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Disseminated neoplasia causes changes in ploidy and apoptosis frequency in cockles *Cerastoderma edule*

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ABSTRACT

A proliferative disease, usually referred as disseminated neoplasia (DN), shows high prevalence in some cockle *Cerastoderma edule* beds of Galicia (NW Spain). Chromosome counts, examination of chromosome morphology, DNA quantification by flow cytometry and estimation of apoptosis frequency by TUNEL assay and flow cytometry were performed in cockles with different DN severity. Metaphases obtained from gills of DN-affected cockles displayed a chromosome number ranging from 41 to 145, while normal number is 38; changes in chromosome morphology were also evident, with numerous microchromosomes occurring. Haemolymph flow cytometry analysis revealed difference in DNA content between healthy and DN-affected cockles. Aneuploid peaks ranged from 1.3n to 8.9n. Apoptosis frequency was determined on histological sections (TUNEL assay) and haemolymph samples (flow cytometry). Both techniques revealed neoplastic cells in apoptosis. The higher DN severity, the lower the percentage of apoptotic cells. According to flow cytometry results, the negative association between DN severity and apoptosis frequency only affected the neoplastic cells, whereas DN did not significantly affect the percentage of apoptotic hyalinocytes or apoptotic granulocytes.

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1. Introduction

Disseminated neoplasia (DN) has been reported in various marine bivalve mollusc world-wide (Peters, 1988; Barber, 2004). In cockles *Cerastoderma edule* DN was first reported in Ireland (Twomey and Mulcahy, 1984; 1988) and then in France (Poder and Auffret, 1986) and Spain (Villalba et al., 2001). In Galicia (NW Spain), Villalba et al. (2001) reported high mortalities in natural populations of *C. edule* associated with high prevalence and severity of DN. Romalde et al. (2007) reported evidence of a possible implication of a retroviral agent in the cockle DN.

Cell transformation in mollusc neoplasia involves changes in nucleic acids (Reno et al., 1994). Chromosomal abnormalities related to DN have been described in clams (Reno et al., 1994; Thiriot-Quiévreux and Wolowicz, 2001; Smolarz et al., 2005). These chromosomal disturbances have been related with abnormal ploidy (Reno et al., 1994; Smolarz et al., 2005). Hyperploidy and hypoploidy were detected in various mollusc species analysing the DNA content of neoplastic cells with flow cytomety (Moore et al., 1991; da Silva et al., 2005; Smolarz et al., 2005; Delaporte et al., 2008; Siah et al., 2008a, 2008b; Le Grand et al., 2010). Cell cycle analysis of DN-affected bivalves by flow cytometry has allowed establishing that relative amounts of DNA vary between normal and neoplastic cells, and these differences are not the same among species (Delaporte et al., 2008).

In normal conditions, there is a balance between cell proliferation and apoptosis. Apoptosis, also called programmed cell death, is an important factor in the progression of animal diseases. In healthy animals, apoptosis usually occurs when a cell is damaged, infected, senescent or otherwise of little use to the animal (Cohen et al., 1992; Sunila and LaBanca, 2003). Apoptosis has been described as a cell mechanism to prevent the proliferation of malignant neoplasia in vertebrates (Elmore, 2007). Differences in apoptosis between healthy and DN-affected individuals of the soft-shell clam *Mya arenaria* (Böttger et al., 2008) and the blue mussel *Mytilus edulis* (Galimany and Sunila, 2008) have been reported.

The present study evaluated changes in DNA content (combining flow cytometry and chromosome analysis), chromosome

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morphology and apoptosis frequency between DN-affected and non-affected cockles to further characterise this disease in cockles.

2. Materials and methods

2.1. Biological material and diagnosis

Cockles, *C. edule*, were collected from a natural bed in Cambados (Galicia, NW Spain, N 42° 30′, W 8° 49′) where a high DN prevalence had been detected (Carballal et al., 2001). All samples were maintained in a tank with running sea water for 24 h for the diagnosis and analyses. DN was diagnosed by examination of histology sections and haemolymph cell monolayers. Haemolymph was withdrawn from the adductor muscle of every cockle using a 21 gauge needle attached to a 2 ml syringe. Fifty µl of haemolymph were mixed with 100 µl of cold modified Alseveŕs anti-aggregate solution (Bachère et al., 1988) and cytocentrifuged onto slides (92×*g*, 5 min, 4 °C). The haemolymph cell monolayers were fixed and stained with the kit Hemacolor (Merck) and examined on an Olympus BX50 light microscope (bright field) for DN diagnosis. The cockles were ranked according to a scale of disease severity: non-affected (N0); low severity (N1), when individuals showed proportion of neoplastic cells lower than 15% in the haemolymph cell monolayers; moderate severity (N2), when the proportion ranged from 15% to 75%; and high severity (N3), when the proportion was higher than 75% (Díaz et al., 2010). In addition, a piece (5 mm thick) of tissue including visceral mass, gills, mantle and foot, was taken from every cockle; it was fixed in Davidson's solution, embedded in paraffin, sectioned with a rotary microtome and 5 µm thick sections were stained with Harriś haematoxylin and eosin. Histological sections were examined with light microscopy to diagnose.

2.2. Chromosome analysis

Metaphase chromosomes were obtained from gill tissue of five DN non-affected cockles and six cockles with high DN severity according to Insua and Thirioy-Quiévreux (1992). Neoplastic cells massively invade the connective tissue of many organs, gills among them, in advances stages of DN (Villalba et al., 2001; Díaz et al., 2010). The animals were maintained in 0.005% colchicine in sea water for 8 h. Then the gills were excised, placed in 50% and 25% seawater solution for 30 min each and fixed by three incubations of 20 min each in a freshly prepared mixture of absolute ethanol and acetic acid (3:1). Gill cells were dissociated in 50% acetic acid and the suspension obtained was dropped onto slides heated at 42 °C (Thiriot-Quiévreux and Ayraud, 1982). Metaphase chromosomes were stained with Giemsa (4%, pH 6.8) for 10 min. Chromosomes were examined on an Olympus BX50 light microscope (bright field).

2.3. Ploidy analysis by flow cytometry

Ploidy of haemolymph cells was analysed by flow cytometry with propidium iodide (PI, Sigma P4170) according to da Silva et al. (2005). PI binds DNA by intercalating between the bases with a stoichiometry of one dye per 4–5 base pairs of DNA. Haemolymph of 97 previously diagnosed cockles was fixed in 100% ethanol and stored at −20 °C. The haemolymph samples were distributed according to DN severity category: 30 cockles N0, 37 N1, 15 N2 and 15 N3. The samples were centrifuged (800*g*, 10 min, 4 °C) and the pellets were resuspended in 0.01 M Phosphate Buffer Saline (PBS) (2% NaCl, pH = 7.4) for 30 min at room temperature. Samples were centrifuged again and the pellet was resuspended in PBS. The cells were treated with PI (50 µm mL^{-1}) and Dnase-free Rnase A (Sigma R4875, 50 µm mL^{-1}) and incubated for 60 min at room temperature in the dark; then they were analysed using a BD FACS Calibur Flow Cytometer. Results were reported in lineal scale fluorescence levels. A total of 10,000 haemolymph cells were analysed for each sample. Flow cytometer data were analysed with WinMDI 2.8 software according to the procedure of da Silva et al. (2005).

2.4. Apoptosis assays

Apoptosis was quantified in histological sections and haemolymph samples with two different methods. Apoptotic cells in histological sections were detected through the TUNEL assay (Gavrieli et al., 1992), using the ApopTag® Plus Peroxidase *in situ* hybridization kit (Chemicon International). Histological sections involving gills, digestive gland, gonad, kidney and foot, of five cockles from each DN severity category were used to detect apoptotic cells *in situ*. Tissue sections (5 mm) were placed on positively charged slides; the sections were deparaffinised and rehydrated with ethanol, rinsed with phosphate buffered saline and digested with Proteinase K (20 µg mL^{-1}) for 15 min at room temperature. Samples were permeabilised with 0.5% of Triton X-100 for 10 min and quenched with 3% hydrogen peroxide for 5 min. The slides were treated with ApopTag® equilibration buffer, and incubated with terminal deoxynucleotidyl tranferase (TdT) enzyme and digoxigenin-conjugated nucleotides, in a humidified chamber, at 37 °C for 1 h. The reaction was stopped with ApopTag® strength stop/wash buffer. The tissues were treated with an anti-digoxigenin peroxidase conjugate in a humidified chamber at room temperature for 30 min. After wash in PBS, tissues were stained with peroxidase substrate and counterstained in 0.5% methyl green. The sections were dehydrated in N-butanol and mounted. The positive control was a histological section of normal female rodent mammary gland included in the ApopTag® Kit. Four replicates of negative control were used, one for each DN severity category, where TdT enzyme reagent was substituted for PBS. The apoptotic index was defined as the average percentage of positive staining cells after counting 100 cells in each of five microscope fields, that is counting 500 cells per cockle.

In addition, the percentage of apoptotic cells in haemolymph samples was analysed by flow cytometry using the PE-Annexin-V apoptosis detection kit (BD Pharmingen). Annexin-V conjugated to phycoerythrin flourochrome (PE-Annexin-V) recognises externalised phosphatil serine, a membrane phospholip restricted to the inner leaflet in normal cell but expressed on external surfaces of early stage apoptotic cells. 7-Amino-Actinomycin (7-AAD) was used in conjunction with PE-Annexin-V to identify apoptotic cells. Early apoptotic cells were labelled with PE-Annexin-V and dead and late apoptotic cells were labelled both with PE-Annexin-V and 7-ADD. In this study, 45 cockles were used; diagnosis for DN-severity showed that 20 cockles were N0, 9 N1, 7 N2 and 9 N3. The cell density (number of cells per ml) of the haemolymph of each cockle was estimated with a haemocytometer (Mallasez chamber) and a haemolymph volume containing 10^5 cells was taken from each cockle; then the cells were separated from plasma by centrifugation (800×*g*, 15 min, 4 °C), resuspended in 100 µl of binding buffer (BD Pharmigen) and incubated with 5 µl PE-Annexin-V and 5 µl of 7-AAD for 15 min at 4 °C in the dark. Following incubation, the cell suspension was diluted with 400 µl of binding buffer. Negative control involved cells that were incubated with binding buffer instead of 7-AAD and / or PE-Annexin-V and positive control consisted of Jurkat T cells and haemolymph cells treated with Camptothecin solution in DMSO (1 mM) for 6 h at 37 °C. A BD FACS Calibur Flow Cytometer was used for the analyses. Apoptotic cells were defined as PE-Annexin-V-positive staining and 7-AAD-negative staining. Additionally three cell types (granulocyte,

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