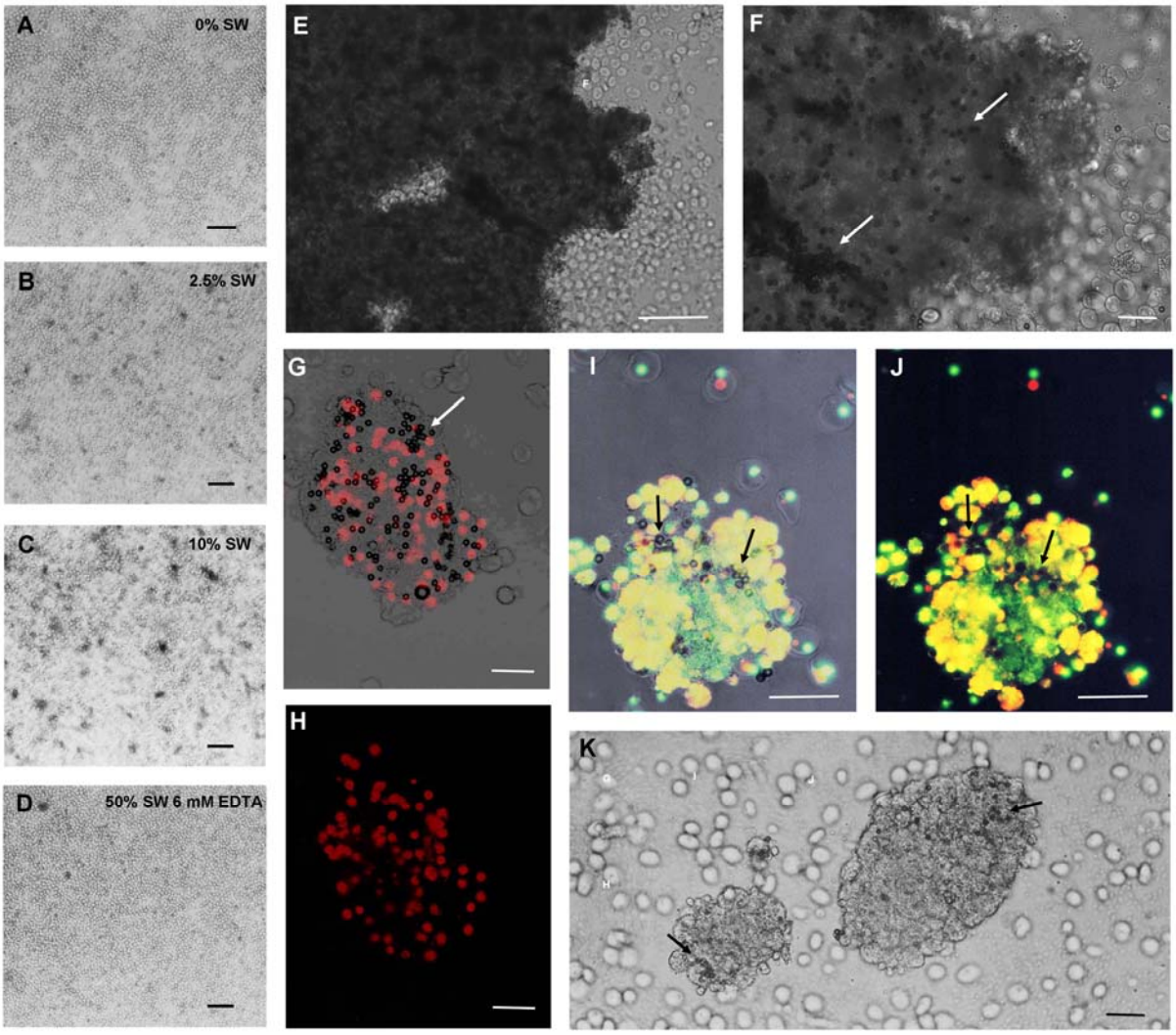

Fig. 4 Celomic fluid harvested in Ca^{++} free saline solution is shown in (A). The effect of exposure to increasing concentrations of sea water (SW) is shown in (B) and (C). Multiple LGL aggregates are observed as dark spots. In panel (D) LGL activation by 50 % SW was blocked by 6mM EDTA. Panel (E) shows a large clot entrapping magnetic beads obtained in vitro and (F) shows a magnified image of (E) where beads are observed (arrows). In (G) and (H) a small clot entrapping beads (arrows) in vitro was stained with propidium iodide. In (I) and (J) a small clot entrapping magnetic beads (arrows) was stained with acridine orange. Note loss of acid granules indicated by green fluorescence at the clot core and preservation at peripheral areas indicated by orange fluorescence. Panel (K) shows small clots formed in vivo after injection of magnetic beads into the celom. No massive clots as shown in (E) were formed in vivo. These small clots entrapping beads coincide with morphological description of brown bodies (multicellular masses demonstrated to entrap particles injected in vivo in other species of sipunculans). Bar = 100 μm in (A) through (E) and Bar = 30 μm in (F) through (K) (Modified from Blanco, 2007; Blanco *et al.*, 2008).

since extracellular strands formed by hemolymph coagulation proteins form the main clot structure (Theopold *et al.*, 2002; Scherfer *et al.*, 2004; Bidla *et al.*, 2005). Even though cell-free celomic fluid does not form strands we speculated that sipunculans could yet form a clot mass, to some extent reminiscent of a platelet thrombus, by means of activation, adhesion and rapid death of nucleated cells. Scherfer (2004) introduced a method to isolate clots from *Drosophila* that was based on creating a clot over a suspension of magnetic beads and further separating the clot with a magnet (Scherfer *et al.*, 2004). Using this so called pullout method we could isolate a massive clot ex vivo formed by LGLs entrapping beads that could be further studied regarding several aspects of clot formation and structure (Figs 4E-F). The hemostatic purpose of this cellular reaction was inferred upon the rapid formation of an insoluble macroscopic mass. However immediate entrapment of particles within the clot was also a strong evidence of being a first line immune response (Blanco *et al.*, 2008).

Our first aim was to demonstrate that the reaction was indeed hemostatic. Since a system of celomic canals allows sipunculan celomic cells to reach the dermis (Fig. 1), we speculated that a body wall injury would create a sudden contact of celomic cells with sea water, causing LGLs to activate, further aggregate, and demonstrate a systematic and rapid death to create a hemostatic plug. To prove this hypothesis we harvested celomic fluid and allow it to flow through a glass tube with an open end in contact with sea water. A massive clot was formed at the open end in contact with sea water which stopped the flow of the liquid column. When observed by light microscopy the clot was formed by aggregated LGLs (Cavaliere *et al.*, 2010).

The immune significance of cellular clotting and LGL death in T. petricola

The immune significance of clot formation was made evident not only due to entrapment of magnetic beads but of several dissimilar biotic particles including bacteria, yeast and leukemic cells (Cavaliere *et al.*, 2010). By eliciting smaller clots over particle suspensions with controlled amounts of sea water we could observe that clots were formed by aggregating LGLs that extended filopodia and adhered to each other creating a mesh where particles became entrapped (Figs 4G-J). DNA degradation as indicated by supravital propidium iodide staining of small clots was observed to occur quite fast at the core of LGL aggregates, while the peripheral layers tend to remain viable for a longer time (Figs 4G, H).

Staining with lysosomotropic dyes like acridine orange showed that acid granules of LGLs were released at the clot core, while most peripheral LGLs showed intact granules indicating that there was a gradient of granule content release from the centre to the periphery (Figs 4I, J). PS exposure as indicated by Annexin V binding followed a similar gradient pattern from the centre to the periphery (Blanco *et al.*, 2008). DNA degradation within the clot centre was further evidenced by the DNA nick-end labelling method (TUNEL) (Cavaliere *et al.*, 2010). Thus we concluded that almost immediate entrapment of potential pathogens such as bacteria or fungi within the clot was followed by formation of a hostile environment within the clot core consisting of degradative enzymes derived from acid granules, and potentially from other enzymes activated as part of programmed cell death such as caspases, calpains and DNAses (Pasquet *et al.*, 1996; Shcherbina and Remold-O'Donnell, 1999; Wolf *et al.*, 1999).

Invertebrate cellular responses often utilize pattern-recognition receptors in the hemolymph or on the immune cell surface to identify pathogen-associated molecular patterns (Kurata, 2010). Members of the peptidoglycan recognition protein (PGRP) family recognize diverse bacteria-derived peptidoglycans and initiate appropriate immune reactions (Kurata *et al.*, 2006; Goto and Kurata, 2006). To further explore the immune significance of LGL clotting as a first line immune response we evaluated the presence of PGRP (Blanco *et al.*, 2008). Using antibodies raised against different human recombinant PGRP we explored the expression of these pattern recognition proteins as indicated by immunofluorescence detection finding that LGLs stained positive to anti PGRP-S (Blanco *et al.*, 2008).

By harvesting celomic fluid in EDTA-containing saline solutions we were able to isolate resting LGLs and evaluate its light dispersion properties through flow cytometry (Blanco *et al.*, 2008). These cells form a cluster of high side light scattering (SSC) due to the high granule content (Fig. 5A). A high expression of PGRP-S was observed in resting LGLs when stained with anti PGRP-S and evaluated by flow cytometry (Blanco *et al.*, 2008), Since the pullout method allowed us to isolate clots entrapping magnetic beads we explored the presence of PGRP-S within these clot masses and also in clot supernatants noting that both the clot and the supernatant had high PGRP-S content (Blanco *et al.*, 2008). Altogether these findings reinforce the role of clot formation as a first line immune cellular response with a role in pathogen-associated molecular pattern recognition.

Clot formation and the second line cellular immune response

Clot formation is not a single and isolated response in *T. petricola* but it is connected to a second line cellular immune response. Light microscopic preparations of small clots were suggestive of two cell types involved in phagocytosis of LGL remnants, namely LHAs and a second type of granulocyte that we designated small granular leukocyte (SGL) (Fig. 3E). These granulocytes remained live as evidenced with viability probes, did not release granule content, and were often seen at the clot edges actively phagocytosing self remnants and particles shed from the clot (Cavaliere *et al.*, 2010). Cytoskeletal arrangement of SGLs was often suggestive of a polarized motile cell with uropodia and lamellipodia. Thus these cells were coincident with type II category of sipunculan granulocytes in the classification proposed by Lunetta (2004). Through flow cytometry SGLs are found in the same cluster of resting LGLs (Fig. 5A). The cluster disappears when cell suspension is exposed to sea water due to activation, aggregation, and death of LGLs, while many SGLs may be washout with elicited LGL clots. Thus sea water presence in harvested celomic cell suspensions that are further filtered through a small pore mesh, yields suspensions almost depleted of LGLs and SGLs (Fig. 5B). In addition the cluster of non-clotting cells formed mainly by LHAs and hemerythrocytes decreases its forward light side scatter properties most probably owing to cell shape changes associated with activation (Fig. 5B).

Clot formation induces shedding of cytoplasmic remnants of a number of disintegrated LGLs that are not completely retained at the clot. These cytoplasmic remnants can be stained and observed by flow cytometry (Cavaliere *et al.*, 2010). In addition, labelled cytoplasmic remnants were demonstrated to be phagocytosed by SGLs, often at the clot neighbourhood (Cavaliere *et al.*, 2010). LHAs were also detected to phagocytose these remnants although they appear to be loaded in the large lysosomic vacuoles and digested more

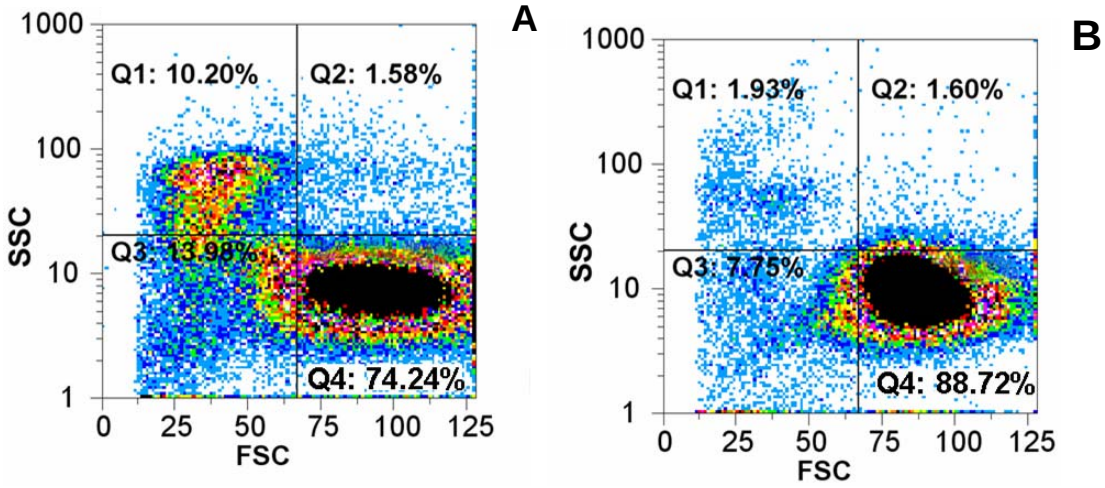

Fig. 5 (A) Flow cytometry dot plot of front (FSC) vs side (SSC) light scatter of celomic fluid harvested in EDTA-containing saline solution. Q1 quadrant shows resting LGLs while Q4 quadrant shows LHAs, and hemerythrocytes that together encompass the most abundant cell types. (B) Celomic fluid harvested and washed in Ca^{++} or sea water-containing saline solutions becomes depleted of LGLs as observed in Q1 quadrant, and contains mainly LHAs and hemeryhtrocytes observed in Q4 quadrant. A decrease in FSC of this cluster is also observed as compared to (A). Percentages were determined after running 80,000 celomic cells (Modified from Blanco *et al.*, 2008).

efficiently as occurs with most targets phagocytosed by these cells (Figs 3D, E). DNA remnants were similarly stained by DNA labels and demonstrated to be engulfed by SGLs and LHAs (Fig. 3E). In addition the TUNEL method demonstrated fragmentation of DNA remnants that were engulfed by SGLs and LHAs (Cavaliere *et al.*, 2010). When clots were formed over suspensions of labelled bacteria the fate of these targets was similar to self remnants. Bacteria appeared entrapped within the clot and SGLs could be seen at the clot edges phagocytosing bacteria. LHAs were also found to phagocytose bacteria although in areas not in much proximity to clot edges (Cavaliere *et al.*, 2010).

Clot formation *in vivo* and the origin of brown bodies

Since a large clot was formed in vitro by exposing the whole content of celomic fluid to a bead suspension, we expected that beads injected directly within the celom would elicit a similar massive clot in vivo. However injected beads recovered from the whole celomic fluid of an injected worm by the pullout method after 24 h showed several small clots of about 50 to 150 μm that entrapped magnetic beads (Blanco, 2007). These small clots resembled brown bodies described in *Sipunculus nudus* (Lunetta, 2004) or similar multicellular masses described in other sipunculans (Hyman, 1959; Rice 1993). Granulocytes could be barely recognized at the outer layers, while the core of multicellular masses was formed by an amorphous mass with entrapped beads interspersed (Fig. 4K). These findings are in agreement with Lunetta (2004) who proposed that formation of brown bodies, which is considered an immune response of sipunculans, would be somehow related to type I granulocytes since they were found in peripheral areas of brown bodies. We can now assert that amorphous material may not be derived from secretion of live cells but from activation, aggregation and active death of LGLs as part of a cellular defence reaction.

Conclusion

Cell death of LGLs is a central process of clot formation in *T. petricola* which involves both a hemostatic and an immune purpose. Cell death is elicited following activation, occurs rapidly, and participates in a sequence of well orchestrated events. When evaluated in LGL aggregates, cell death occurs first at the clot core and later at the periphery. LGL death within the clot core provides a hostile environment to entrapped pathogens while containment at the periphery preserves neighbouring bystander cells and tissues. Activation followed by cell death is responsible of several morphological changes in granulocytes. Since granulocyte activation, aggregation and death are elicited when sea water is present in harvested celomic cells, failure to recognize this fact in the past may have contributed to the plethora of granulocyte types and multicellular structures described in sipunculan species. However brown bodies formation appears to result from LGL clotting within the celom and is definitely a first line immune cellular response of sipunculans. Thus programmed cell death in celomic cells may not only occur through classic apoptosis due to external insults such as oxidative stress or toxic substances, but

may have peculiar cell death mechanisms tailored to efficiently and simultaneously achieve a hemostatic and immune purpose.

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