Mutation Research 654 (2008) 69–75

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxic damage in polychaetes: A study of species and cell-type sensitivities

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ARTICLE INFO

Article history:
Received 3 January 2008
Received in revised form 25 April 2008
Accepted 6 May 2008
Available online 23 May 2008

Keywords:
DNA damage
Coelomocytes
Sperm
Nereis virens
Nereis diversicolor
Arenicola marina

ABSTRACT

The marine environment is becoming increasingly contaminated by environmental pollutants with the potential to damage DNA, with marine sediments acting as a sink for many of these contaminants. Understanding genotoxic responses in sediment-dwelling marine organisms, such as polychaetes, is therefore of increasing importance. This study is an exploration of species-specific and cell-specific differences in cell sensitivities to DNA-damaging agents in polychaete worms, aimed at increasing fundamental knowledge of their responses to genotoxic damage. The sensitivities of coelomocytes from three polychaetes species of high ecological relevance, i.e. the lugworm *Arenicola marina*, the harbour ragworm *Nereis diversicolor* and the king ragworm *Nereis virens* to genotoxic damage are compared, and differences in sensitivities of their different coelomic cell types determined by use of the comet assay. *A. marina* was found to be the most sensitive to genotoxic damage induced by the direct-acting mutagen methyl methanesulfonate (MMS), and showed dose-dependent responses to MMS and the polycyclic aromatic hydrocarbon benzo(*a*)pyrene. Significant differences in sensitivity were also measured for the different types of coelomocyte. Eleocytes were more sensitive to induction of DNA damage than amoebocytes in both *N. virens* and *N. diversicolor*. Spermatozoa from *A. marina* showed significant DNA damage following *in vitro* exposure to MMS, but were less sensitive to DNA damage than coelomocytes. This investigation has clearly demonstrated that different cell types within the same species and different species within the polychaetes show significantly different responses to genotoxic insult. These findings are discussed in terms of the relationship between cell function and sensitivity and their implications for the use of polychaetes in environmental genotoxicity studies.

1. Introduction

A significant proportion of the chemicals entering aquatic environments have the potential to induce DNA damage or interfere with the processes involved in cell division [1,2]. These include, amongst others, persistent organics such as polycyclic aromatic hydrocarbons (PAHs) and metals, which can damage DNA either directly or indirectly via the production of free radicals or after metabolic activation. Whilst the body of evidence documenting these effects in vertebrates is large, there has been less attention given to the invertebrate species that occupy key ecological niches in marine habitats and for which genetic damage may have great potential for harm. The increasingly widespread aquatic distribution of chemicals with genotoxic potential has meant that the measurement of genotoxicity in the marine environment is fast becoming an area of great concern [3–7]. Sediments have long been recognised as a sink for organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), which by virtue of their hydrophobic nature can strongly adsorb onto sediments, affecting the benthic community that inhabits them. Sediment-dwelling macrofauna (infauna) are important vectors for the transfer of sediment-associated contaminants to higher trophic levels, since they form the primary food source for many commercial fish and crustacean species. Understanding and monitoring the genotoxic impacts of pollutants in sediment-dwelling organisms is therefore of great importance for both the environment and human health.

Polychaete worms tend to form the dominant sediment-dwelling fauna of most mud flats and estuaries, yet despite their obvious importance for environmental monitoring purposes they have received surprising little attention with regards to their genotoxic responses to environmental pollutants. The main study is that of De Boeck and Kirsch-Volders [8], who investigated the use of *Nereis virens* as a sentinel species for measuring genotoxic responses to PAHs. The authors used an intra-coelomic injection of benzo(*a*)pyrene (0.3–45 mg/ml), harvesting coelomocytes for assessment of DNA damage using the comet assay 1 h after injection. Based on the negative results obtained using this protocol, De Boeck and Kirsch-Volders conclude that this species is

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doi:10.1016/j.mrgentox.2008.05.008

tolerant to PAHs and therefore not suitable for environmental monitoring. Studies using alternative endpoints in other polychaete species, however, have suggested that certain polychaetes are highly sensitive to genotoxic damage (e.g. *Platynereis dumerilii* [9,10]; *Pomatoceros lamarckii* [11]). Studies using the terrestrial earth worms *Lumbricus terrestris* and *Eisenia foetida* [12–15] also found increased comet-tail lengths in response to PAHs, benzene and dioxins in the soil. A greater understanding of the genotoxic responses of marine polychaetes is required to aid our understanding of their survival in polluted environments and hence their role in environmental monitoring.

The *in vivo* comet assay in its alkaline form (pH > 13) is increasingly used in genotoxicity testing of substances such as industrial chemicals, biocides and pharmaceuticals [16]. Genotoxicity studies using the comet assay are often based on free cell preparations, i.e. blood cells or sperm in humans and vertebrates or haemocytes or coelomocytes – invertebrate equivalents to blood cells – in invertebrate species, due to their ease of collection. Polychaetes are morphologically and physiologically an extremely diverse group of animals and their free cells exhibit a similar diversity, which has confounded attempts to apply a simple classification system to describe the cell types of this group [17]. Certain types of coelomocytes are present throughout the group, however, the most widely used terms to describe them being 'amoebocytes' and 'eleocytes'. *N. virens* and *Nereis diversicolor* both have several types of amoebocyte, which have varying functions such as immunity and wound healing, as well as nutritive eleocytes that produce vitellogenin for developing oocytes and nucleotides for developing spermatids [17,18].

The overall response of a cell to a genotoxic substance will result from a combination of exposure, uptake, metabolic activation, defence mechanisms and repair efficiency, and may differ significantly for different cell types. Any differences in the responses of these different cell types would lead to large individual variation in the genotoxicity data collected, making it more difficult to determine any significant effects caused by environmental exposure to a low genotoxic dose. The coelomic cavities of most polychaete species also contain developing gametes during some or most of the year, depending on species and location. These cells may also have differing responses from somatic cells, and therefore have the potential to interfere with investigations of genotoxicity if they are not taken into consideration when cells are sampled from the coelomic cavity.

This study is an exploration of species-specific and cell-specific differences in cellular sensitivity to DNA-damaging agents in three polychaete species, aimed at improving the fundamental knowledge of genotoxic responses in these animals. Using three abundant polychaete species, i.e. the lugworm *Arenicola marina*, the king ragworm *N. virens* and the harbour ragworm *N. diversicolor*, the responses (i) between coelomic cell types (i.e. amoebocytes, eleocytes and spermatozoa) and (ii) between species, to the direct-acting genotoxin methyl methanesulfonate are compared to test the hypothesis that different cell types will exhibit different sensitivities to genotoxic damage. The time and dose responses of *A. marina* to methyl methanesulfonate (a direct-acting mutagen) and the metabolically activated PAH benzo(*a*)pyrene are also compared and a comparison of *N. virens* from different locations is made to address the hypothesis that this species has a high tolerance to DNA damage from PAH-contaminated habitats.

2. Materials and methods

2.1. Collection and maintenance of animals

Animals were chosen from sites considered to be relatively 'clean' and free of any significant contamination (Environment Agency, 2007), and were collected outside of their natural breeding season (except for the experiments using spermatozoa, as described below). Adult *A. marina* were collected from the beach at Mothercombe estuary, South Devon (50°18′41″N, 3°56′45″W), during September–December 2006. Individual animals were collected according to the methods described by Lewis et al. [19] and transferred to the laboratory were they were checked for maturity (gravid animals were not used except in the experiment using spermatozoa), then stored at 15 °C in individual containers in filtered (0.2 μm) seawater (FSW). Animals were maintained in 10-l glass aquarium tanks in well-aerated FSW for 2 days post-digging, in order to allow their gut contents to be voided.

Adult *N. diversicolor* were dug from the muddy estuary at Exmouth, South Devon (50°36′51″N, 3°26′43″W) during September 2006. Animals were collected and transferred to the laboratory where they were maintained in aquaria in well-aerated FSW at 15 °C until use. *N. virens* specimens were purchased from an aquaculture supplier (Seabait Ltd.), for the initial comet-assay work, since local populations tend to occur in contaminated areas. For the population comparison experiment, animals were also collected from the muddy shore at Torpoint, in the Tamar estuary, Cornwall (50°22′14″N, 4°11′44″W), and Poole harbour, Dorset (50°42′14″N, 1°58′43″W). Large immature adults were collected and transferred to the laboratory, where they were maintained in aquaria in well-aerated FSW at 15 °C until use. Torpoint and the lower regions of the Tamar estuary are well documented as being heavily contaminated with PAH, metals and tributyltin (TBT) [20,21], whilst Poole harbour appears to have much lower PAH levels [22].

2.2. Comet-assay procedure

Coelomic fluid samples were collected using a 1-ml syringe fitted with a 21-gauge hypodermic needle (chilled prior to use), carefully inserted into the posterior region of the body avoiding the gut, and stored on ice until use. All samples were checked for cell viability prior to the comet-assay procedure by use of Eosin Y staining. For each assay 50 μL of coelomic fluid was used from each animal. Coelomic fluid was gently centrifuged at 78 × *g* (1000 rpm) for 4 min and the excess fluid removed. The cell concentrate was then gently mixed with 1% low melting point agarose (heated to 37 °C) and dropped onto slides previously coated with 1% normal melting point agarose. The slides were protected with coverslips while they set for 10 min at 4 °C, then the coverslips were carefully removed. The Comet assay was then performed according to the method described by Singh et al. [23] with modifications, using alkaline conditions at 5 °C. Briefly: 1 h lysis was followed by 45 min denaturation in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, at pH 13) and then electrophoresis for 30 min at 25 V and 300 mA, followed by neutralisation. Cells were stained with 20 mg L^{-1} ethidium bromide and examined with a fluorescence microscope using a 420–490-nm excitation filter and a 520-nm emission filter. One hundred cells per preparation were quantified by use of Kinetic COMET Software.

2.3. Effects of using anticoagulant

A. marina coelomocytes aggregate spontaneously to form large clumps as soon as they are removed from the body [17] and anticoagulants are often required for cell-based investigations. However, the use of anticoagulant in marine invertebrates has only previously been reported in Crustaceae (where the pH of haemolymph is generally around 4.6, [24]) with no reports for polychaetes. Optimisation of the assay, therefore, included an investigation into the impact of anticoagulant on polychaete cells. Anticoagulant solution (0.45 M sodium chloride; 0.1 M glucose; 30 mM sodium citrate; 10 mM citric acid; 10 mM EDTA) was made up according to Soderhall and Smith [24] at pH 4.6. Half of this solution was then adjusted to pH 7.3 – the pH of *A. marina* coelomic fluid, [25] – by adding NaOH. To confirm the anticoagulant properties of these solutions, coelomic samples were collected from *A. marina* using a 1-ml syringe fitted with a 21-gauge hypodermic needle containing 0.5 ml of treatment solution: (i) PBS; (ii) anticoagulant at pH 4.6 or (iii) anticoagulant at pH 7. The samples were examined under a light microscope after 10 min to determine the degree of cellular aggregation for each solution. Three samples of coelomic fluid were then collected from each of six *A. marina* specimens into 0.5 ml of each (chilled) treatment solution and the comet assay performed on each sample as described above.

2.4. Cell-type sensitivities

Nereids generally have two distinct types of coelomocytes present during gametogenesis: amoebocytes and eleocytes [18,26]. To determine their individual sensitivities to genotoxins these cells were separated with a combination of filtering and a density-gradient technique. Coelomic samples were collected from five *N. diversicolor* and six *N. virens* specimens into a small volume of PBS buffer using a 21-gauge hypodermic needle and syringe, and stored on ice. Approximately 1 ml of coelomic fluid in PBS was collected for each specimen. To separate out the amoebocytes from the larger eleocytes, the coelomic fluid was first passed through a 30-μm mesh filter. This retains most of the larger, 'sticky' eleocytes, which were then backwashed and retrieved into a small Petri dish before being split into two microcentrifuge tubes for the *in vitro* exposures. Five hundred microliters of the remaining coelomic fluid (that had passed through the mesh) was then pipetted onto a 40:60 (v:v) Lympho Separation Medium (ICN Biomedicals Ltd, OH) in PBS and centrifuged at 78 × *g* for 15 min to further separate any remaining eleocytes