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Hovering between death and life: Natural apoptosis and phagocytes in the blastogenetic cycle of the colonial ascidian *Botryllus schlosseri*

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ABSTRACT

Colonies of the compound ascidian *Botryllus schlosseri* undergo recurrent generation changes during which massive, natural apoptosis occurs in zooid tissues: for this reason the species is emerging as an interesting model of invertebrate chordate, phylogenetically related to vertebrates, for studies of apoptosis during development.

In the present work, we carried out a series of morphological, cytofluorimetrical and biochemical analyses, useful for a better characterization of *Botryllus* apoptosis. Results are consistent with the following viewpoints: (i) both intrinsic and extrinsic pathways, probably connected by the BH3-only protein Bid, are involved in cell death induction; (ii) phagocytes, once loaded with senescent cells, frequently undergo apoptosis, probably as a consequence of oxidative stress caused by prolonged respiratory burst, and (iii) senescent phagocytes are easily recognized and ingested by other phagocytes, responsible for their clearance. In addition, results suggest the conservation of apoptosis induction mechanisms throughout chordate evolution.

1. Introduction

Cell death by apoptosis is a fundamental biological process required for the correct sculpturing of developing organs and the controlled elimination of unwanted cells in morphogenesis, regeneration, tissue renewal and maturation of the immune system [1–5]. Apoptosis is characterized by a series of morphological changes, such as cytoplasm and nuclear condensation, leading to cell shrinking, blebbing, internucleosomal cleavage of chromatin and exposure of cell surface molecules enabling the recognition and the removal of dying cells by phagocytes [6]. It implies the triggering of a series of biochemical events culminating in the activation of initiator and effector cysteinyl aspartate proteases, known as caspases. The latter are normally present in healthy cells as inactive zymogens, with little or no protease activity, and are converted to active enzymes during apoptosis so that extensive proteolysis occurs during the process [7–9]. Pro-apoptotic stimuli favor the assembly of large complexes that allow the clustering of initiator zymogens and their cleavage to active enzymes which, in turn, convert effector pro-enzymes to active caspases [8]. Initiator procaspases are characterized by the presence of the caspase recruitment domain (CARD) which allows their aggregation by adaptor proteins such as Fas-associated death domain protein (FADD) or apoptotic protease-activating factor-1 (APAF-1), and, lastly, their autoactivation [7–9].

In mammals, apoptosis can be either intrinsically induced by mitochondrial damage, leading to the release of cytochrome *c* in the cytosol and the setting up of apoptosomes which activate the initiator caspase-9, or extrinsically triggered by the interaction of death receptors, such as Fas (CD95), with their ligands, e.g., FasL (CD95L), that allows the formation of the death-inducing signaling complex (DISC) and the activation of the initiator caspase-8. In both cases, initiator caspase-9 and -8 activate effector caspases which are responsible of the majority of the events occurring in cells having entered an apoptotic pathway [7,8].

Tunicates are invertebrate chordates, mainly represented by ascidians, phylogenetically related to vertebrates. In the solitary ascidian *Ciona intestinalis*, a reference species for developmental and cell biology studies, the genome has been fully sequenced and homologous of vertebrate genes involved in apoptosis have been identified [10–14]. This suggests a high degree of conservation of the apoptotic machinery throughout the evolution of chordates.

Botryllus schlosseri is a cosmopolitan colonial ascidian, easily found in shallow temperate waters, which is emerging as a valuable model organism for the study of variety of biological processes such as sexual and asexual reproduction, regeneration, allorecognition, immunobiology [15].

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A colony is a clone as it derives from the settlement and metamorphosis of a tadpole-like larva into a founder zooid, and grows by asexual reproduction through the continuous production of new buds. Colonies are formed by many filter-feeding zooids, grouped in star-shaped systems, bearing palleal buds which, in turn, produce budlets, so that three blastogenetic generations are usually present. Zooids, buds and budlets are connected by the colonial circulatory system which assures well-synchronized development and tight coordination among colony members [15].

At regular intervals (weekly at the temperature of 20 °C), colonies undergo a generation change or take-over, lasting 24–36 h, during which adult zooids are progressively replaced by growing buds; in the meantime budlets become buds and a new budlet generation appears. Therefore, a colonial blastogenetic cycle can be defined beginning with the opening of the siphons of a new zooid generation, which starts its filtering activity, and ending with the take-over, when adult zooids cease filtering, contract and are gradually resorbed [15].

During take-over, diffuse, natural apoptosis occurs in zooid tissues, which renders this species an interesting model also for the study of this process. In addition, a colony is virtually immortal so that many cyclical apoptotic events can be studied during its lifespan. The clearance of dying cells is assured by circulating phagocytes which are massively recruited and infiltrate zooid tissues and engulf senescent cells. Phagocytes are represented by spreading hyaline amoebocytes (HA), able to recognize and ingest foreign particles or cells, and round macrophage-like cells (MLC) deriving from HA which withdraw their cytoplasmic projections after the ingestion of non-self material [16].

At the generation change, a significant change in the distribution of circulating phagocytes with respect to mid-cycle, i.e., phases of the blastogenetic cycle that are more than one day from the preceding and following take-over [15,17], occurs: the frequency of circulating HA falls from 25–42% to 12–25%; at the same time, the percentage of MLC with ingested materials inside their vacuoles rises from 4–10% to 20–30% [16,18]. In addition, during the blastogenetic cycle, the amount of dying hemocytes with apoptotic features increases abruptly from 2–5% at mid-cycle to about 30% at take-over. They are characterized by DNA fragmentation, phosphatidylserine (PS) exposure on the outer layer of plasma membrane and activation of caspase-9 and -3 [18,19]; recognition of PS is required for the phagocytes to clear dying cells [18]. New young, undifferentiated cells, released from unidentified hemopoietic sites, enter the circulation at this phase [19].

However, despite the increasing number of studies on apoptosis in *Botryllus*, uncertainties and doubts still persist on the biochemical pathways involved, the modalities of recognition of effete cells and the fate of phagocytes. With the aim to clarify some of these aspects, we carried out new morphological, cytofluorimetrical and biochemical studies, useful for a better characterization of *Botryllus* apoptosis. Our results indicate that both intrinsic and extrinsic pathways are involved in cell death induction and are probably connected by Bid, a member of the "BH3-only protein" subgroup of the Bcl-2 family [20]. In addition, they suggest that oxidative stress represent the key event in triggering the apoptotic cascade, at least in phagocytes.

2. Materials and methods

2.1. Animals

Colonies of *B. schlosseri* were collected in the Lagoon of Venice and allowed to adhere to glass slides (5 cm × 5 cm). They were reared in aquaria filled with filtered seawater (FSW) at the temperature of 19 °C, fed with Liquifry Marine (Interpet, Dorking, England) and the water was changed every other day.

2.2. Hemocyte collection

Blood was collected with glass micropipettes from the peripheral vessel of colonies (which had been previously rinsed in 12.9 mM Na-citrate in FSW, pH 7.5, to prevent hemocyte clumping), that were punctured with fine tungsten needles. Cells were collected with glass micropipettes and centrifuged at 780 × *g* for 10 min. Pellets were resuspended in FSW, in 1.5-ml vials, to a final concentration of 10^6 cells/ml. Sixty microlitres of hemocyte suspension were placed in the centre of culture chambers prepared as described elsewhere [22,23] and left to adhere to coverslips for 30 min at room temperature. Cell mortality was evaluated with the Trypan blue exclusion assay [24] and was less than 5% after 2 h of incubation at room temperature.

At least three colonies (10–20 systems in size) were used for each experiment; each colony was previously cut into subclones of 4–5 systems and each subclone was bled during the course of the blastogenetic cycle, in order to obtain blood samples from take-over and mid-cycle phases.

2.3. Transmission electron microscopy

Colonies at take-over were fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate buffer (CB), pH 7.4, plus 0.29 M NaCl. After washing in CB and postfixation in 1% OsO_4 in 0.2 M cacodylate buffer, specimens were dehydrated and embedded in Epon Araldite. Transversal and sagittal serial sections (1 μm) of zooids were counterstained with Toluidine blue; thin sections (90 nm) were stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope operating at 75 kV. Photographs were digitalized with an Epson Perfections Scanner 1200S and were collected and typeset in Corel Draw X3.

2.4. Immunocytochemistry on hemocytes

Hemocyte monolayers were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde in 0.4 M cacodylate buffer containing 0.29 M NaCl and 29 mM sucrose, treated for 30 min with 1% H_2O_2 in phosphate-buffered saline (PBS: 0.13 M NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.7 mM KH_2PO_4; pH 7.4) to block endogenous peroxidase, incubated for 30 min in 5% powdered milk and 5% fetal calf serum in PBS to reduce nonspecific binding and finally incubated overnight with 10 μg/ml primary polyclonal antibodies. The following primary polyclonal antibodies, raised against human antigens, were assayed: anti-Bcl-2, anti-Bax, anti-caspase-3, anti-caspase-7, anti-caspase-8, anti-Fas and anti-FasL. They were purchased from Santa Cruz Biotech (anti-Bcl-2 and anti-Bax), Oncogene (anti-Fas, anti-caspase-3), GeneTex (anti-FasL), Sigma (anti-caspase-7), Calbiochem (anti-caspase-8). In control preparations, antibodies were pre-incubated for 2 h with colony lysates or HEP-G2 cells as described below.

Hemocytes were then washed in PBS, incubated in goat biotinylated anti-rabbit-IgG antibody (Santa Cruz Biotech, 10 μg/ml) for 30 min, washed again and incubated for 30 min in avidin–biotin–peroxidase complex (ABC, Vector Laboratories). After thorough washing in PBS, they were finally incubated for 5 min in a solution of 0.63 mM 3,3′-diaminobenzidine (DAB), containing 4% hydrogen peroxide, and mounted with Acquovitrex (Carlo Erba). In controls, primary antibodies were substituted with absorbed primary antibodies prepared as described below; pre-immune serum was used in negative controls. Positive sites appeared brown.

2.5. Lectin cytochemistry

Fixed hemocytes were treated for 30 min with 1% H_2O_2 in PBS followed by 30 min in 5% powdered milk and 5% fetal calf serum in