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Putative midkine family protein up-regulation in *Patella caerulea* (Mollusca, Gastropoda) exposed to sublethal concentrations of cadmium

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Abstract

A cDNA sequence of a putative midkine (MK) family protein was identified and characterised in the mollusc *Patella caerulea*. The midkine family consists of two members, midkine and pleiotrophin (PTN), and it is one of the recently discovered cytokines. Our results show that this putative midkine protein is up-regulated in specimens of *P. caerulea* exposed to sublethal cadmium concentrations (i.e. 0.5 and 1 mg l^{-1} Cd) over a 10-day exposure period. Semiquantitative RT-PCR and quantitative Real time RT-PCR estimations indicate elevated expression of midkine mRNA in exposed specimens compared to controls. Moreover, RT-PCR Real time values were higher in the viscera (here defined as the part of the soft tissue including digestive gland plus gills) than in the foot (i.e. foot plus head plus heart) of the limpets. At present, information on the functional signalling significance of the midkine family proteins suggests that the up-regulation of *P. caerulea* putative midkine family protein is a distress signal likely with informative value on health status of the organism and with potential prognostic capability.

Keywords: *Patella caerulea*; Mollusc; Midkine; Pleiotrophin; Distress signal; Cadmium; RT-PCR Real time

In the last decade, coastal environments have been subjected to increasing monitoring and efforts have been spent to study distress signals in order to develop biomarkers with warning prognostic capability (Moore, 2002). Cadmium is one of the most toxic and widespread heavy metals found in the marine environment (Romeo et al., 1995); it promotes cellular

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oxidative stress and induction of anti-oxidant systems in many marine molluscs (e.g. Leung and Furness, 1999; Geret et al., 2002). Moreover, cadmium is recognised to be carcinogenic in mammals (IARC, 1999), exerting pronounced co-mutagenic effects. At present, these effects are explained not only by increasing the level of DNA strand breakage induced by oxidative stress, but also by impairing cell recovery (Hartwing and Schwerdtle, 2002; Fatur et al., 2003), and delaying the onset of apoptosis (Waalkes et al., 2000). Recently, it has been showed that in the mussel *Mytilus edulis* cadmium enhances genotoxicity by mechanisms similar to those reported for mammals (Pruski and Dixon, 2002).

The midkine (MK) is a recently discovered family of cytokines (see reviews Deuel et al., 2002; Kadomatsu and Muramatsu, 2004) and consists of only two members, namely heparin-binding growth factors MK and pleiotrophin (PTN). Information on PTN and MK function is mostly from mammals; MK and PTN share receptors and show similar biological activities that include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, chemotactic, and protooncogenic ones. In adults, a characteristic property of MK/PTN genes is a striking induction of gene expression in limited emergency conditions, such as wound repair following injuries. In stark contrast, MK and PTN are strongly expressed in neurodegenerative diseases and in malignant tumours. The blood MK level in human carcinomas is correlated with prognostic factors; thus, it is as a good marker for evaluating the progress of carcinomas (Ikematsu et al., 2003).

In this work, we report on isolation and characterisation of a cDNA sequence of a putative MK family protein that is up-regulated in *Patella caerulea* exposed to sublethal concentrations of cadmium. To our knowledge, this is the first report regarding MK/PTN identification in molluscs and on the involvement of MK/PTN in response to heavy metal exposure. We also report data on the mRNA tissue/organ quantitative expression feature in terms of both RT-PCR and RT-PCR Real time estimations. We chose the gastropod limpet *P. caerulea* as it is widely distributed over Mediterranean coastal areas, accumulates cadmium and is considered as a sentinel organism for the Mediterranean sea (Campanella et al., 2001).

Samples of *P. caerulea* were collected from the external side of a breakwater at the Marina di Cala Galera (42°26′50″N, 11°26′00″E, Tyrrhenian sea) on 7 June 2003. To reduce size/age-related variability, limpets of similar wet flesh weight (1 ± 0.4 g without shell) were selected. Measurements taken in the surface water layer in front of the breakwater gave an average salinity of 35 g l^{-1} and a temperature of 20 °C. Immediately after collection, limpets were transported to the laboratory (Florence) and acclimated for 2 days before being exposed to cadmium (Cd). Three holding tanks, containing 20 specimens each, with each specimen singly placed in an open Petridish (diameter 6 cm, height 1 cm), were prepared and supplied with continuously aerated artificial seawater (35 g l^{-1} Reef Crystals™ salt, Aquarium Systems). The three tanks were located in a temperature-controlled room (20 ± 0.5 °C) with a natural daylight cycle. Exposure concentrations of cadmium were chosen with reference to sublethal concentrations based on 96-h acute toxicity tests carried out on *P. caerulea* in the same laboratory (Parenti, 2003). The three groups of limpets were exposed for 10 days to 0 (control), 0.50 and 1.0 mg l^{-1} Cd, respectively. Cadmium concentrations were prepared using a 100 mg l^{-1} Cd stock solution obtained by adding an appropriate quantity of $CdCl_2$ (Fluka Chemie®). Water was changed every second day to ensure no build-up of toxic materials from animals, changing water quality. The animals were not fed for the entire exposure period. Mortalities were recorded daily and dead animals were discarded; at the end of the 10th day mortalities were 4, 20 and 39% for 0 (control), 0.5 and 1.0 mg l^{-1} Cd treated animals, respectively. Following exposure, the tissues of the control and cadmium treated limpets were separated from the shells, and for each specimen the whole soft body was dissected into two parts: the foot (i.e. the foot plus head plus heart) and the viscera (i.e. the remain soft tissue including digestive gland plus gills); foot (F) and viscera (V) were frozen in liquid nitrogen and stored at −70 °C until analyses.

Total RNA was extracted from 500 mg of tissues (viscera plus foot) of *P. caerulea* specimens exposed to 1 mg l^{-1} cadmium using Trizol (Invitrogen) solution according to the manufacturer's protocol. The generation of cDNA was carried out by 3′RACE technique using a sense degenerated primer (Mtfw: 5′-GTGTGGNAGCVVGTGC-3′) designed on cDNA consensus sequences from gastropod metallothioneins (the original objective was focused on MT, with unexpected isolation of MK). SMART PCR cDNA was