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Evidence of retroviral etiology for disseminated neoplasia in cockles (*Cerastoderma edule*)

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Abstract

Epizootiologic outbreaks of disseminated neoplasia have been reported in association with massive mortalities of various bivalve species. In cockles, *Cerastoderma edule*, this pathological condition was described in Ireland and France. Since 1997, different populations affected by this pathology have been detected in Galicia (NW Spain). Transmission electron microscopy allowed the visualization of virus-like particles in neoplastic cells, resembling a retrovirus-like agent. To confirm this hypothesis, we used a commercial kit for detection and quantification of reverse transcriptase (RT) activity, based on the use of bromo-deoxyuridine triphosphate (BrdUTP) and a BrdU binding antibody conjugated to alkaline phosphatase. In addition, we developed a product-enhanced RT assay using RNA of hepatitis A virus as a template. These two assays showed positive RT activity in 90.9 and 81.8% of samples, respectively, from cockles displaying disseminated neoplasia as determined by light microscopy. These results strongly support the hypothesis of retroviral etiology for this pathological condition.

Keywords: Disseminated neoplasia; Cockle; *Cerastoderma edule*; Reverse transcriptase; Retrovirus

1. Introduction

Disseminated neoplasia (DN) is a progressive and lethal condition of bivalves characterized by the presence of neoplastic cells in the animal's circulatory system and tissue spaces (Barber, 2004; Elston et al., 1992; Peters, 1988). The disease was first detected in oysters (*Crassostrea virginica* and *C. gigas*) by Farley (1969a), who described it as a "probable neoplastic disease of the hematopoietic system". Since then, similar cases have been reported worldwide in at least 20 other bivalve species, including the flat oyster (*Ostrea edulis*), mussel (*M. edulis*, *M. galloprovincialis* and *M. trossulus*), soft shell clams (*Mya arenaria* and *M. truncata*), and macoma clams (*Macoma* spp.) (Balouet et al., 1986; Barber, 2004; Bower et al., 1994; Ciocan and Sunila, 2005; Elston et al., 1992; Farley, 1969b; Leavitt et al., 1990; Mix, 1983; Moore and Elston, 1993; Neff et al., 1987; Peters, 1988).

In cockles (*Cerastoderma edule*), DN was first described in 1982 in Cork Harbour, Ireland (Twomey and Mulcahy, 1988a) and, at approximately the same time, it was also found in cockle populations from the north coast of Brittany, France (Auffret and Poder, 1986; Poder and Auffret, 1986). In recent years, this pathological condition was detected in this species in samples from Galicia (NW Spain) (Carballal et al., 2001; Villalba et al., 2001; Ordás and Figueras, 2005). In all of these cases, affected individuals showed a characteristic infiltration of the connective tissue in various organs by abnormal large anaplastic cells with high nucleus/cytoplasm ratios, diffuse chromatin patterns and pleomorphic nuclei (Auffret and Poder, 1986; Villalba et al., 2001). Swollen mitochondria and altered Golgi complexes are ultrastructural features also observed in these cells (Auffret and Poder, 1986). These alterations have also been detected in other mollusc species affected by DN (Barber, 2004).

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The factors responsible for causing DN have not been determined. It has been proposed that various pollutants (i.e. fuel or chlordane) might induce the development of DN (Farley et al., 1991; Yevich and Barszcz, 1976). Other authors hypothesized that sublethal levels of biotoxins or the presence of stressors, may play a role in the appearance of the disease (Balouet et al., 1986; Landsberg, 1996; Twomey and Mulcahy, 1988a), although, unfortunately, none of these hypotheses were supported by experimental data.

On the other hand, evidence of a possible viral etiology was observed by several authors. First of all, DN has proven to be transmissible between individuals in different bivalve species including mussel, soft-shell clam and cockle (Collins and Mulcahy, 2003; Elston et al., 1988; Kent et al., 1991; Sunila, 1994; Twomey and Mulcahy, 1988b; Weinberg et al., 1997) indicating that an infective agent could be involved. To our knowledge, few works described viral agents associated with this disorder. Oprandy et al. (1981) reported the isolation of a virus, similar to retrovirus type-B, from neoplastic soft shell clams, which was able to reproduce the disease in healthy clams. These authors also reported the induction of viral replication and induction of neoplasia in clams with 5-bromodeoxyuridine, an inductor of the expression of retroviral particles in some mammalian cell culture systems (Oprandy and Chang, 1983). Some years later, Sunila (1994) isolated an icosahedral viral agent from the ova of soft shell clams with DN, being another potential candidate for the disease etiology. However, similar descriptions are lacking for other bivalve species. Thus, ultrastructural examination of neoplastic cells from mussels and cockles revealed no pathogenic agents of any type (Auffret and Poder, 1986; Elston et al., 1992).

Infectious retroviruses are important causative agents of human and animal disease (Coffin et al., 1997). They all possess a characteristic enzyme essential for replication, reverse transcriptase (RT), and can thus be detected by assays for this activity (Coffin et al., 1997; Pyra et al., 1994). RT is a retroviral-RNA dependent DNA-polymerase that transcribes the viral genomic RNA into a double-stranded DNA copy, which integrates into the host genome as a provirus. Investigations on DN in bivalves suggested the presence of reverse transcriptase activity in tissues from neoplastic individuals, but not in control samples, reinforcing again the hypothesis of a potential retroviral etiology of the disorder (House et al., 1998; Medina et al., 1993).

In this work, different direct and indirect techniques were employed to obtain evidence of the possible implication of a retroviral agent in the appearance and development of DN in cockles from Galicia.

2. Material and methods

2.1. Samples

A total of 41 cockles, *Cerastoderma edule*, obtained from Galicia (NW Spain) from November 2002 to March 2004 were included in this study. Cockles were collected from 5 sites located in Ría de Camariñas (site 1), Ría de Noia (sites 2 and 3), and Ría de Vilagarcía (sites 4 and 5). Upon arrival in the lab, hemolymph samples (0.1 ml) were collected from the anterior adductor muscle sinus and kept at −20 °C until use. Then, cockles were opened shucked and dissected to separate the different tissues that were divided proportionally for the subsequent studies.

2.2. Histological techniques and transmission electron microscopy (TEM)

Five millimeter thick sections were taken from every specimen, fixed in Davidson's solution and embedded in paraffin as previously described (Villalba et al., 2001). Five micrometer sections were stained with Harris' hematoxylin and eosin (Howard et al., 1983). In addition, small pieces (1–2 mm thick) were cut and fixed in Carson's solution (Pearse, 1980). After the examination of histological sections by light microcopy (LM), those cockles affected by DN were selected and processed for electron microscopy.

For TEM, small fragments of the different tissues were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.6 for 2 h at 4 °C, washed for 2 h at 4 °C in the same buffer, and postfixed in buffered 2% osmium tetroxide for 2 h at 4 °C. The fragments were dehydrated through a graded series of ethanol and embedded in Epon. In the case of hemolymph samples, pools of hemolymph from several individuals (6–10) previously classified as neoplastic by cytospin smear (Carballal et al., 1997), were treated as before with the exception that after fixation in osmium, these samples were pre-embedded in agar, cut in small pieces, and then embedded in Epon. Ultrathin sections (70–90 nm) were double stained with uranyl acetate and lead citrate and observed under a JEOL 100 CXII transmission electron microscope operating at 80 kV.

2.3. Non-radiactive detection and quantification of RT activity

Detection of RT activity was performed employing the RetroSys™ RT activity kit (Innovagen), a commercial kit developed for the quantification of retroviral RT activity in cell culture media and plasma samples. It is a non-radioactive 96-well microtitre plate reverse transcriptase (RT) assay, based on the use of covalently bound riboadenosine homopolymer in the wells and 5-bromodeoxyuridined 5′-triphosphate (BrdUTP) as dNTP (Ekstrand et al., 1996). The kit was used following the manufacturer's instructions.

Pieces of gonad and mantle (50–100 mg) from cockles were separately homogenized in sterile saline solution (0.85% NaCl; 1:1 wt/vol), centrifuged at 12,000 rpm for 5 min, and the supernatants collected. Hemolymph samples were centrifuged as above and the supernatants saved. All supernatants were stored at −20 °C until use.

For screening for RT actvity, 40 μl of each filtrate was added to the respective well in the poly(A) plate, previously filled with 150 μl of the reaction mixture (containing all the