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Expression of mutant protein p53 and Hsp70 and Hsp90 chaperones in cockles *Cerastoderma edule* affected by neoplasia

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ABSTRACT: High prevalence of disseminated neoplasia has been found in cockles *Cerastoderma edule* of Galicia (NW Spain). Disseminated neoplasia has been associated with high mortalities of various bivalve species. In vertebrates, proteins such as p53 and heat shock proteins (HSPs) play important roles in carcinogenesis. The protein p53 has been detected in neoplastic cells of bivalve molluscs such as *Mytilus edulis*, *Mytilus trossulus*, *Mya arenaria*, *Spisula solidissima*, *Crassostrea rhizophorae* and *Crassostrea gigas*. In this study, western blotting analyses were used to test the expression of Hsp70, Hsp90 and mutant p53 proteins in the cells and plasma of the haemolymph of cockles showing various intensities of neoplasia. Disseminated neoplasia was previously diagnosed by examination of stained haemolymph monolayers with light microscopy. In the present study, mutant p53 was detected in haemolymph cells of cockles diagnosed as affected by moderate and heavy neoplasia intensity, whereas it was not detected in cockles with either no or light neoplasia. The higher the neoplasia intensity, the higher the levels of Hsp70 and Hsp90. These proteins were not found in plasma. The results reveal the possible association between p53 and HSPs in neoplastic cells of cockles, which could prevent p53 from carrying out its functions, as occurs in human cancers.

KEY WORDS: Disseminated neoplasia · p53 · Hsp70 · Hsp90 · *Cerastoderma edule*

INTRODUCTION

Disseminated neoplasia affects various bivalve mollusc species worldwide (Peters 1988, Barber 2004). It is characterised by infiltration of the connective tissue of various organs by neoplastic cells, whose tissue of origin is controversial in many cases, having been proposed by some authors as deriving from haemocytes or with haemopoietic origin (Barber 2004). In cockles *Cerastoderma edule*, this disease was first detected in 1982 in Cork Harbour, Ireland (Twomey & Mulcahy 1984), and was later found in cockle populations from the north coast of Brittany, France (Auffret & Poder 1986, Poder & Auffret 1986). The disease was also detected in cockles from Galicia (NW Spain) (Carballal et al. 2001) and high cockle mortalities were associated with high prevalence and severity of this pathology (Villalba et al. 2001). In cockles, neoplastic cells are larger than normal haemocytes, and show a rounded and enlarged nucleus with a prominent nucleolus and stippled chromatin and a high nucleus/cytoplasm ratio. Numerous mitotic figures are seen in these cells. In early stages of the disease, few neoplastic cells are seen in the haemolymph as they are infiltrated in the connective tissue of some organs; as disease progresses, these cells divide actively, replace haemocytes and fill blood vessels, and massively infiltrate the connective tissue of most organs (Carballal et al. 2001, Villalba et al. 2001). With regard to the aetiology, some studies have related this mollusc condition with various pollutants (Yevich & Barszcz 1976, Farley et al. 1991) and others have suggested a viral aetiology (Oprandy et al. 1981, Oprandy & Chang 1983, Sunila 1994). Recently, Romalde et al. (2007) detected virus-like particles and reverse transcriptase activity in Galician cockles affected by disseminated neoplasia, thus

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supporting a viral aetiology. This disease has been proven to be transmissible between cockles (Twomey & Mulcahy 1988, Collins & Mulcahy 2003), which is consistent with a viral aetiology.

Tumour suppressor protein p53 has been widely studied in human cancers, and is related to the control of the cell cycle, DNA binding, transcriptional activation and repression and apoptosis. This tumour suppressor activity can be neutralized by mutations (Royds & Iacopetta 2006) or the interaction with other cellular proteins, such as MDM2, E1 and E6 (Barker et al. 1997). Mutated p53 has been detected in more than 50% of human cancers.

Heat shock proteins (HSPs) are synthesized by cells in response to various stress situations: oxidative stress, exposure to heavy metals, pathological conditions, inflammations, mutant proteins (Jolly & Morimoto 2000), variation in temperature, deficiency of nutrients, UV radiation and chemicals (Pockley 2003). The functions attributed to HSPs include: preventing protein aggregates under physical stress, serving as molecular chaperones in protein transport between cell organelles, and contributing to the folding of nascent and altered proteins (Robert 2003). HSP interference with apoptotic signalling was confirmed with observations that high levels of HSPs are often detected in human tumours (Jolly & Morimoto 2000). For example, lower levels of Hsp70 and Hsp90 are expressed in non-cancerous tissues compared to tumorous tissues (Helmbrecht et al. 2000), and Hsp70 inhibition stopped the proliferation of tumoral cells and induced apoptosis (Jäättelä et al. 1998). The Hsp70 family has been associated with key molecules in the cell cycle such as p53, Cdk4, pRb and p27. The activity of these chaperones could influence the tumour genesis-regulating proteins of the cell cycle. Studies in humans (Helmbrecht et al. 2000, Zylicz et al. 2001) have shown that mutant p53 tumour suppressor protein co-immunoprecipitates with members of the Hsp70 and Hsp90 families. Mutant p53 could form simple chaperones with Hsp70 and Hsp40, but the conformational mutant of p53, which possesses a low affinity towards Hsp90, can form a stable multichaperone complex in which Hsp90 is bound to mutant p53 indirectly (mut p53-Hsp40-Hsp70-Hop-Hsp90) (Zylicz et al. 2001). These chaperones are responsible for the stabilization and sequestration of p53 to the cytoplasm, and they inhibit the ability of the murine double minute oncogene (Mdm2) to promote p53 ubiquitination and degradation (Helmbrecht et al. 2000).

Homologues for human p53 and the p53 family have been cloned in several bivalves, e.g. *Mya arenaria* (Kelley et al. 2001), *Spisula solidissima* (Cox et al. 2003), *Crassostrea rhizohorae* (GenBank accession no. AY442309), *Crassostrea gigas* (GenBank AM236465), *Mytilus edulis* (Ciocan & Rotchel 2005, Muttray et al. 2005) and *Mytilus trossulus* (Muttray et al. 2005), and have shown highly conserved regions. This indicates that these proteins may function similarly to human proteins. Other genes related to p53 or cell cycle control have been sequenced in molluscs, such as the *ras* gene in *Mytilus edulis* (Ciocan & Rotchell 2005) and *Mytilus trossulus* (Ciocan et al. 2006) and E3 ubiquitin-protein ligase in *Mya arenaria*, which mediates degradation of cell-cycle regulatory proteins, such as p53 (Kelley & Van Beneden 2000).

As in humans, mutated p53 was expressed by tumour cells from *Mya arenaria* with advanced disseminated neoplasia, but it was not present in cells from non-affected clams (Barker et al. 1997). Conversely, normal p53 occurred in lower levels in the reproductive tissue of gonad neoplasia-affected *Mya arenaria* than in non-affected clams, concomitant with a higher concentration of E3 in the gonad neoplasia-affected clams (Olberding et al. 2004). The proteins p63 and p73, both in the p53 family, have been previously analysed in *Mya arenaria* (Kelley et al. 2001, Stephens et al. 2001) and *Mytilus edulis* (St-Jean et al. 2005, Muttray et al. 2007), and differences in the proteins levels between neoplasia affected and non-affected individuals were found.

HSPs have been studied in molluscs (Sanders 1993, Snyder et al. 2001), but their association with particular proteins has been scarcely reported in molluscs. The chaperoning function of mortalin, a member of the Hsp70 family of proteins, has been described in leukemic clams *Mya arenaria* (Walker et al. 2006). Two variants of mortalin protein have been found in the clam—a full-length and a truncated variant—both of which are complexed in the cytoplasm of leukemic clam hemocytes with p53 (Böttger et al. 2008).

In the present study, the expression of mutated p53, Hsp70 and Hsp90 in both the cytosol of haemolymph cells and plasma of cockles with different stages of disseminated neoplasia was analyzed to evaluate the involvement of these proteins in carcinogenesis.

MATERIALS AND METHODS

Biological material and diagnosis. Cockles *Cerastoderma edule* (n = 452) were collected in July 2006 from a sand flat in Cambados (Galicia, Spain) where a high disseminated neoplasia prevalence was previously detected (Carballal et al. 2001). Water temperature and salinity at sampling were 21°C and 34 ppt, respectively. Haemolymph was withdrawn from the adductor muscle of every cockle using a 21 gauge needle attached to a 2 ml syringe. An aliquot of the haemolymph was used to count the number of cells in the haemo-

lymph with a Malassez haemocytometer. Another aliquot was used to produce a cell monolayer onto a slide by cytocentrifugation. The haemolymph cell monolayers were fixed and stained with the Hemacolor® kit (Merck) and examined with light microscopy for disease diagnosis. The remaining haemolymph was divided into volumes containing 10^6 cells; cells were then separated from plasma by centrifugation (800 × *g*, 15 min, 4°C). Samples of plasma and cells were frozen and lyophilised (Telstar Cryodos-80) and then stored at −80°C until used for analyses. The cockles were ranked according to the disseminated neoplasia intensity using the following scale: non-affected (N0); low severity (N1), when individuals showed proportions 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% (Fig. 1). This scale was established according to the correspondence between previous observations of histological sections and haemolymph cell monolayers of the same individuals using a modification of the scale proposed by Cooper et al. (1982). A similar scale was utilised to diagnose disseminated neoplasia in *Mya arenaria* by indirect peroxidise staining of haemocyte preparations treated with monoclonal antibodies specific for neoplastic cells (Leavitt et al. 1990). In addition, a piece of meat (5 mm thick) including visceral mass, gills, mantle and foot was taken from every cockle, fixed in Davidson's solution and embedded in paraffin; 5 µm thick sections were stained with Harris' hematoxylin and eosin and examined with light microscopy to diagnose other pathological conditions that could interfere with the analyses.

Reagents for western blotting. Mouse monoclonal antibody Pab240 raised against human mutant p53 was purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody raised against heat stress cognate 72 of the oyster *Crassostrea gigas* (anti-CgHsc72) (Boutet et al. 2003a) was provided by Dr. Boutet. Mouse monoclonal antibody raised against human Hsp90 was purchased from Stressgen Bioreagents; this antibody also binds Hsp 90 from mussels and scallops according to the manufacturer's specification sheet. Anti-mouse IgG and anti-rabbit IgG peroxidase conjugated monoclonal antibodies were purchased from Sigma. The chemiluminiscence kit ECL® western detection system was purchased from Amersham. All other reagents were of the highest quality available.

Western blot analysis. At least 3 cockles of each intensity (total 15 cockles) were analysed for each antibody by western blotting. Protein concentrations in samples of cells and plasma were determined by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard and Bio-safe Coomassie stain (Bio-Rad). For the western blotting analysis, cells and plasma were resuspended in 150 µl of lysis buffer (20 mM N-[2-hydroxyethyl] piperazine-N′-[2-athanosulfonic acid] [HEPES], 5 mM EDTA, 20 mM pyrophosphate, 30 mM NaF and 0.5% Triton X-100, pH 7.4), kept at 4°C for 15 min and then centrifuged at 3200 × *g*, 10 min, 4°C ; this lysis buffer allows the removal of the cytosolic proteins from cells (Cao et al. 2004). A volume of supernatant containing 15 µg of proteins was applied to each well of an electrophoresis device. After separation by 10% sodium dodecyl sulfate-poliacrylamide gel electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane (Immobilon-P Millipore) at 4°C using a Bio-Rad transfer blot apparatus (90 V, 4 h). Non-specific sites were blocked by incubation of the nitrocellulose membranes with skimmed milk. Antibodies dilutions were as follows: Pab240, 1:250; anti-CgHsc72, 1:125; anti-Hsp90, 1:500; and mouse or rabbit anti-IgG peroxidase conjugated, 1:1000. For signal detection the ECL detection system (Amersham Biosciences) was used. For the analysis of the bands obtained, the densitometer GS-800 (Bio-Rad) and Quantity-One software were used.

RESULTS

Histological examination did not show relevant pathological conditions or parasites other than disseminated neoplasia. Oocysts of the protozoan *Nematopsis* sp. were observed in all cockles, without obvious differences in infection intensity among cockles.

Western blot analysis revealed various bands when using the antibody Pab240 raised against human mutant p53; one band corresponding to 53 kDa was detected in haemolymph cells of cockles with heavy and moderate neoplasia intensity (Fig. 2A). The level of mutant p53 (the intensity of the band of 53 kDa) was similar for intensities N2 and N3, whereas it was not detected in cockles with N0 and N1 intensities (Fig. 2B). This protein was not detected in the plasma of cockles in any of the intensity categories.

A band corresponding to 70 kDa was detected by western blot analysis in haemolymph cells of cockles with different stages of disseminated neoplasia when using the anti-CgHsc72 antibody (Fig. 3A). The higher the neoplasia intensity, the higher the levels of Hsp70 (Fig. 3B). Hsp70 level was virtually nil in the cases of low-severity disseminated neoplasia. Hsp 70 protein was not detected in the plasma of cockles in any of the intensity categories. Western blots also revealed a second band of ~90 kDa in neoplasia-affected cockles that could be a p70-related protein.

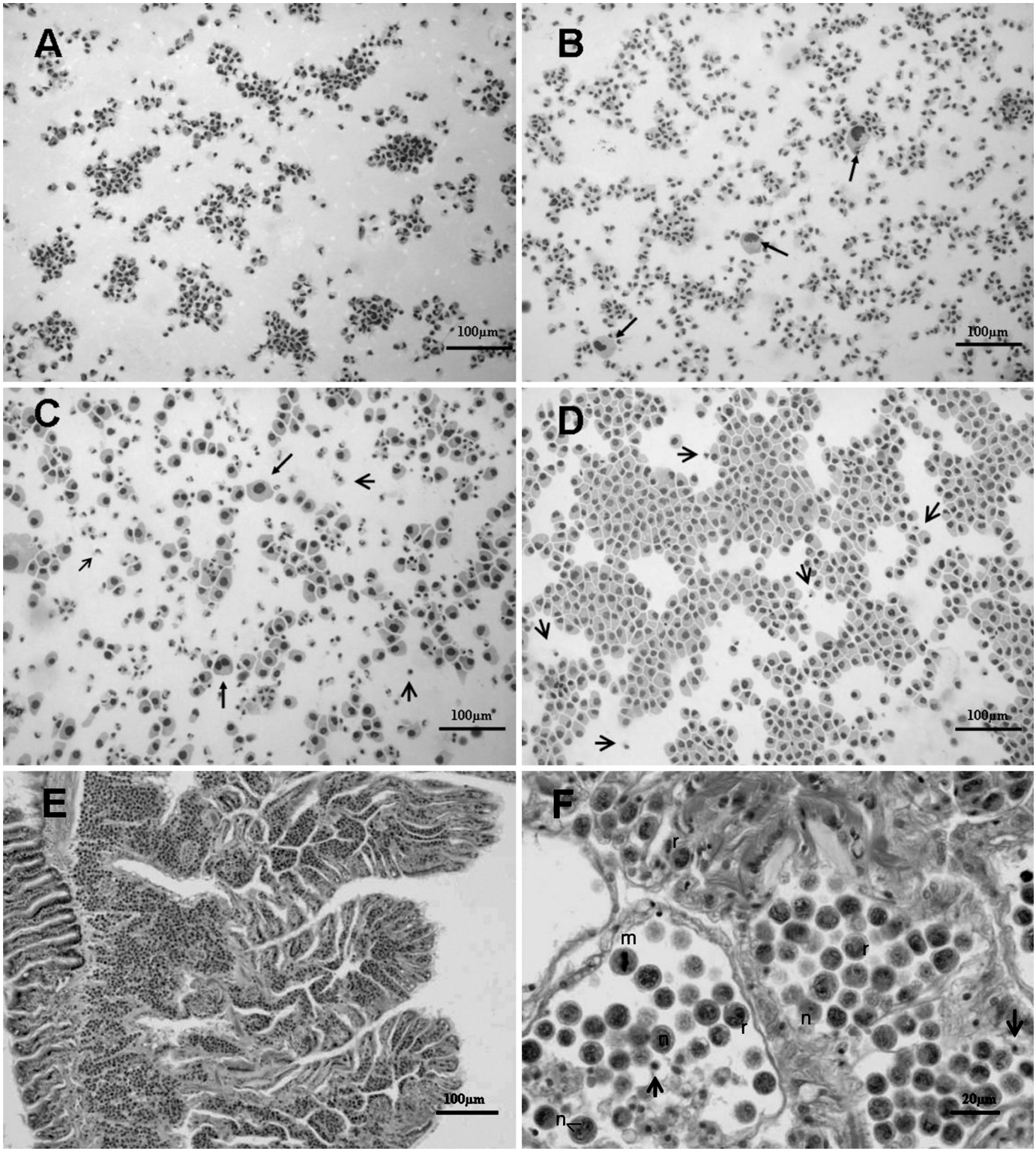

Fig. 1. Haemolymph cell monolayers of cockles with different severity of disseminated neoplasia: (A) non-affected, (B) low severity, (C) moderate severity and (D) high severity. (E) Histological section through the gills of a cockle with heavy infiltration of connective tissue by neoplastic cells, stained with hematoxylin and eosin. (F) Detail of neoplastic cells in the connective tissue of the gills, showing enlarged nucleus with characteristic kidney shape (r), prominent nucleolus (n), many cells with 2 nucleoli and mitotic figures (m). Arrows: neoplastic cells; arrowheads: haemocytes

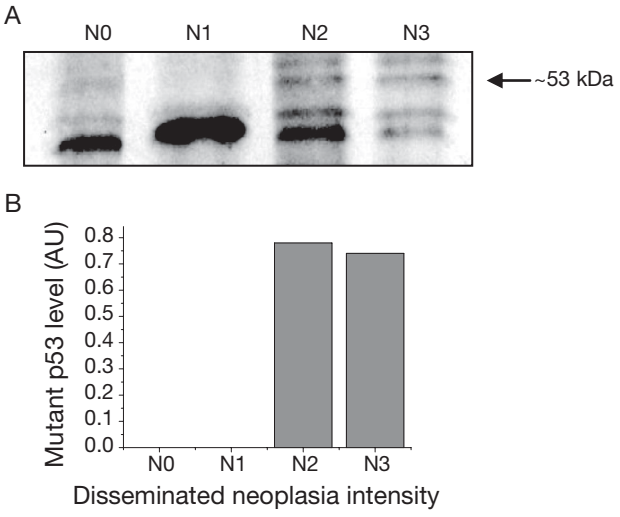

Fig. 2. (A) Western blotting and (B) intensity of mutant p53 protein expression measured by densitometric scanning (AU: arbitrary units) in haemolymph cells of *Cerastoderma edule* distributed in disseminated neoplasia intensity classes: non-disseminated neoplasia (N0); low-severity disseminated neoplasia (N1); moderate-severity disseminated neoplasia (N2); and high-severity disseminated neoplasia (N3)

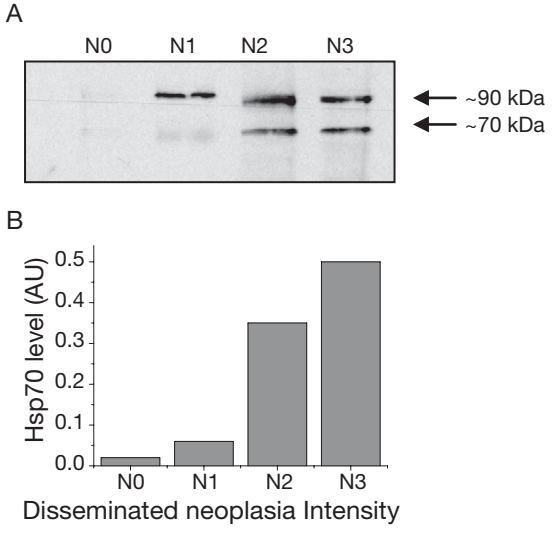

Fig. 3. (A) Western blotting and (B) intensity of Hsp70 protein expression measured by densitometric scanning (AU: arbitrary units) in haemolymph cells of *Cerastoderma edule* distributed in disseminated neoplasia intensity classes (see Fig. 2)

A band corresponding to 90 kDa was detected by western blot analysis in haemolymph cells of cockles with different stages of disseminated neoplasia, when using the antibody raised against Hsp90 (Fig. 4A). The protein levels detected were higher in neoplastic cockles than in cockles without this condition (Fig. 4B). The level of Hsp90 in the different stages of disseminated

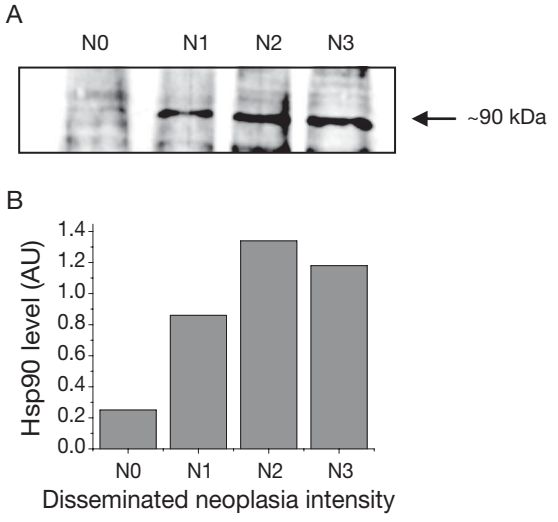

Fig. 4. (A) Western blotting and (B) intensity of Hsp90 protein expression measured by densitometric scanning (AU: arbitrary units) in haemolymph cells of *Cerastoderma edule* distributed in disseminated neoplasia intensity classes (see Fig. 2)

neoplasia (N0, N1, N2, N3) was higher than that of Hsp70. Hsp90 was not detected in the plasma of cockles in any of the intensity categories.

DISCUSSION

Western blot analysis revealed differential expression of mutant p53, Hsp 70 and Hsp 90 in cockles *Cerastoderma edule* according to the intensity of disseminated neoplasia. The quick proliferation of neoplastic cells in cockles with disseminated neoplasia leads to progressive replacement of normal haemocytes with the abnormal cells, which invade the connective tissue of most organs. Cockles become immunocompromised with a frequent fatal outcome, and the cell cycle in neoplastic cells is altered. Results from the present study demonstrate that cockles with moderate and heavy disseminated neoplasia express a 53 kDa protein corresponding to mutant p53, which may be related to alteration of the cell cycle. Alteration of p53 was found in softshell clams *Mya arenaria* in advanced stages of the disease (Barker et al. 1997). Nevertheless, mutant p53 was not detected in non-affected cockles or in those in the initial stage of disseminated neoplasia, as it occurred in *Mya arenaria*. Normal p53 was found in *Mya arenaria* normal haemocytes and in malignant cells (Kelley et al. 2001, Stephens et al. 2001), but the level of the normal protein was lower in neoplastic clams than in non-affected ones (Butler et al. 2004, Olberding et al. 2004). In the case of mussels *Mytilus edulis*, no 53 kDa protein was

detected in immunoblots when using antibodies against p53 in the haemolymph of either disseminated neoplasia-affected or non-affected mussels (St-Jean et al. 2005).

The p53 gene has been cloned in *Mya arenaria* (GenBank 45237–U45238), *Mytilus edulis* (Ciocan & Rotchel 2005) and *Mytilus trossulus* (Muttray et al. 2005). The p53 genes of humans and molluscs are closely related and include highly conserved regions such as the transcriptional activation domain, Mdm2 binding site, phosphorylation site, proline-rich domain, DNA binding domains, nuclear import and export signals and the tetramerization domain (Kelley et al. 2001). Other studies suggest sequence conservation between human and clam p53 proteins could have functional similarities. Holbrook et al. (2009) compared the activity of both proteins in a human p53 null cell line. Whereas human p53 induced markers of growth arrest or apoptosis, clam p53 has the capacity to interact with the human Mdm2 ubiquitin ligase, implying some functional similarity in p53 regulation (Holbrook et al. 2009). Most p53 mutations occur in the regions of the gene which are highly conserved through evolution (Greenblatt et al. 1994). Barker et al. (1997) detected a transversion of the p53 gene in clams *Mya arenaria* with advanced stage of disease, but mutations were not detected in *Mytilus trossulus* (Ciocan et al. 2006).

The procedure used in the present study involved isolation of cytosol proteins but not nuclear proteins. Therefore, the high mutant p53 levels detected corresponded to the cytosol of haemolymph cells of cockles with moderate or heavy disseminated neoplasia. Nuclear localization of p53 is essential for its function (Nikolaev et al. 2003). Accumulation of p53 in the cytoplasm of tumour cells, where it is not functional, was demonstrated in humans (Moll et al. 1992, 1995). In molluscs, Kelley et al. (2001) found that p53 and another protein of p53 family (p73) were located in the cytoplasm of leukemic *Mya arenaria* rather than in the nucleus as they were in normal cells. Walker et al. (2006) demonstrated that p53 and mortalin (an Hsp70 family protein) are localized and complexed in the cytoplasm of clam neoplastic cells, and that p53 is translocated to the nucleus after treatment with a cationic inhibitor of mortalin. Another way to reactivate p53 functions in *Mya arenaria* is by inducing a genotoxic stress, resulting in DNA damage that promotes transcription of the novo-p53 protein which, in turn, results in a nuclear translocation of the protein and promotion of the apoptosis of the damaged cell (Böttger et al. 2008).

HSPs are encoded by genes whose expression is substantially increased during stress conditions such as heat shock, inhibitors of energy metabolism, heavy metals, oxidative stress, fever or inflammation. During these conditions, HSPs increase cell survival by protecting and disaggregating stress-labile proteins (Skowyra et al. 1990), as well as through the proteolysis of the damaged proteins (Wickner et al. 1999). Under non-stress conditions, HSPs have multiple housekeeping functions, such as folding and translocating newly synthesized proteins; activation of specific regulatory proteins, including transcription factors, replication proteins and kinases; protein signalling; and tumour immunogenicity (Helmbrecht et al. 2000, Jolly & Morimoto 2000). Our results showed an increase of Hsp70 and Hsp90 synthesis in cockles affected by disseminated neoplasia. The levels of Hsp70 and Hsp90 in neoplastic cockles were higher than in normal ones. Two bands appeared on the membrane incubated with the polyclonal antibody anti-CgHsc72 at molecular weight of 70 and 90 kDa. The antibody anti-CgHsc72 was produced with isolated CgHsc72 protein of *Crassostrea gigas* (Boutet et al. 2003a), and it was tested to confirm its specificity of *C. gigas* oyster (Boutet et al. 2003a). Western blot analysis performed with this antibody in *Ostrea edulis* proteins showed reaction with 2 proteins of different molecular weight (68 and 70 kDa) (Boutet et al. 2003b).

HSP synthesis is rapidly up-regulated by various stressors (Robert 2003). Likely, the disseminated neoplasia was the cause of the increase in HSP level in the cockles in the present study. In humans, some HSPs may have become specialized as a response modality to stress associated with cancer (Robert 2003). HSPs are overexpressed in a wide range of human cancers (Ciocca & Calderwood 2005), but it is not known how and why HSPs become overexpressed in cancer. One hypothesis is that the physiopathological features of the tumour microenvironment tend toward HSP induction. Another hypothesis is that oncoproteins may appear during carcinogenesis and these mutated and conformationally altered proteins may elicit an HSP response (Ciocca & Calderwood 2005).

With regard to the involvement of HSPs in cell proliferation, Hsp70 and Hsp90 interact with important kinases of the mitogen-activated signal cascades, such as proto-oncogenic tyrosine kinases and mitogen-activated protein kinases (Gabai et al. 2000, Helmbrecht et al. 2000, Song et al. 2001). Interestingly, observations have also been made with regards to the ability of Hsp70 to negatively regulate various stages of the p53-dependent or -independent apoptotic pathways (Beere et al. 2000, Gabai et al. 2000, Li et al. 2000, Mosser et al. 2000, Nylandsted et al. 2000).

With regard to connection between p53 and HSPs, human mutant p53 tumour suppressor protein co-immunoprecipitates with Hsp70 and Hsp90. Such interactions lead to the formation of a p53 multi-chaperone complex that is responsible for the stabiliza-

tion and sequestration of p53 into the cytoplasm (Zylicz et al. 2001). The formation of these complexes prevents the mutant p53 from joining with Mdm2 by its ubiquitination (Helmbrecht et al. 2000). This fact could explain the high concentrations of p53, Hsp70 and Hsp90 found in cockles affected by disseminated neoplasia. Various studies have suggested that disseminated neoplasia in molluscs is associated with viral infection (Medina et al. 1993, House et al. 1998, Romalde et al. 2007), but it is not known how the virus changes the host metabolism. In some human cancers, the Simian virus 40, which encodes proteins that target p53 protein causes an indirect inactivation of p53 (Dobbelstein & Roth 1998).

The results obtained in the present study reveal the possible association between p53 and HSPs in neoplastic cells of cockles, which could prevent p53 from carrying out its functions, as occurs in human cancers. Further study is needed to confirm the association, including colocalisation in histological sections through immunohistochemistry and co-immunoprecipitation. To our knowledge, the present study is the first of its kind to study the modifications produced in the cell cycle of neoplastic cockles. More cell cycle regulators such as p21, p16, p57 and cyclin-dependent kinases have to be studied in order to understand which factors contribute to the abnormalities in the cell cycle of disseminated neoplasia-affected cockles.

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