CARYOLOGIA Vol. 59, no. 4: 350-353, 2006

Apoptosis and recognition of apoptotic cells in colonial ascidians

Ballarin Loriano* and Francesca Cima

Dipartimento di Biologia, Università di Padova - Via Ugo Bassi 58/B Padova

Abstract — Colonies of the compound ascidian *Botryllus schlosseri* undergo a cyclical generation change, known as take-over, during which cell death occurs and senescent cells are recognised and rapidly ingested by circulating phagocytes. We re-examined, by means of morphological and cytochemical approaches, the cell death process which occurs during take-over, focussing on circulating haemocytes as reference tissue. Since dying cells are actively ingested by circulating phagocytes, we studied the expression of phosphatidylserine and CD36 in senescent cells and phagocytes, respectively, and their role in the recognition of effete cells by phagocytes.

Key words: apoptosis, *Botryllus*, phagocytosis, recognition.

INTRODUCTION

Invertebrate Chordata, collectively named Protochordata, are filter-feeding marine animals and include Cephalochordata and Urochordata. The latter are characterised by a planktonic or sedentary life-style and owe their alternative name of Tunicates to the test or tunic, the peculiar covering in which the body is embedded.

Ascidians, or sea-squirts, are sessile, marine Tunicates diffuse throughout the world, mainly in shallow tropical and temperate waters. About 3,000 species have been reported so far, both solitary and colonial. In colonial ascidians, zooids share a common tunic and are frequently interconnected by a common circulation.

In recent years, solitary ascidian species (*Ciona intestinalis*, *Ciona savignyi*, *Halocynthia roretzi*) have emerged as model organisms for the study of the molecular control of embryogenesis and differentiation of specific cell lines (Oda-Ishii *et al.* 2005; Passamanek and Di Gregorio 2005; Satoh and Levine 2005) and their genome has been partially or fully sequenced (Dehal et al. 2002; Yokobori *et al.* 2003).

Although less fully investigated at the molecular level, compound ascidians offer the advantage of asexual reproduction, which means that, in the same organism and at various levels (morphological, biochemical, molecular), different developmental pathways (embryogenesis and blastogenesis) lead to the same end product: the adult, filter-feeding zooid can be compared

The colonial ascidian *Botryllus schlosseri* has emerged as a model organism for the study of asexual reproduction, natural apoptosis, and clearance of senescent cells (Lauzon *et al.* 1992; 1993; Cima *et al.* 2003), allorecognition (Rinkevich 1992; Cima et al. 2004), immunobiology (Ballarin *et al.* 2002; Ballarin and Burighel 2006) and regeneration (Tiozzo *et al.* 2005).

B. schlosseri colonies form new zooids by blastogenesis, through the formation of palleal buds which progressively grow and mature until new adults are formed. Three blastogenic generations are commonly found in *Botryllus* colonies: adult, filtering zooids, their buds and budlets on buds. At a temperature of 19°C, adult zooids remain active for about one week; then they contract, close their siphons, and are gradually resorbed, being replaced by a new generation of adult zooids, represented by buds which reach functional maturity, open their siphons and begin their filtering activity (Berrill 1941; Sabbadin 1955; Burighel and Schiavinato 1984; Lauzon *et al.* 1992). During resorption, or take-over, massive cell death occurs in tissues of regressing zooid which, according to morphological data (Burighel and Schiavinato 1984; Lauzon *et al.* 1992) has been ascribed to apoptosis. In addition, effete cells are identified and ingested by circulating phagocytes which infiltrate zooid tissues.

* Corresponding author: e-mail loriano.ballarin@unipd.it

The present report re-examines the main cellular events occurring during take-over, considering circulating phagocytes as reference tissue, using both morphological and immunocytochemical approaches, with particular attention to the interaction between senescent cells and phagocytes.

MATERIALS AND METHODS

Colonies of the ascidian *Botryllus schlosseri* were collected in the Lagoon of Venice and reared, attached to glass slides, in aerated aquaria filled with filtered sea water (FSW) at a temperature of 19°C, and fed with Liquifry Marine (Liquifry, U.K.). Haemocytes were collected from colonies previously rinsed in FSW containing 0.38% Na citrate as anticlotting agent, after puncture of the tunic marginal vessel with a fine tungsten needle. Cells were centrifuged at 780 x g for 10 min and pellets were resuspended in FSW to yield a final concentration of 5 x 10^6 cells/ml.

Short-term haemocyte cultures were prepared as described elsewhere (Ballarin *et al.* 1994); phagocytes were classified according to Ballarin and Cima (2005). Haemocytes were fixed for 30 min in Sanfelice solution, stained with Pfitzner's safranin solution, and counterstained with Mayer's haematoxylin. Chromatin fragmentation was detected by the TUNEL reaction, whereas FITC-coupled annexin-V was used to detect cells expressing phosphatidylserine (PS) on their surface. Immunocytochemical staining with anti-CD36 monoclonal antibody was also performed on haemocytes fixed for 30 min in 4% paraformaldehyde in FSW, to evaluate the extent of the expression of the antigen on haemocytes during the blastogenetic cycle and the type of cells expressing it. The same antibody and the soluble PS agonist phospho-L-serine were used to study the role of CD36 and PS in the recognition of apoptotic cells by phagocytes.

RESULTS AND DISCUSSION

The cyclical generation change in colonies of *B. schlosseri*, known as regression, or take-over, allows the definition of a weekly blastogenetic cycle, beginning from the opening of the siphons of a new adult generation and the appearance of a new blastogenetic generation, and ending with resorption of adult zooids.

During take-over, circulating phagocytes massively infiltrate zooid tissues and engulf senescent cells (fig. 1a). As a consequence, with respect to intermediate or mid-cycle stages, as defined by Lauzon *et al.* (1992), take-over is characterised by a significant increase in the frequency of circulating phagocytes, showing globular morphology and containing ingested cells or cell debris (fig. 1b), whereas the frequency of amoeboid phagocytes (fig. 1c), representing mobile and active phagocytes, significantly decreases (fig. 2). In addition, the number of haemocytes showing nuclear condensation and recognised by annexin-V, and positive to the TUNEL reaction (all indices of apoptosis) significantly increases (fig. 3), as well as the frequency of circulating macrophage-like cells containing TUNEL-positive cells (Cima *et al.* 1996; 2003).

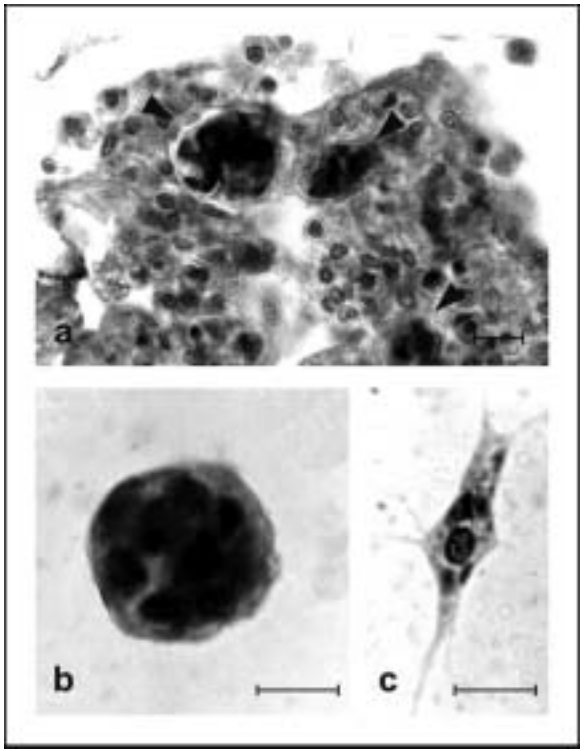

Fig. 1 — **a**: phagocytes containing ingested cells (arrowheads) in the tissues of senescent zooids; **b**: globular phagocyte containing ingested material inside its vacuoles; c: amoeboid phagocyte. Cells and tissues were stained with Pfitzner's safranin and Mayer's haematoxylin. Scale bar: 10 µm.

Phagocytes actively recognise senescent cells and ingest them. When living haemocytes were labelled with the fluorescent stain carboxyfluorescein diacetate and matched *in vitro* with haemocytes from the same colony, but at different stages of the colonial life-cycle, the number of phagocytes ingesting fluorescent cells was significantly

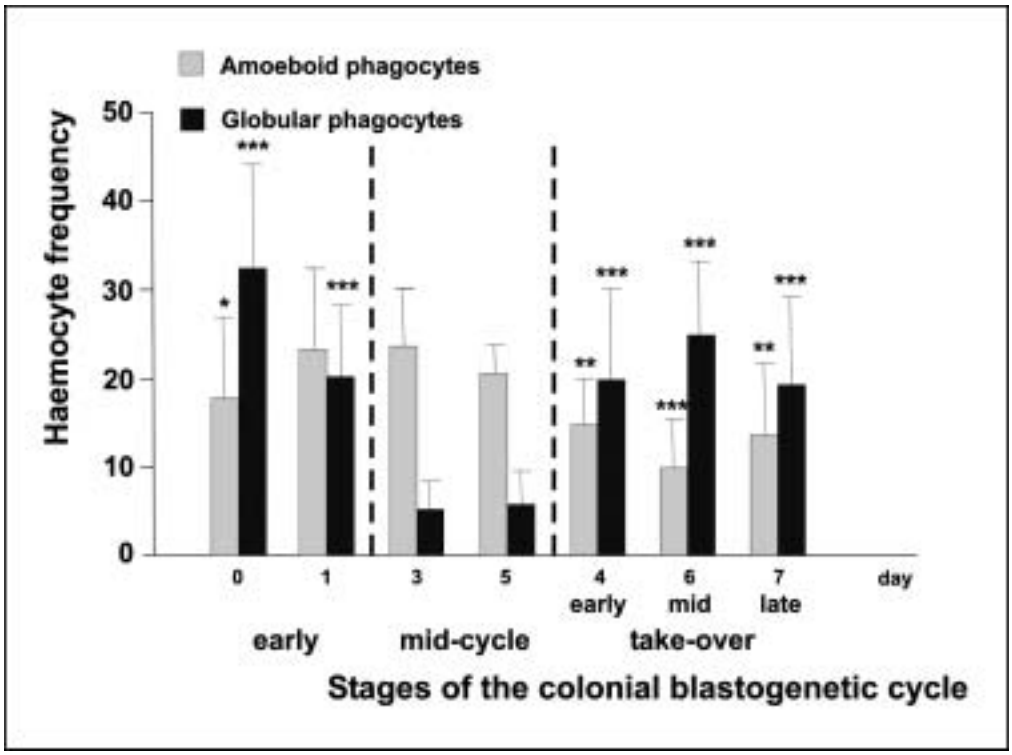

Fig. 2 — Frequency of amoeboid and globular phagocytes during various stages of the colonial blastogenetic cycle, starting from day 0 (beginning of a new cycle) to day 7 (end of the take-over). Significant differences with respect to mid-cycle stages (day 3-5) are marked by asterisks. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

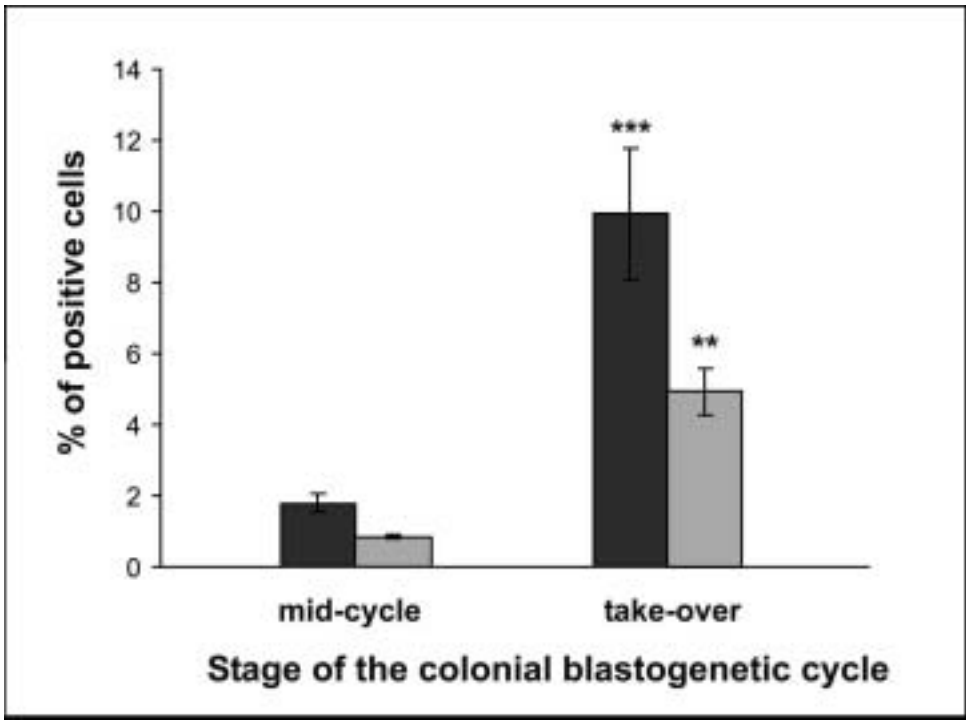

Fig. 3 — Frequency of circulating haemocytes showing positivity to annexin-V (dark grey) and TUNEL reaction (light grey) during mid-cycle and take-over. Asterisks indicate significant differences with respect to mid-cycle stages. **: $p < 0.01$; ***: $p < 0.001$.

higher if unlabelled haemocytes from mid-cycle stages were incubated together with labelled haemocytes from take-over, than in the case of the opposite combination (fig. 3). Non-professional phagocytes, mainly epithelial cells, also occasionally ingest senescent cells.

As regards the "eat-me" signals on effete cells allowing their recognition and clearance by circulating and occasional phagocytes, there is a progressive increase in haemocytes recognised by annexin-V from the beginning of the colonial life-cycle to take-over. PS seems to be involved in recognition, as the addition of phospho-L-serine, a soluble analogue of PS, inhibits *in vitro* phagocytosis of apoptotic cells. Oxidised plasma membrane lipids are also important in the interaction between phagocytes and senescent cells, as phagocytes cannot ingest effete cells in the presence of antioxidants, (Voskoboynik *et al*. 2004). CD36, part of the receptorial complex binding thrombospondin, a bridging molecule between phagocyte surface and apoptotic cells, is expressed in *Botryllus* phagocytes: the frequency of cells recognised by anti-CD36 antibodies significantly increases during take-over with respect to mid-cycle and the expression pattern changes from patchy distribution on the plasma membrane in mid-cycle to uniform staining of the phagocyte surface during take-over. In addition, anti-CD36 antibody significantly decreases the phagocytosis of effete cells, suggesting that the thrombospondin receptor plays a role in apoptotic cell removal by phagocytes in a manner similar to that described in Vertebrates (Cima *et al*. 2003).

On the whole, data obtained until now supports the idea that fundamental mechanisms for the recognition of apoptotic cells are well conserved throughout the evolution of Chordates.

REFERENCES

Ballarin L., Cima F., 2005 — *Cytochemical properties of* Botryllus schlosseri *haemocytes: indications for morpho-functional characterisation.* Eur. J. Histochem., 49: 255-264.

Ballarin L., Burighel P., 2006 — *RGD-containing molecules induce macropinocytosis in ascidian hyaline amoebocytes*. J. Invertebr. Pathol., 91: 124-130.

Ballarin L., Cima F., Sabbadin A., 1994 — *Phagocytosis in the colonial ascidian* Botryllus schlosseri. Dev. Comp. Immunol., 18: 467-481.

Ballarin L., Scanferla M., Cima F., Sabbadin A., 2002 — *Phagocyte spreading and phagocytosis in the compound ascidian* Botryllus schlosseri*: evidence for an integrin-like, RGD-dependent recognition mechanism*. Dev. Comp. Immunol., 26: 39-48.

Berrill N.J., 1941 — *The development of the bud in* Botryllus. Biol. Bull., 80: 169-184.

Burighel P, Schiavinato A., 1984 — *Degenerative regression of the digestive tract in the colonial ascidian* Botryllus schlosseri *(Pallas).* Cell Tissue Res., 235: 309-318.

Cima F., Basso G., Ballarin L., 2003 — *Apoptosis and phosphatidylserine-mediated recognition during the take-over phase of the colonial life-cycle in the ascidian* Botryllus schlosseri. Cell Tissue Res., 312: 369-376.

Cima F., Sabbadin A., Ballarin L., 2004 — *Cellular aspects of allorecognition in the compound ascidian* Botryllus schlosseri. Dev. Comp. Immunol., 28: 881-889.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A et al., 2002 - *The draft genome of* Ciona intestinalis*: insights into chordate and vertebrate origins.* Science, 298: 2157–2167.

Lauzon R.J., Ishizuka K.J., Weissman I.L., 1992 — *A cyclical, developmentally-regulated death phenomenon in a colonial urochordate.* Dev. Dyn., 194: 71–83.

Lauzon R.J., Patton C.W., Weissman I.L., 1993 — *A morphological and immunohistochemical study of programmed cell death in* Botryllus schlosseri *(Tunicata, Ascidiacea)*. Cell Tissue Res., 272: 115–127.

Oda-Ishii I., Bertrand V., Matsuo I., Lemaire P., Saiga H., 2005 — *Making very similar embryos with divergent genomes: conservation of regulatory mechanisms of Otx between the ascidians* Halocynthia roretzi *and* Ciona intestinalis. Development, 132: 1663-1674.

Passamaneck Y.J., Di Gregorio A., 2005 — Ciona intestinalis*: chordate development made simple*. Dev. Dyn., 233: 1-19.

Rinkevich B., 1992 — *Aspects of the incompatibility nature in botryllid ascidians*. Anim. Biol., 1: 17-28.

Sabbadin A., 1955 — *Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di* Botryllus schlosseri *(Pallas), in condizioni di laboratorio*. Boll. Zool., 22: 243-265.

Satoh N., Levine M., 2005 — *Surfing with the tunicates into the post-genome era*. Genes Dev., 19: 2407-2411.

Tiozzo S., Christiaen L., Deyts C., Manni L., Joly J.-S., Burighel P., 2005 — *Embryonic versus blastogenetic development in the compound ascidian* Botryllus schlosseri*: insights from Pitx expression patterns.* Dev. Dyn., 232: 468-478.

Voskoboynik A., Rinkevich B., Weiss A., Moiseeva E., Reznick A.Z., 2004 — *Macrophage involvement for successful degeneration of apoptotic organs in the colonial urochordate* Botryllus schlosseri. J. Exp. Biol., 207: 2409-2416.

Yokobori S.-I., Watanabe Y., Oshima T., 2003 — *Mitochondrial genome of* Ciona savignyi *(Urochordata, Ascidiacea, Enterogona): comparison of gene arrangement and tRNA genes with* Halocynthia roretzi *mitochondrial genome.* J. Mol. Evol., 57: 574–587.