Marine Biology (2006) 149: 25–34
DOI 10.1007/s00227-005-0212-1

RESEARCH ARTICLE

V. Matranga · A. Pinsino · M. Celi · G. Di Bella
A. Natoli

Impacts of UV-B radiation on short-term cultures of sea urchin coelomocytes

Received: 15 March 2005 / Accepted: 25 July 2005 / Published online: 10 January 2006

Abstract Three specialized cell types constitute the heterogeneous population present in the coelomic cavity fluid of sea urchins. The list includes: phagocytes, which undergo a stress-induced petaloid-filopodial transition, white or red amoebocytes and vibratile cells. As a whole, they act as the immune defense system of the sea urchin and respond to environmental and experimental challenge triggering specific stress markers. Here we extended our studies on coelomocytes short-term cultures by describing the morphology and occurrence of each cell type and analyzing their response to UV-B radiation at the biochemical level and with respect to DNA damage. The effects of different doses, ranging from 500 to 2,000 J/m^2, on cells, which were then cultured in vitro for 1–6 h were tested. As early as 1 h after irradiation we found an increase in the levels of the heat shock protein70 (hsp) stress marker. A peak at 1,000 J/m^2 corresponding to a 3.4-fold hsp70 increase over the levels of control coelomocytes was observed. Concurrently, we found an increase in the number of apoptotic nuclei detected by Hoechst staining, which varied from 7.1 to 15.6% in cells that were exposed to 500 and 2,000 J/m^2, respectively. On the basis of our findings we confirm sea urchin coelomocytes as a sensitive cell culture system for environmental studies and propose their use for the analysis on the effects of UV-B radiation.

Communicated by R. Cattaneo-Vietti, Genova

Physical and Chemical Impacts on Marine Organisms, a Bilateral Seminar Italy-Japan held in November 2004.

This paper is dedicated to the memory of Aurelia Koros, Ph. D., University of Pittsburg, who committed her scientific life to the study of natural killer cell antigens expressed in small lung carcinoma cells and sea urchin coelomocytes.

V. Matranga (✉) · A. Pinsino · M. Celi · G. D. Bella · A. Natoli
Consiglio Nazionale delle Ricerche, Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy", Palermo, Italy
E-mail: matranga@ibim.cnr.it
Tel.: +39-091-6809551
Fax: +39-091-6809557

Introduction

It is well known that solar radiation is filtered by the stratospheric ozone layer, which, functioning as a protective screen, reduces the amount of harmful ultraviolet radiation reaching the Earth's surface. The potential impacts of UV radiation on organisms living on our lands and oceans received little attention until significant depletion of stratospheric ozone, the so called ozone hole, due to the release of artificial damaging materials into the atmosphere, was observed in Antarctica in the early 80's (Farman et al. 1985; Nigel and Gwynn-Jones 2003). Although more recently a less severe ozone depletion has been observed (Kerr and McElroy 1993; Rex et al. 1997), a general increased awareness in world environmental issues has called for innovative and sensible methods of detection of putative harmful effects on living organisms. So far, the ultraviolet radiations UV-A (320–400 nm) and UV-B (280–320 nm) have been implicated as causing skin cancers (reviewed by Matsumura and Ananthaswamy 2004) and some eye disorders (reviewed by Cejkova et al. 2004), with UV-B being more damaging to skin and eyes than UV-A. In addition, it has been demonstrated that exposure to UV-B modulates immune responses in humans, causing opposite beneficial and adverse consequences (reviewed by Garssen and van Loveren 2001). One result known is that the exposure to UV-B decreases the severity of some immunologic diseases/conditions such as psoriasis and nickel allergy, but several adverse effects have also been determined such as: depressed resistance to tumors and infectious diseases, potential impairment of vaccination responses, and even increased severity of some autoimmune and allergic responses (Garssen and van Loveren 2001).

For millions of years marine animals, which were the first to appear on the Earth, have developed strong defense mechanisms and strategies for their survival against environmental pressure, host attacks and diseases. Nowadays, researchers are investigating on a

number of marine model systems whose ancient presumptive prototypes of the immune system could be instrumental in gaining insights on how to cope with adverse extreme conditions (Smith and Davidson 1992, 1994). Among marine invertebrates, the population of cells that live in the coelomic cavity fluid of adult echinoids, referred to as coelomocytes, has been considered as their genuine immune system, one of the most studied among higher invertebrate deuterostomes. Indeed, a variety of different functions are displayed by coelomocytes, which include: cell motility, cytotoxicity, phagocytosis, encapsulation, clotting, graft rejection, chemiotaxis, as well as the secretion of antifungal or antibacterial factors, lysins, agglutinins, and clotting factors (reviewed by Matranga 1996; Matranga et al. 2005). Coelomocytes are present as a heterogenous population whose composition in different cell types has been postulated to depend on the species used, as well as on patho-physiological conditions specific to each individual. Unfortunately, because of the controversial reports present in the literature displaying different nomenclatures, a failure in providing an unequivocal correspondence between names and functions of each of the morphotypes is still evident. In addition, a number of mistaken results, possibly due to the methodology used for obtaining cells, contributed to maintaining a certain ambiguity on the matter.

We have previously shown for the first time that sea urchin coelomocytes are responsive to a variety of stresses induced in vivo or ex vivo in the laboratory, such as high or low temperatures, acidic pH, heavy metals (cadmium) (Matranga et al. 2000, 2002, 2005; Angelini et al. 2003). Interestingly, we showed that coelomocytes ''sense'' environmental adverse conditions such as chemically polluted seawaters in field campaigns (Matranga et al. 2000, 2005; Pinsino et al. 2006). In most of the above mentioned studies, their response to stress was measured at the biochemical level by an increase in the expression of the hsp70 protein and characterized, at the cellular level, by the observation of a rise in the number of a subset of coelomocytes, containing a red pigment, earlier referred to as red spherule cells (Matranga et al. 2000).

The present study was undertaken to assess the potential impacts of UV-B radiation on sea urchin coelomocytes cultures that, representing a suitable marine model system, could be used as biological indicators of stress and DNA-damaging effects. Cells were first analyzed for their different morphologies and occurrence in order to better detail their classification into different subpopulations. Then, the levels of the hsp70 stress marker were measured in short-term cultures exposed to different UV-B doses. The number of apoptotic nuclei was determined and correlated to the time cells were maintained in culture and to the UV-B exposure doses used.

Materials and methods

Coelomocytes collection and exposure to UV-B radiation

Paracentrotus lividus were collected from the seacoast of the Palermo gulf and maintained in aquaria under controlled conditions. Short-term cultures of sea urchin coleomocytes were obtained by a modification of the procedure previously reported (Matranga et al. 2002). Briefly, sea urchins were bled individually making a cut in the peristomal membrane by scissors. Usually between 5 and 10 ml of coelomic fluid were obtained, depending on the size of the sea urchin. The fluid, containing the total coelomocyte population, was poured on an equivalent amount of a 2× ice-cold anticoagulant solution (AS), composed of 0.5 M NaCl-5 mM, $MgCl_2$ 20 mM, Hepes-1 mM EGTA pH7.2 (Henson et al. 1992). The suspension was immediately diluted 1:2.5 with 1× AS, with gentle agitation to avoid clotting. Usually, between 1 and 2×10^6 cells per ml were contained in this medium. The cell suspension was divided in 60 mm plastic dishes (Kartell, Milan, Italy), usually 6 ml per dish, at a dilution of 2.0×10^5 cell/ml and irradiated with a 312 nm UVB lamp (Labortechnik, model VL-6.M) placed at a distance of 6 cm. UV-B doses used were 0, 500, 1,000, and 2,000 J/m^2. After irradiation cells were kept in the dark and harvested at different time intervals for the detection of apoptotic cells or 1 h later for WB analysis. All treatments were performed at 16–18°C controlled temperature unless otherwise specified.

Optical microscopy and nuclear staining

Fresh preparation of cells were immediately observed under the microscope in order to monitor changes in their morphology and/or movements. For permanent preparations, we obtained a good preservation of cell morphology utilizing a technique previously reported (D'Andrea et al. 1994) with some modifications. Briefly, after dilution in the anticoagulant solution (Henson et al. 1992), cells were first disposed onto 0.01% polylisine-coated (SIGMA P8920) glass slides for 10 min, fixed in 3%gluteraldheide in SW for 1 h and washed twice for 5 min with sea water. Nuclear staining was performed according to Moser et al. (1975), with minor changes. Briefly, cells, disposed onto polylisine-coated glass slides described as above, were fixed in methanol at −20°C for 20 min, washed twice for 5 min with sea water, incubated at 4°C in the dark for 15 min in 0.12 μg/ml Hoechst 33258 (SIGMA, B2883) diluted in sea water. Slides were washed three times for 10 min with seawater at 4°C in the dark. Fresh preparations, fixed and stained cells were observed using a Zeiss Axioscop2 plus inverted microscope; images were recorded by a digital camera, using the program AxioVision 3.1.

SDS-PAGE and western blot

Cell suspensions to be used for SDS-PAGE were collected using a rubber scraper in order to facilitate removal of all the cells from the plastic, centrifuged at 1,500 rpm for 10 min at 4°C. Supernatants were discarded and coelomocyte pellets were lysed in 500 µl of lysis buffer pH 7.5 (20 mM Tris, 2 mM EDTA, 1% NP-40, 15% glycerol, 2 mM DTT), supplemented with a cocktail of protease inhibitors: 2 µg/µl antipain and leupeptin; 1 µg/µl aprotinin and pepstatin, 1 mM benzamidine, and 0.1 mM PMSF, using a glass Dounce homogenizer. Lysates were centrifuged at 10,000 rpm for 10′ at 4°C, supernatants were collected, dyalized against 50 mM Tris pH7.5 and total protein concentrations determined by the Lowry method (Lowry et al. 1951). The equivalent of 15 µg of total coelomocyte lysates per each sample were separated on 7.5% SDS-PAGE under reducing conditions, according to Laemmli (1970). The SDS-polyacrylamide minigels were transferred to nitrocellulose paper as reported by Towbin et al. (1979). Western blots were performed using an anti-bovine brain 70 kDa hsp monoclonal antibody (hsp70 McAb), commercially available (Sigma Chemical Company, H-5147, St Louis, MO, USA), diluted 1:4,000. The second antibody was a peroxidase-conjugated anti-mouse IgG purchased from Amersham, diluted 1:6,000. Bands were detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Hyperfilm ECL films (Amersham). After detection, band intensities were quantified by scanning, with a Bio-rad imaging densitometer (Gel Doc 1000) equipped with an analysis program automatic integrator (MultiAnalyst, Version 1.1).

Results

Morphological analysis of live coelomocytes

There is a growing evidence that various procedures used for collection, together with inappropriate preservation media developed in the past 50 years by different laboratories, probably produced false outcomes, which led to a contradictory coelomocytes nomenclature. This notion is valid for many of the species used; specifically, this is the case for the sea urchin species *P. lividus* in which a number of phenotypes have been described after cells were either fixed or placed in AS (for a review see Matranga 1996). In order to find a common nomenclature that could be of help in the understanding of common biological phenomena, we revisited our previous morphological description of different cell types. To this purpose, cells were immediately inspected under the microscope just after collection from healthy and freshly harvested sea urchins. Under these conditions, the most numerous morphology we found was represented by cells with a dendritic-like phenotype, which, within 5–10 min, underwent a striking morphological transition from a so called "petaloid" to a "filopodial" shape (Fig. 1). At first, all members of this category of cells appeared as big flowers with petals formed by cytoplasmic sheets arranged around the nucleus (Fig. 1a). The first sign of transition was given by the formation of microspikes at the edge of each petal (Fig. 2b). Subsequentely, their cytoplasm retracted, spikes became more evident, and nuclei appeared slightly bigger (Fig. 2c). By about 10 min out of the body cavity phagocytes bearing filopodia were the only cells present in our fresh preparations (Fig. 2d). Although this phenotype is apparently not very mobile, its function in clotting and phagocytosis (from which they derived the name) occurring upon host invasion has been well documented in the past (reviewed by Smith 1981). In recent times, their massive adhesion has been elegantly described in ex vivo experiments by Hillier and Vacquier (2003). As above mentioned, we found that phagocytes are the most abundant category of cells observed, constituting about 80–85% of the total population, in good agreement with previous reports on different sea urchin species (Table 1).

The second major represented population we observed, constituting about 15% of the total, was composed of fast moving cells, having an irregular shape and a clear or red color (Fig. 2, Table 1). We know now that the relative percentages of the two differently pigmented cell types greatly depend on the health state of the sea urchin from which they were extracted, as we have shown it can vary very much depending, for example, on pollution and injuries (Matranga et al. 2000; Pinsino et al. 2006). We followed under the microscope their locomotion and recorded it by time laps cinematography (Fig. 2). Peculiar to this cell type was its characteristic locomotion (about 0.5 µm/s) achieved by rapid changes in their body shape, in which a leading cytoplasmic edge of a cell was protruded towards its march direction, the nucleus being always last. Consequently, these cells have been referred to as amoebocytes, from the Greek term *amoeba*, meaning *change*, referring to their cell shape. The red pigment, named echinochrome, which characterizes almost half of the amoebocyte category, is thought to be utilized by echinoderms as an anti-bactericidal agent. Actually, this notion comes from the only report present to our knowledge in the literature, describing the release of a red pigment by sand dollar (*Mellita quinquiesperforata*) coelomocytes in response to stress (Smith and Smith 1985). Accidentally, because of the EDTA containing medium used for cell collection, which caused a complete block of motility and consequently a spherical appearance, amoebocytes have been referred to as colorless or red spherule cells (Pagliara and Canicatti 1993; Cervello et al. 1994; Matranga et al 2000). On the other hand, a similar nomenclature, namely red and white spherulocytes, is still in use to identify coelomocyte subpopulations in *Lythechinus variegatus* (Borges et al. 2005). We now believe that they do not exist as a distinct population of cells because we never observed such round (spherule) cells in fresh

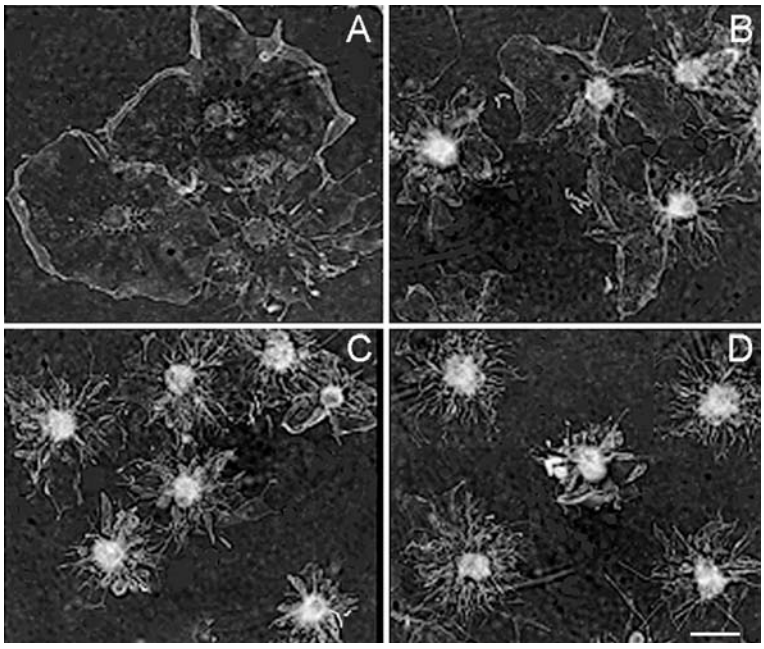

Fig. 1 Morphological transition from petaloid to filopodial phagocytes. Cells were attached to polylisine coated glass slides (see text for more details) and observed after 1 (**a**), 2 (**b**), 4 (**c**), and 8 (**d**) minutes. Interference contrast observed on a Zeiss Axioscope 2 microscope using a 63× objective lent. *Bar* 10 μm

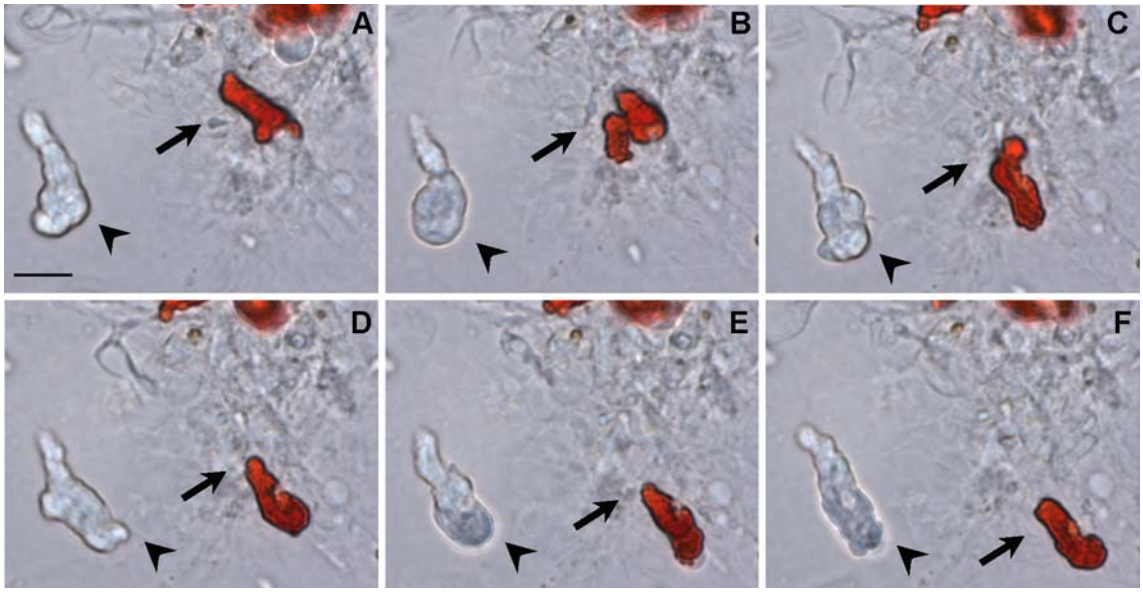

Fig. 2 Itinerant red and white amoebocytes from *P. lividus*. Time lapse recording of freshly collected red and white amoebocytes (see text for more details). Pictures were taken every 8 s. Bright fields viewed on a Zeiss Axioscope two plus, using a 63× objective lent. *Arrows* point to *red* amoebocytes, *arrow* heads point to *white* amoebocytes. *Bar* 10 μm

preparations. It has been reported that amoebocytes migrate to sites of injury and clotting and remain trapped in phagotyte clots (Johnson 1969). This phenomenon was consistently observed when fresh preparations were inspected for a several minute lag of time under the microscope (Fig. 2). We found that both red and white amoebocytes remained trapped in clotting phagocytes, but eventually they tended to escape from them (see pointing arrows Fig. 2a–f). It could be assumed that in analogy with phagocytes, red and white amoebocytes could result from their inter-conversion, although the experimental confirm of this hypothesis in still awaited.

Another type of very motile cells we observed, representing 5–6% of the total cell population, are the so-called vibratile cells; these are round cells which, thanks to a long flagellum, can move in a straight direction along a helicoidal pattern. Because of their fast movement, it is not possible to record their image without fixing the cells (not shown, Matranga and Bonaventura 2002; Matranga et al. 2005). The function of this less

Table 1 Summary of sea urchin coelomocyte morphotypes

	S. droebachiensis (Bertheussen and Seljelid 1978)	*S. purpuratus* (Smith et al. 1992)	*P. lividus* (Matranga and Bonaventura 2002; this paper)
Phagocytes (petaloid/filopodial)	67.1 ± 3.0	66.3 ± 11.6	80.03 ± 1.77
Amoebocytes			
White	6.5 ± 2.5	5.1 ± 4.6	7.82 ± 1.46
Red	8.1 ± 2.0	14.8 ± 8.5	4.70 ± 1.48
Vibratile cells	18.5 ± 3.5	13.9 ± 9.8	7.45 ± 0.86

Numbers refer to the percentage of a particular cell type ± standard error

studied cell type is at present unknown and no hypotheses have been made so far.

These three/four major cell types have been originally described and later consistently observed in at least three different species, i.e. *Strongylocentrotus droebachiensis* from the North Sea (Bertheussen and Seljelid 1978), *Strongylocentrotus purpuratus* from the Pacific Ocean (Smith et al. 1992) and *P. lividus* from the Mediterranean Sea (Matranga and Bonaventura 2002; this paper). A comparative analysis of circulating coelomocytes from the above mentioned species, reported in Table 1, shows minor variations in their relative proportions.

Expression of hsp70 in short-term cultures of sea urchin coelomocytes exposed to UV-B

It has become evident that under stressful conditions, such as exposure to elevated temperatures, xenobiotics, heavy metals, free radicals, UV light etc., cells react by synthesizing a class of proteins, the so called heat shock proteins (hsp). Initially found to be expressed in response to heat, they are now acknowledged markers of phato-physiological states in a broad range of organisms, including man (Mukhopadhyay et al. 2003). We have previously shown that ceolomocytes respond to temperature stress, heavy metal, acidic pH, pollution, and injuries elevating the hsp70 levels (Matranga et al. 2000, 2002; Matranga and Bonaventura 2002; Pinsino et al. 2006). It was then important to assess putative changes in the levels of the hsp70 stress maker as a consequence of UV-B radiation, in short-term cultures of sea urchin coelomocytes. To this purpose, cells were exposed shortly after collection to different doses of UV-B radiation (312 nm) and then given a 1 h recovery period in the dark, to prevent repair mechanisms linked to the activation of the photolyase system (Sancar 1990). The UV-B doses used were comparable to those to which vertebrate cell lines are commonly exposed in laboratory experiments (Wong et al. 2000). Figure 3 shows a representative Western blot experiment in which equal amounts of proteins were loaded onto SDS-PAGE and analyzed for the expression of hsp70 using a commercially available antibody. We found about a threefold increase in hsp70 protein expression in coelomocytes exposed to 500 and 1,000 J/m^2, as compared to control cells. When we used the highest dose of 2,000 J/m^2 we found a modest drop in hsp70 levels, which, on the other hand, were always over a twofold value bigger than controls. In another series of experiments, in order to compare with conditions used for the assessment of DNA damage (see below), cells were cultured for 4–6 h after UV-B exposure and then used for WB analysis (not shown). In all cases, the results obtained matched with those observed for the 1-h recovery period.

The number of apoptotic cells increases with the increase in the UV-B radiation dose

In our previous studies we challenged coelomocytes with a variety of experimental stresses and always found a good correlation between the dose of the stressor used (temperature, pH, heavy metals), and the hsp70 levels obtained. Interestingly, this holds true for all but the highest doses, which always showed a small drop, although values were always very far beyond those of

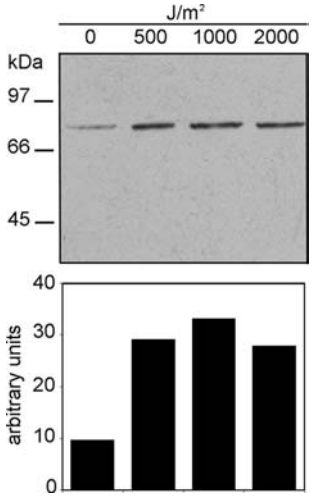

Fig. 3 Sea urchin coelomocytes respond to UV-B by increasing hsp70 levels. Western blot analysis of hsp70 expression in total ceolomocytes exposed to 0, 500, 1,000, and 2,000 J/m^2 UV-B doses and collected 1 h after recovery in the dark. *Upper panel* equal amounts (15 μg) of lysates from control and UV-B exposed coelomocytes were loaded on 7.5% SDS-PAGE and immunoblotted with anti-hsp70 antibody. *Lower panel* quantification of hsp70 expression in UV-B exposed coelomocytes. Densitometric analysis of Western blot with anti-hsp70 antibody of lysates from coelomocytes exposed to different UV-B doses. Values are expressed in arbitrary units

control levels. This paradoxical reduction was interpreted as an increase in the number of cells that, failing to cope with the induced stress, were going into apoptosis thus reducing the overall levels of hsp70. Bearing in mind this interpretation and considering that DNA damage is specifically caused by UV-B radiation, it was important to assess putative apoptotic events induced by UV-B in coelomocyte total populations. It is well known that one of the most striking appearance of damaged chromatin is the formation of apoptotic nuclei that can be easily observed by optical microscopy (Moser et al. 1975). In a first series of experiments, it was necessary to evaluate the percentage of nuclei fragmentation in cells that were not exposed to UV-B radiation, but maintained in culture for different periods of time, in order to check for their viability during the following UV-B exposure experiments. We selected the following periods of time in culture to be tested: 0, 4, 6, 8, 12, 14, and 16 h. After the indicated periods of time, cells were fixed and stained with the DNA-fluorochrome Hoechst 33258, a dye widely used for staining cells nuclei. Apoptotic nuclei were counted on an average of 200 cells per experimental point; the number of apoptotic nuclei, expressed as percentage of the total number of cells scored, was reported (Fig. 4). We found that the number of apoptotic cells increased after long-lasting times of culture. Specifically, a little amount of apoptotic nuclei (lower than 5%) was detected for culture times up to 6 h; the percentage gradually increased (higher than 5%) for culture times over 8 h, in a time-dependent fashion, reaching the maximum of approximately 25% after 16 h. The nuclear fragmentation of apoptotic cells was very evident under the microscope; examples of typical apoptotic nuclei scored are shown in Fig. 5, where the brightly fluorescent (condensed) chromatin areas are well distinguishable from the sparse, evenly distributed, weak fluorescence occurring in control nuclei. We found compacted chromatin in dense masses at the periphery of the nucleus (Fig. 5b, c) or, in other cases, we observed the condensation of the entire nucleus into a dense ball (Fig. 5d). Areas of no fluorescent signals were also indicative of apoptotic cellular events, thus resembling very much to apoptotic nuclei features described in cell cultures of higher vertebrates (Wyllie et al. 1980). The three different cell types equally contributed to the overall picture, i.e. apoptosis occurred proportionally in phagocytes, amoebocytes and vibratile cells (not shown). The specific nuclear staining was obtained using the Hoechst 33258 dye, that, preferentially binding to AT regions, is quite selective for DNA. Hoechst–stained cells showed virtually no cytoplasmic staining. Negative control, performed omitting, after fixation, the DNA dye, gave no fluorescent signal.

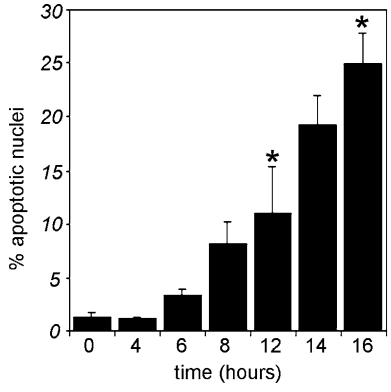

Fig. 4 Time-dependent apoptosis in short-term cultures. Apoptotic nuclei were counted on an average of 200 cells per each experimental point and expressed as percentage of the total number of cells scored. Quantitative data (mean ± SE) usually represent eight independent experiments, data from four experiments are marked with an asterisk

In order to monitor changes in nuclear integrity as a consequence of exposure to UV-B, cells were exposed to doses from 500 to 2,000 J/m^2 and a similar analysis on nuclear morphology, by Hoechst 33258 staining, was performed. Since the percentage of apoptotic cells found in short-term cultures (4–6 h) was consistently lower than 5% (see Fig. 4), we selected the recovery period of 6 h after exposure to UV-B for the following experiments. As shown in Fig. 6, we consistently found a significant dose-dependent increase in the number of apoptotic cells; namely nuclear fragmentation was 7.1 ± 1.3 and $10.1 \pm 3.9\%$ in cells exposed to 500 and 1,000 J/m^2, respectively. The $15.6 \pm 5.8\%$ of apoptotic cells found at the highest dose tested (2,000 J/m^2) accounts for the plateau in the hsp70 levels found for this UV-B dose in Western blot experiments and is indicative of the tendency that cells have with respect to their survival. Morphological appearance of apoptotic nuclei scored was at all similar to that reported in Fig. 5.

Discussion

In this work we describe the use of short-term cultures of sea urchin coelomocytes for the analysis of UV-B stress induced in the laboratory and the effects on both hsp70 expression and DNA integrity. The three populations of coelomocytes, here described in detail, altogether account for the evident increase in the hsp70 levels already 1 h after exposure to UV-B, in dose dependent fashion. Additionally, exposed cells fail to cope with dangerous UV-B, as the number of apoptotic cells increases with the increase in the UV-B radiation dose used. Thus, our results reinforce the notion that sea urchin coelomocytes can be used in in vitro cultures as bio-indicators of experimentally-induced stress and suggest their use for the analysis of cell and DNA damages occurring upon UV-B radiation in marine invertebrates.

It is still under debate if each of the many functions performed by coelomocytes are carried out by a single specific cell type or if maturation/differentiation mechanisms can be elicited in response to environmental factors. Here we describe three ceolomocyte cell types contained in the perivisceral coelomic fluid of the sea urchin *P. lividus*, which we refer to as phagocytes, white

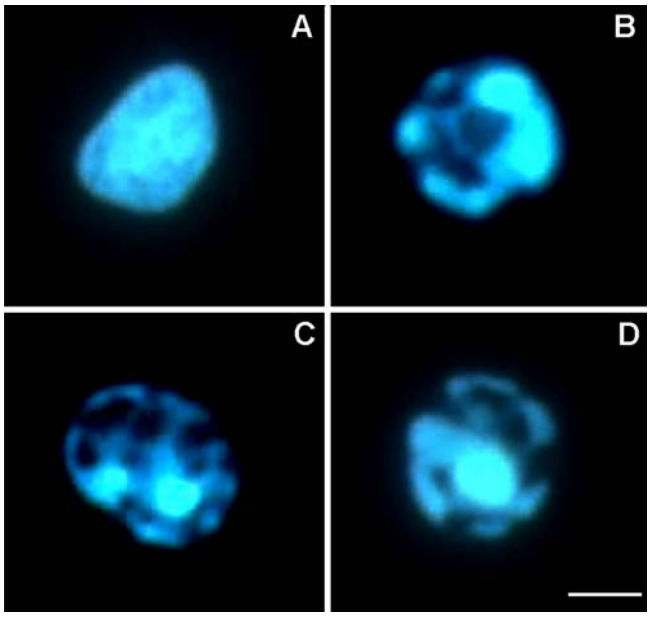

Fig. 5 Apoptotic nuclei in sea urchin ceolomocytes. Morphological analysis of nuclear fragmentation by Hoechst 33258 staining (see text for more details). **a** control; **b–d** UV-B exposed cells. Pictures were taken using a mercury lamp, on a Zeiss Axioscope 2 plus, using a 63× objective lens. *Bar* 5 μm

or red amoebocytes and vibratile cells. Phagocytes, deriving their name from their ability to engulf (phagocyte) foreign particles, undergo a stress-induced (out-of-the-body) petaloid-filopodial transition. This result is in agreement with previous reports by several authors using another sea urchin species, which postulated that petaloid and filopodial cells are different functional states of the same cell type (Edds 1985, 1993; Henson et al. 1999). Although the formation and elongation of filopodia have been described in *S. droebachiensis* by the use of scanning electron microscopy

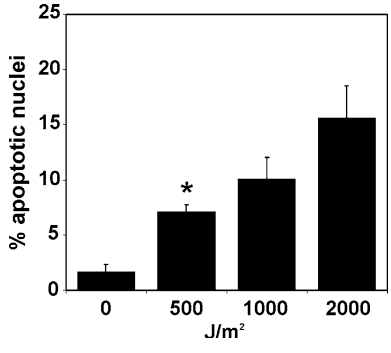

Fig. 6 Dose-dependent effects of UV-B radiation on nuclear fragmentation in sea urchin ceolomocytes. Apoptotic nuclei were counted on an average of 200 cells per experimental point and expressed as percentage of the total number of cells scored. Each bar represents the mean ± SE of eight independent experiments. Bar with asterisk refers to the mean of three independent experiments

(Edds 1980), to the best of our knowledge, this is the first report describing in detail *P. lividus* coelomocyte morphologies. In addition, as claimed in old fashioned experiments in hanging drops (Johnson 1969), our observations confirm that no other cell type but phagocytes are responsible for clot formation, while all the others, namely amoebocytes and vibratile cells, are passively trapped in clots from which they eventually escape. The biochemistry occurring upon sea urchin coelomocytes clotting, involving well regulated and highly responsive cell-cell adhesion mechanisms, has been recently described (Hillier and Vacquier 2003). It seems that amassin, a 75 kDa coelomic plasma protein, plasma-free coelomocytes and calcium ions are the only three components needed for clotting. The authors also suggest integrins, whose expression has been shown in coelomocytes (Burke 1999), as good candidates for receptorial and clot triggering functions. Although promising, more studies are awaited in this direction.

The second most abundant cells we found were amoebocytes, red or white fast moving cells, probably utilized as defense elements participating in wound healing or promptly attacking foreign materials (Smith 1981). It seems they produce most of the soluble factors of the sea urchin immune system (for a review see Matranga 1996; Matranga et al. 2005). Certainly, their granular cytoplasmic structures together with the spherical shape produced by the use of inappropriate fixatives and/or anticoagulant media, induced several authors, including ourselves, to assign the name of spherule cells to this cell type. A systematic re-consideration of all the reports in which these cells were referred to as spherule (red or colorless), would probably demonstrate that amoebocytes are indeed the true secretory cells of the sea urchin immune system. Interestingly, we observed that the cytoplasmic granules, which fill up red and white amoebocytes were positively stained by monoclonal antibodies specific for proteins known to be contained in the coelomic fluid (Cervello et al. 1994). Additionally, in analogy with the petaloid-filopodial phagocyte transition, we suggest that red amoebocytes arise from a maturation/differentiation process occurring to white amoebocytes in response to stress. This hypothesis would agree with the notion that sea urchins from polluted sea waters or exposed to cold stress or injuries show an increased number of red amoebocytes (Matranga et al. 2000, 2005; Pinsino et al. 2006). Although attractive, this hypothesis needs more experimental confirms. Finally, it should be recalled that phagocytes were often referred to as phagocytic amoebocytes (see Matranga et al. 2000; Borges et al. 2005), thus leading to great confusion about the classification of cell types.

The last coelomocyte type found in *P. lividus* is the vibratile cell, a very fast moving cell whose function is at present unknown. An attractive hypothesis could involve them in contributing to the continuous circulation of the perivisceral coelomic fluid generated by the ciliated coelomic epithelium (Ruppert and Barnes 1994).

It should be taken into consideration that, with the introduction of new biotechnological tools, many of the hypothesized functions can now be approached. For example, the analysis of differentially expressed genes could be performed at the proteomic and genomic levels in coelomocytes subpopulations, which could be easily separated by density gradients. Studies in this direction are greatly awaited.

In synthesis, on the basis of current results and other papers found in the literature (see Table 1), we propose to reduce the list of coelomocyte morphologies present in the sea urchin nomenclature to three types: phagocytes, amoebocytes, and vibratile cells. This is consistent with the fact that the above mentioned morphotypes have been repeatedly found in *S. droebachiensis* from the North Sea, *S. purpuratus* from the Pacific Ocean and *P. lividus* from the Mediterranean Sea.

We believe that different morphological annotations, reported for coelomocytes belonging to other classes of the Echinoderm phylum, such as *Asteria rubens* (Coteur et al. 2003) and *Holothuria polii* (Pagliara et al. 2003), could be re-conducted to the above mentioned nomenclature. We have some preliminary evidence that this seems to hold true at least for the *A. rubens* species (Pinsino, personal communication).

The molecular mechanisms underlying the toxic action of many environmental stresses are not well understood; at present, very little data are available on the effects of UV radiation on marine organisms (see Epel 2003). In this paper, we showed for the first time that short-term cultures of sea urchin coelomocytes are able to respond to UV-B radiation elevating their hsp70 levels and triggering apoptosis. Since about 85% of coelomocytes are phagocytes, it appears evident that they account for most of the biochemical features; however, the contribution of the other cell types is also present, in agreement with our previous report (Matranga et al. 2000). The finding that coelomocytes elevate the hsp70 levels well matched with our previous reports, which describe the same type of biochemical increase in response to a few experimentally induced stresses (Matranga et al. 2000, 2002). The elevation of hsp70 levels in response to a variety of stresses has been reported in other marine invertebrates (Koziol et al. 1997; Müller et al. 1998; Schröder et al. 1999; Chiang et al. 2003; Hamer et al. 2004). Our previous findings showed that a combination of heavy metal (cadmium), added first, and UV-B exposure stresses did not cause a proportional increase in the hsp70 levels (Schröder et al. 2005). This apparent paradox reinforces the notion that hsps increase resistance and induce tolerance in agreement with what is known in other systems (Samali and Cotter 1996).

To the best of our knowledge, there have been no studies describing the use of sea urchin coelomocytes for the analysis of DNA damage. The establishment of good conditions for cell biology experiments, thus providing a good compromise between cell fixation and preservation of cellular morphology, was a prerequisite for the proposed analysis. In our experiments, apoptosis was identified by the altered nuclear morphology of cells. We found that about one-fourth of the cells, apparently exposed to no stress, underwent apoptosis after 16 h in culture. This should be taken into consideration when attempting the establishment of continuous cell lines from celomocytes (see Matranga et al. 2005). At present, very little information is available on the activation of apoptotic mechanisms in marine invertebrates in response to UV-B radiation. This is the first report describing a dose-dependent UV-B induced increase in the number of apoptotic nuclei occurring in sea urchin coelomocytes; the only other report relates to UV-exposed embryos (Lesser et al. 2003). We have recently proposed that an increase in hsp70 levels together with the activation of the p38MAPk, might provide an adaptive cellular response of UV-B exposed embryos thus increasing their survival (Bonaventura et al. 2005). However, if we consider that, although at the highest UV-B dose used (2,000 J/m^2), hsp70 levels underwent a reduction, coupled to an increase in the number of apoptotic cells, our findings on coelomocytes do not seem to support this hypothesis. Further studies are needed to determine the cascade of cellular and biochemical events occurring in coelomocytes and the precise role played by the UV-B activated stress proteins.

The impact of elevated UV-B on marine ecosystems has been first documented in Antarctica, as a significant DNA damage measured by cyclobutane pyrimidine dimers formation (Malloy et al. 1997). However, reduction of ozone concentration, and the probable increase in UV-B radiation reaching the Earth, is also occurring at temperate and tropical latitudes (Stolarski et al. 1992). In fact, negative UV-B effects have been noted in fish eggs and larvae (Malloy et al. 1997), and proposed as a contributing factor in worldwide decline of sea urchins and corals (Hader et al. 2003). An essential problem in evaluating genotoxic effects is the lack of appropriate techniques to sample and quantify genetic damage in field populations. In addition, it is currently not feasible to estimate UV-B exposure levels for organisms in their natural habitats (Karentz 1994). Thus, the need to conduct UV-B radiation measurements at sites where the animals are living (Hakkinen and Oikari 2004; Hamer et al. 2004).

It is quite obvious that coelomocytes would never be exposed to UV-B radiation in nature, since they are contained inside the body of sea urchins, which are not at all transparent. Nevertheless, our findings confirm once again that coelomocytes are sensitive biosensors for studies on stress; their use is recommended for the analysis on the effects of UV-B radiation. In addition, due to their acknowledged immune competences, coelomocyte cultures constitute valid tools for the analysis of innate and/or adaptive responses elicited by extreme adverse conditions. Interestingly, a recent report demonstrates that sea urchin embryos use a *bona fide* complement-based immune system for defense against

pathogens in their aquatic environment (Shah et al. 2003). This probably suggests that some cells in the embryo have at least a coelomocyte-like function.

The possibility to use sea urchin coelomocyte cells as a stress model system under controlled laboratory conditions was first proposed by Koros (1993) and later exploited by Matranga et al. (2000, 2002, 2005). Recently, using a medium developed from the one used for mollusk heart cells (Le Marrec-Croq et al. 1999), we obtained coelomocyte primary cultures that lasted up to 20 days (Matranga et al. 2005). The exploitation of marine invertebrate cell cultures is now considered the leading edge of marine biotechnology. Its central importance is acknowledged not only for studies on environmental pollution, but also for the production of biologically active compounds from taxonomically diverse groups of marine invertebrates (see review by Mayer and Gustafson 2004) and in particular echinoderms (see review by Zito et al. 2005). However, since no established cell line is at present available for any marine invertebrate, more studies are awaited in this direction.

Acknowledgments This research has been supported by the EU program UV-TOX to V. Matranga, Contract No. EVK3-CT-1999-00005. Partial support was also obtained by the ASI Contract No. I/R/073/01. We are grateful to all the other members of the group for their helpful contributions and suggestions, and the stimulating working environment. The authors thank Mr. Davide Comacchio for assistance in maintenance of sea urchins in aquaria.

References

Angelini C, Amaroli A, Falugi C, Di Bella G, Matranga V (2003) Acetylcholinesterase activity is affected by stress conditions in paracentrotus lividus coelomocytes. Mar Biol 143:623–628

Bertheussen K, Seljelid R (1978) Echinoid phagocytes in vitro. Exp Cell Res 111:401–412

Bonaventura R, Poma V, Costa C, Matranga V (2005) UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochem Biophys Res Commun 328:150–157

Borges JC, Jensch-Junior BE, Garrido PA, Mangiaterra MB, Silva JR (2005) Phagocytic amoebocyte sub populations in the perivisceral coelom of the sea urchin *Lytechinus variegatus* (Lamarck, 1816). J Exp Zoolog A Comp Exp Biol 303:241–248

Burke RD (1999) Invertebrate integrins: structure, function, and evolution. Int Rev Cytol 191:257–284

Cejkova J, Stipek S, Crkovska J, Ardan T, Platenik J, Cejka C, Midelfart A (2004) UV Rays, the prooxidant/antioxidant imbalance in the cornea and oxidative eye damage. Physiol Res 53:1–10

Cervello M, Arizza V, Lattuca G, Parrinello N, Matranga V (1994) Detection of vitellogenin in a subpopulation of sea urchin coelomocytes. Eur J Cell Biol 64:314–319

Chiang WL, Au DW, Yu PK, Wu RS (2003) UV-B damages eyes of barnacle larvae and impairs their photoresponses and settlement success. Environ Sci Technol 37:1089–1092

Coteur G, Pernet P, Gillan D, Joly G, Maage A, Dubois P (2003) Field contamination of the starfish Asterias rubens by metals. Part 1: short- and long-term accumulation along a pollution gradient. Environ Toxicol Chem 22:2136–2144

D'Andrea L, Danon MA, Sgourdas GP, Bonder EM (1994) Identification of coelomocyte unconventional myosin and its association with in vivo particle/vesicle motility. J Cell Sci 107:2081–2094

Edds KT (1980) The formation and elongation of filopodia during transformation of sea urchin coelomocytes. Cell Motil 1:131–140

Edds KT (1985) Morphological and cytoskeletal transformation in sea urchin coelomocytes. In: Cohen WD (ed) Blood cells of marine invertebrates: experimental systems in cell biology and comparative physiology. R Liss Inc., NY, pp 53–74

Edds KT (1993) Effects of cytochalasin and colcemid on cortical flow in coelomocytes. Cell Motil Cytosk 26:262–273

Epel D (2003) Protection of DNA during early development: adaptations and evolutionary consequences. Evol Dev 5:83–88

Farman JC, Gardiner BG, Shanklin JD (1985) Large losses of total ozone in Antarctica reveal seasonal ClOx/NOx interaction. Nature 315:207–210

Garssen J, van Loveren H (2001) Effects of ultraviolet exposure on the immune system. Crit Rev Immunol 21:359–397

Hader DP, Kumar HD, Smith RC, Worrest RC (2003) Aquatic ecosystems: effects of solar ultraviolet radiation and interactions with other climatic change factors. Photochem Photobiol Sci 2:39–50

Hakkinen J, Oikari A (2004) A field methodology to study effects of UV radiation on fish larvae. Water Res 38:2891–2897

Hamer B, Hamer DP, Muller WE, Batel R (2004) Stress-70 proteins in marine mussel *Mytilus galloprovincialis* as biomarkers of environmental pollution: a field study. Environ Int 30:873–882

Henson JH, Nesbitt D, Wright BD, Scholey JS (1992) Immunolocalisation of kinesin in sea urchin coelomocytes: association of kinesis with intracellular organelles. J Cell Sci 103:309–320

Henson JH, Svitkina TM, Burns AR, Hughes HE, MacPartland KJ, Nazarian R, Borisy GG (1999) Two components of actin-based retrograde flow in sea urchin coelomocytes. Mol Biol Cell 10:4075–4090

Hillier BJ, Vacquier VD (2003) Amassin, an olfactomedin protein, mediates the massive intercellular adhesion of sea urchin coelomocytes. J Cell Biol 160:597–604

Johnson PT (1969) The coelomic elements of sea urchins (*Strongylocentrotus*). I. The normal coelomocytes; their morphology and dynamics in hanging drops. J Invertebr Pathol 13:25–41

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Le Marrec-Croq F, Glaise D, Guguen-Guillouzo C, Chesne C, Guillouzo A, Boulo V, Dorange G (1999) Primary cultures of heart cells from the scallop *Pecten maximus* (molluscabivalvia). In Vitro Cell Dev Biol Anim 35:289–295

Lesser MP, Kruse VA, Barry TM (2003) Exposure to ultraviolet radiation causes apoptosis in developing sea urchin embryos. J Exp Biol 206:4097–4103

Lowry OH, Rosebrough NY, Farr LA, Randall R (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–276

Karentz D (1994) Considerations for evaluating ultraviolet radiation-induced genetic damage relative to Antarctic ozone depletion. Environ Health Perspect 102(Suppl 12):61–64

Kerr JB, McElroy CT (1993) Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. Science 262:1032–1034

Koros AMC (1993) Neuroendocrine marker expression on sea urchin coelomocytes and other immunoregulatory parameters may be used to monitor environmental changes. Mar Environ Res 35:137–140

Koziol C, Batel R, Arinc E, Schröder HC, Müller WEG (1997) Expression of the potential biomarker heat shock protein 70 and its regulator, the metazoan DnaJ homolog, by temperature stress in the sponge *Geodia cydonium*. Mar Ecol Prog Ser 154:261–268

Malloy KD, Holman MA, Mitchell D, Detrich HW III (1997) Solar UVB-induced DNA damage and photoenzymatic DNA repair in antarctic zooplankton. Proc Natl Acad Sci USA 94:1258–1263

Matranga V (1996) Molecular aspects of immune reactions in Echinodermata. In: Müller WEG, Rinkevich B (eds) Invertebrate immunology. Prog Mol Subcell Biol Series, vol 15. Spinger, Berlin Heidelberg New York, pp 235–247

Matranga V, Toia G, Bonaventura R, Muller WEG (2000) Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. Cell Stress Chaperones 5:158–165

Matranga V, Bonaventura R (2002) Sea urchin coelomocytes, the progenitors of vertebrate immune effectors, as bio-indicators of stress and pollution. In: Yokota Y, Matranga V, Smolenicka Z (eds) The sea urchin: from basic biology to aquaculture. Swets and Zeitlinger, Lisse, The Netherlands, pp 161–176

Matranga V, Bonaventura R, Di Bella G (2002) hsp70 as a stress marker of sea urchin coelomocytes in short term cultures. Cell Mol Biol 48:345–359

Matranga V, Pinsino A, Celi M, Natoli A, Bonaventura R, Schröder HC, Müller WEG (2005) Monitoring chemical and physical stress using sea urchin immune cells. In: Matranga V (ed) Echinodermata, vol 39. Springer, Berlin Heidelberg New York, pp 85–110

Matsumura Y, Ananthaswamy HN (2004) Toxic effects of ultraviolet radiation on the skin. Toxicol Appl Pharmacol 195:298–308

Mayer AM, Gustafson KR (2004) Marine pharmacology in 2001–2002: antitumour and cytotoxic compounds. Eur J Cancer 40:2676–2704

Moser FG, Dorman BP, Ruddle FH (1975) Mouse-human heterokaryon analysis with a 33258 Hoechst-Giemsa technique. J Cell Biol 66:676–680

Mukhopadhyay I, Nazir A, Saxena DK, Chowdhuri DK (2003) Heat shock response: hsp70 in environmental monitoring. J Biochem Mol Toxicol 17:2492–2954

Müller WEG, Batel R, Lacorn M, Steinhart H, Simat T, Lauenroth S, Hassanein HMA, Schröder HC (1998) Accumulation of cadmium and zinc in the marine sponge Suberites domuncula and its potential consequences on single-strand breaks and on expression of heat shock protein: a natural field study. Mar Ecol Prog Ser 167:127–135

Nigel DP, Gwynn-Jones D (2003) Ecological roles of solar UV radiation: towards an integrated approach. Trends Ecol Evol 18:48–55

Pagliara P, Canicattì C (1993) Isolation of cytolytic granules from sea urchin amoebocytes. Eur J Cell Biol 60:179–184

Pagliara P, Carnevali C, Burighel P, Ballarin L (2003) The spherule cells of *Holothuria polii* Delle Chiaie, 1823 (Aspidochirota, Holothuroidea) during brown body formation: an ultrastructural study. J Submicrosc Cytol Pathol 35:295–301

Pinsino A, Bonaventura R, Della Torre C, Giuliani S, Matiddi M, Matranga V, Amato E (2006) Hsp70 as a stress marker for the assessment of TNT-exposure in *Paracentrotus lividus*: a case study in the Tremiti Islands Marine protected area. Hydrobiologia (in press)

Rex M, Harris NRP, vonderGathen P, Lehmann R, Braathen GO, Reimer E, Beck A, Chipperfield MP, Alfier R, Allaart M, O'Connor F, Dier H, Dorokhov V, Fast H, Gil M, Kyro E, Litynska Z, Mikkelsen IS, Molyneux MG, Nakane H, Notholt J, Rummukainen M, Viatte P, Wenger J (1997) Prolonged stratospheric ozone loss in the 1995–1996 Arctic winter. Nature 389:835–838

Ruppert EE, Barnes RD (1994) Invertebrate zoology, 6th edn. Saunders College Publishing, Philadelphia

Samali A, Cotter TG (1996) Heat shock proteins increase resistance to apoptosis. Exp Cell Res 223:163–170

Sancar GB (1990) DNA photolyases: physical properties, action mechanism, and roles in dark repair. Mutat Res 236:147–160

Schröder HC, Hassanein HMA, Lauenroth S, Koziol C, Mohamed TA-AA, Lacorn M, Steinhart H, Batel R, Müller WEG (1999) Induction of DNA strand breaks and expression of hsp70 and GRP78 homolog by cadmium in the marine sponge Suberites domuncula. Arch Environ Contam Toxicol 36:47–55

Schröder HC, Di Bella G, Janipour N, Bonaventura R, Russo R, Müller WEG, Matranga V (2005) DNA damage and developmental defects after exposure to UV and heavy metals in sea urchin cells and embryos compared to other invertebrates. In: Matranga V (ed) Echinodermata, vol 39. Springer, Berlin Heidelberg New York, pp 111–138

Shah M, Brown KM, Smith LC (2003) The gene encoding the sea urchin complement protein, pC3, is expressed in embryos and can be upregulated by bacteria. Dev Comp Immunol 27:529–538

Smith VL (1981) The echinoderms. In: Ratcliffe NA, Rowley AT (eds) Invertebrate blood cells 2. Academic Press, London

Smith SL, Smith AC (1985) Sensitization and histamine release by cells of the sand dollar *Mellita quinquesperforata*. Dev Comp Immunol 9:597–603

Smith LC, Davidson EH (1992) The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes. Immunol Today 13:356–362

Smith LC, Davidson EH (1994) The echinoderm immune system. Characters shared with vertebrate immune systems and characters arising later in deuterostome phylogeny. Ann NY Acad Sci 712:213–226

Smith LC, Britten RJ, Davidson EH (1992) SpCoel1: a sea urchin profilin gene expressed specifically in coelomocytes in response to injury. Mol Biol Cel 3:403–414

Stolarski RS, Bojkov R, Bishop L, Zerefos C, Staehelin J, Zawodny J (1992) Measured trends in stratospheric ozone. Science 256:342–349

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Wong JW, Shi B, Farboud B, McClaren M, Shibamoto T, Cross CE, Isseroff RR (2000) Ultraviolet B- mediated phosphorylation of the small heat shock protein HSP27 in human keratinocytes. J Invest Dermatol 115:427–434

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–305

Zito F, Costa C, Sciarrino S, Cavalcante C, Poma V, Matranga V (2005) Cell adhesion and communication: a lesson from echinoderm embryos for the exploitation of new therapeutic tools In: Matranga V (ed) Echinodermata, vol 39. Springer, Berlin Heidelberg New York, pp 7–44