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Cadmium Toxicity Monitoring Using Stress Related Gene Expressions in *Caenorhabditis elegans*

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Abstract

The toxicity of cadmium on *Caenorhabditis elegans* was investigated to identify sensitive biomarkers for environmental monitoring and risk assessment. Stress-related gene expression were estimated as toxic endpoints Cadmium exposure led to an increase in the expression of most of the genes tested. The degree of increase was more significant in heat shock protein-16.1, metallothionein-2, cytochrome p450 family protein 35A2, glutathione S-transferase-4, superoxide dismutase-1, catalase-2, *C. elegans* p53-like protein-1, and apoptosis enhancer-1 than in other genes. The overall results indicate that the stress-related gene expressions of *C. elegans* have considerable potential as sensitive biomarkers for cadmium toxicity monitoring and risk assessment.

Keywords: *Caenorhabditis elegans*, cadmium, stress-related gene expressions, biomarker, toxicity monitoring, environmental risk assessment

Caenorhabditis elegans, a free-living nematode that lives mainly in the liquid phase of soils, constitutes a useful bio-indicator of environmental disturbances. Due to its abundance in soil ecosystems, its key role in decomposition and nutrient cycling, its convenient handling in the laboratory, and its sensitivity to different kinds of stress, *C. elegans* is frequently used in ecotoxicological studies utilizing various exposure media, including soil, aquatic media, and freshwater[1-3]. Since its genome has been completely sequenced, the functional relations of gene expression and phenotypic response have been investigated to a considerable extent. For these reasons, *C. elegans* seems to be an attractive animal model for the study of the ecotoxicological relevance of chemical-induced gene-level responses[4,5].

The current literature on *C. elegans* provides a degree of insight into the relative sensitivity of several of its endpoints[6-8]; only few experiments, however, venture into a direct comparison of multiple endpoints under a common set of experimental conditions. In this study, the toxicological effects of exposure to cadmium were assessed on *C. elegans*. Taking into account the frequent appearance of this metallic compound in the environment and the ecological importance of *C. elegans*, a study on the effects of metals on *C. elegans* could provide valuable information for the biomonitoring or risk assessment of these compounds in the ecosystem.

The aims of the current study were to evaluate the acute toxicity of cadmium on *C. elegans*, to identify the sensitive genes expressed as part of the metal-activated stress responses of *C. elegans* and to validate the ecotoxicological relevance of stress-related gene expression by investigating the physiological-level responses of *C. elegans*.

A lethal toxicity test was conducted to investigate the acute effects of heavy metals on *C. elegans*. Its responses on the molecular / physiological levels were subsequently investigated upon sublethal exposure. As potential stress-related genes, heat shock protein (hsp-16.1, hsp-16.2, hsp-16.48, hsp-70), metallothionein (mt-1, mt-2), vitellogenin (vit-2, vit-6), cytochrome p450 family protein 35A2 (cyp35a2), glutathione S-transferase (gst-4), superoxide dismutase-1 (sod-1), catalase-2 (ctl-2), *C. elegans* p53-like protein (cep-1), and apoptosis enhancer (ape-1) were examined in a semi-quantitative manner. Growth and reproduction were investigated as physiological descriptors of metal toxicity by measuring the body length and by counting the eggs of each worm, respectively.

Acute toxicity was studied using LC50s derived through Probits analysis (Table 1). The 24 h LC50s of Cd in *C. elegans* were 846 mg/L. Based on the results of the acute toxicity test, 3 concentrations-corresponding to 1/1000, 1/100, and 1/10 of the 24 h LC50-were selected for the sublethal exposure conditions, which were 0.85, 8.5, and 85 mg/L .

Fig. 1 shows the stress-related gene expression profile measured in the young adults of *C. elegans* exposed to Cd for 24 h. Cd exposure led to increases in the expression of most of the genes tested, except for hsp-16.48 and mt-1 genes, where no significant

change was observed. The degree of increase was more important in hsp-16.2 (4.5 folds compared to the control), mt-2 (3.7 folds), cyp35a2 (7.3 folds), gst-4 (8.1 folds), ape-1 (7.9 folds), sod-1 (4.5 folds), and ctl-2 (5 folds). In particular, the induction of the cyp35a2 gene expression was inversely concentration -dependent (7.3, 4.7, and 1.9 folds for 0.85, 8.5, and 85 mg/L, respectively). The responses of the stress-related genes to Pb, Cr, and As exposure were not as intense as their responses to Cd exposure. Pb exposure induced hsp-16.1, hsp-16.2, hsp-70, and ape-1 gene expressions, and among these, the increase in the hsp-16.2 expression was most remarkable (about 3.3 folds compared to the control). Only the hsp-16.1 gene expression was increased by Cr exposure, whereas As exposure induced the hsp-16.1 and gst-4 gene expressions. The increase in the hsp-16.1 gene expression was observed in all the worms that had been exposed to the four metals. The degree of increase, however, was less than 1.5 folds than that of the control.

Table 1. Estimation of 24 h-LC50 of Cd in *C. elegans*.

		24 h LC (mg/L)	Interval of confidence (95%)
Cadmium chloride	LC50	846	637 < LC50 < 1064

As a growth descriptor, the changes in the worms' body lengths after metal exposure were presented in Fig. 2a. The worms, which had been exposed to Cd showed a decrease in their body length. The most significant decrease was observed at the lowest concentrations of Cd (0.85 mg/L). The effects of metals on the reproduction of nematodes were estimated by counting the eggs in the control worms and in the metal-treated worms (Fig. 2b). Cd exposure caused a serious decrease in the number of eggs per worm. The most significant decrease was observed at the lowest concentrations of Cd (0.85 mg/L), which were 57.1% of the decreases, compared to the control.

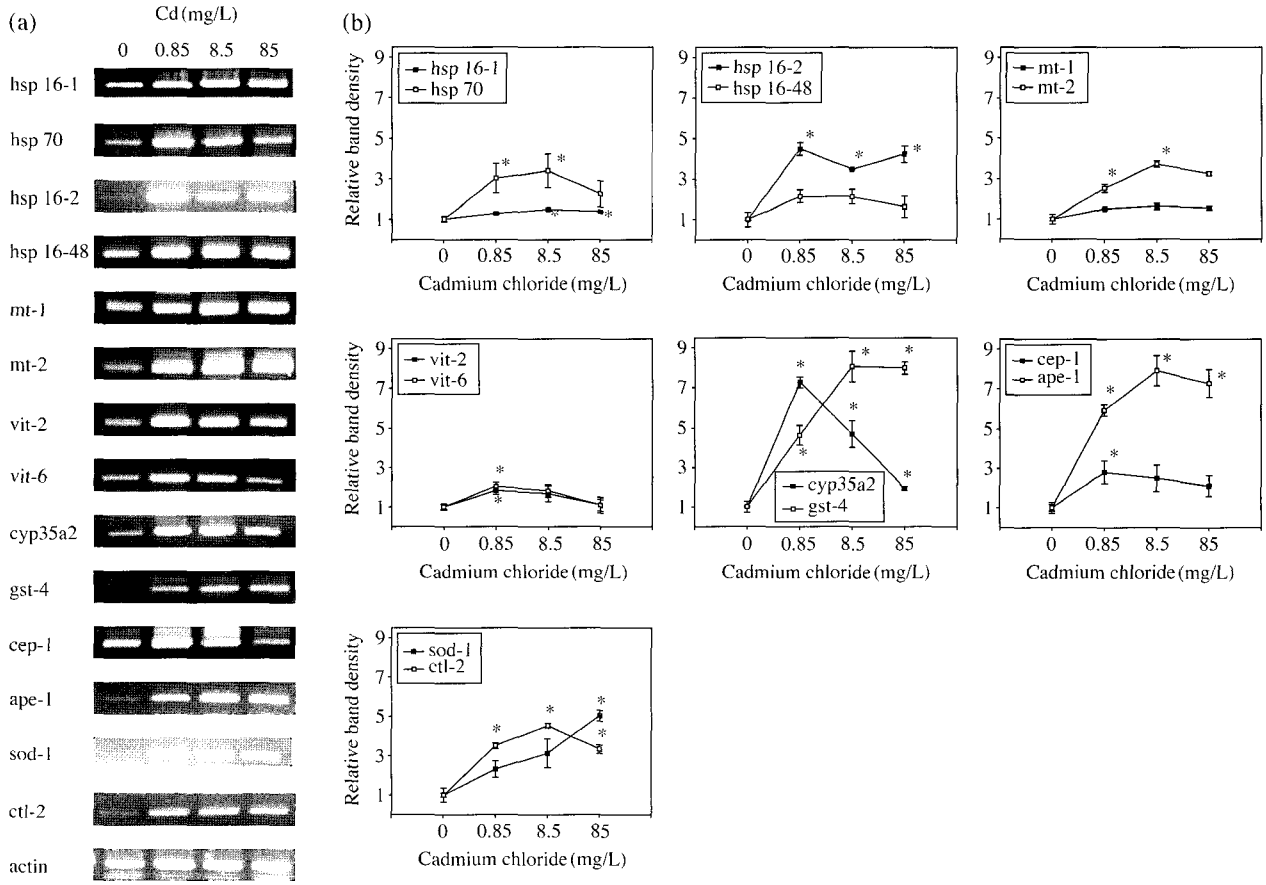

Fig. 1. Stress-related gene expression profiling in the young adult of *C. elegans* exposed to Cd for 24 h (a). Densitometric values were normalized using actin mRNA and are presented in arbitrary unit compared to control (control=1, b; n=3; mean±SEM; $*p < 0.05$).

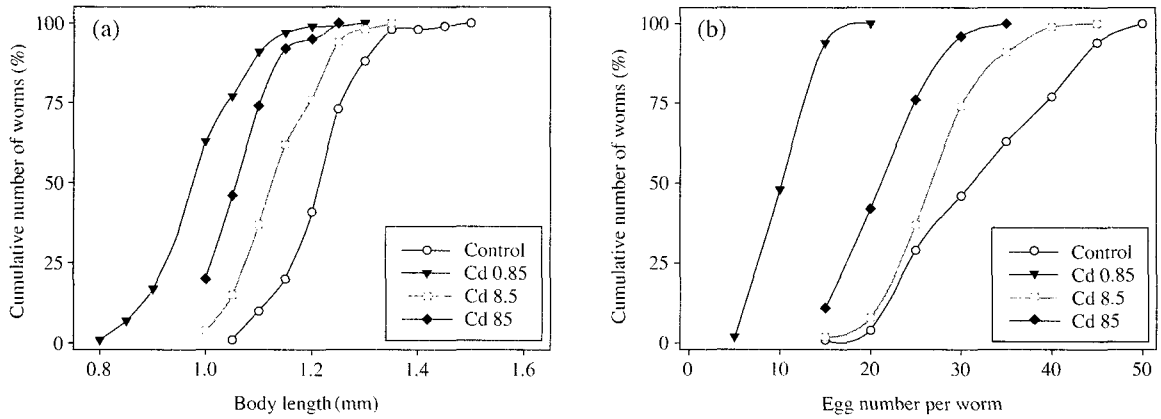

Fig. 2. Body length measured in the young adult of *C. elegans* exposed to Cd for 24 h (a). Egg number per worm measured (b).

Discussion

Nematodes are becoming popular bioindicators of pollution stress because of their ecological significance, their short life cycle, and the convenience of forming cultures from them and of maintaining a large number of them in the laboratory[3,7,9,10]. In this study, sensitive genes expressed as part of metal-activated stress responses were identified in *C. elegans* using Cd, and an attempt was made at validating their ecotoxicological relevance by investigating their physiological-level responses, such as growth and reproduction, and by comparing these with the stress-related gene expression profile in aid of a correlation study.

Acute toxicity tests using *C. elegans* have been performed by many investigators[3,11-14]. The LC50s of Cd obtained in this study are in accordance with the *C. elegans* aquatic toxicity data previously reported by Williams and Dusenbery[13]. In this study, *C. elegans* showed a high level of tolerance of / resistance to Cd exposure. The fact that Cd exhibits such a high level of tolerance compared to the other metals tested may imply that these organisms possess efficient defense equipment that prevent Cd-related damage-i.e., the existence of a specific protein that protects against Cd toxicity in *C. elegans*. The cadmium-responsive gene cdr-1 and its family have recently been identified and characterized in *C. elegans*[15,16]. This protein may contribute to this animal's high tolerance level towards Cd exposure, as observed in this study. Different from mt or hsps, cdr-1 was reported to have been induced only by Cd and not by other metals, organic chemicals, or physical stresses. An acute toxicity study showed that *C. elegans* survived at a high level of Cd exposure. Such level of tolerance suggests an efficient molecular/ biochemical or physiological level of protection against Cd toxicity, which was elucidated in this study. Cd exposure led to increases in the expression of most of the stress-related genes tested, including hsp-16.2, mt-2, cyp35a2, gst-4, ape-1, sod-1, and ctl-2. In particular, it has been reported that almost all cyp35 forms in *C. elegans* are moderately or strongly inducible by different xenobiotics in a cyp450 gene-expression screening experiment[4,17]. A sensitive response at the molecular level may contribute to organism-level resistance, which may be translated into high LC50 values. Physiological-level alterations, such as growth, reproduction, feeding, movement, or behavior, have been used as endpoints for metal-induced toxicity testing in *C. elegans*[7,9,10,13,18]. The effects of xenobiotics on the growth and reproduction of the test organisms are broadly accepted test parameters, and were found to be much more sensitive indicators of toxicity than lethality, as shown in this study (Figs. 2a, 2b). The decreases in body length and egg number per worm observed after Cd exposure may induce alteration in the growth and reproduction of the nematode population in the long term. In particular, for Cd exposure, an increase in many stress-related gene expressions occurred concomitantly with this deterioration on the physiological level.

The statistical tests revealed 27 significant correlations in Cd exposure (data not shown). Their biological/ecological meanings, however, should be interpreted because a correlation study alone cannot provide any causal relationship between these parameters. Direct experimental demonstrations of the wider relationships between molecular/biochemical-level effects and their subsequent consequences at higher levels of biological organization are needed in order to establish causal relationships. The characteri-

zation of the causal relationships between the biomarker responses and the effects at higher biological levels will help to define the sublethal hazards of chemicals in this animal.

Xenobiotically induced gene expression is considered a highly promising tool in biomonitoring for the early detection of environmental contaminants[4,19,20]. Gene expression endpoints are not only sensitive and useful in estimating the effects of toxicants on expected populations, but may also provide insight into the mechanisms underlying these effects. Their field application is still limited, though, because these systems are not capable of completely integrating the physiological status of a living organism, and thus, have low ecological relevance. While the parameters from higher levels of biological organizations, such as growth and reproduction, are accepted as valid and standardized endpoints, their responses are nonetheless not very sensitive or specific. The main advantage of the gene expression test compared to growth or reproduction test is the increased sensitivity and specificity. The parallel determination of a variety of stress-inducible genes (e.g., by using DNA microarray) will considerably promote the implementation of the approach[5,21]. Depending on the selected marker genes, this approach has the potential of identifying substance class-specific effects. In this context, it is important to reveal the potential relationships between the observed toxicity and the induced gene expression. Reliable, sensitive, and specific test systems are therefore needed, particularly for the risk assessment of a low-level mixture of xenobiotics in the environment, which affects both the wildlife and human health on a subcellular level. Using sensitive and reproducible detection methods, it is possible to establish significant pollution-induced changes on specific gene expressions and to generate new and accurate assays.

The overall results suggest that the use of the responses of stress-related gene expression as sensitive biomarkers has considerable potential for the diagnosis of Cd exposure, and that *C. elegans* seems to be a good biological model for this approach.

Methods

Organisms

The wild-type *C. elegans* Bristol strain N2 was used in this study. *C. elegans* were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20°C, using the standard method previously described by Brenner[22].

Metal Exposure and Sample Preparation

Nematodes were exposed to cadmium prepared in a K-medium (0.032 M KCl, 0.051 M NaCl). Young adults (3 days old) from an age-synchronized culture were used in all the experiments. Worms were incubated at 20°C for 24 h without a food source, and were then subjected to each type of analysis. Three replicates for each metal concentration and a control were conducted for all the test types.

Lethal Toxicity Tests

Each test consisted of Cd concentrations and a control. Using a dissecting microscope, 10 of the young *C. elegans* adults were transferred onto 24-well tissue culture plates containing 1 mL of the test solution per well. The worms were exposed for 24 h at 20°C. Following exposure, the numbers of live and dead worms were determined through visual inspection and by probing the worms with a platinum wire

Table 2. Sequence of primers used in the amplification of stress-related gene mRNA in *C. elegans*.

Gene (wormbase accession No)	Primer sequence
hsp 16-1	5′TCTCAATGTCTCGCAGTTCA3′
(T27E4.8)	5′CTTCCTTCTTTGGTGCTTCA3′
hsp 16-2	5′GCTATCAATCCAAGGAGAAC3′
(Y46H3A.3)	5′GAACCGCTTCTTTCTTTGG3′
hsp 16-48	5′ACTCGATGTTTCTCATTTC3′
(T27E4.3)	5′TGGGAATAGAACGAGATGAG3′
hsp 70	5′ACAACGAGATCGAATTAGCTCG3′
(C12C8.1)	5′ATCAACTTCCTCTACAGTAGGTC3′
mt 1	5′GAAATCATGGCTTGCAAGTGTG3′
(K11G9.6)	5′TTTAATGAGCCGCAGCAGTTCC3′
mt 2	5′CAAAAATGGTCTGCAAGTGTG3′
(T08G5.10)	5′ AATGAGCAGCCTGAGCACATTC3′
vit-2	5′TCTGAGCTTTCCCAATCCCG3′
(C42F8.2)	5′TCAAGGAAGGCATCTGCTCG3′
vit-6	5′GACTTCCAGTCCCATCTACC3′
(K07H8.6)	5′CTTGGTGCTCACGGTTCATG3′
cyp35a2	5′TCGATTTGTGGATGACTGG3′
(C03G6.15)	5′AATGGATGCATGACGTTGAA3′
gst-4	5′TTGGAGACTCATTGACTTGG3′
(K08F4.7)	5′AAACAATACTATCCTTTCTTGTTGCC3′
cep-1	5′TTCCGACGCAAGTAGTCTCC3′
(F52B5.5)	5′CGGTAAAAGCTGAGAAACG3′
ape-1	5′GTTTGGTGATAGTCTAGACG3′
(F46F3.4)	5′TGTTGTGGTATCACTACCTAATACC 3′
sod-1	5′CGAGGGAGTCGGAGACAAGG3′
(C15F1.7)	5′GTAGTAGGAGTAGGAACAAC3′
ctl-2	5′GACAATCAGCAACATGCTCC3′
(Y54G11A.5)	5′CTGGCACATTCTCTCCCGAG3′
actin	5′AGAAGAGCACCCAGTCCTCC3′
(T04C12.6)	5′GAAGCGTAGAGGGAGAGGAC3′

under a dissecting microscope.

RNA Preparation and Semi-Quantitative RT-PCR

Following 24-h incubation with exposure to sublethal concentrations of Cd, nematodes were harvested for the preparation of RNA. Standard procedures were followed and a Trizol reagent was used for the total RNA isolation. The two-step reverse transcription-polymerase chain reaction (RT-PCR) method was used with RT Premix (Bioneer Co., Seoul, Korea) and PCR Premix kits (Bioneer Co., Seoul, Korea), using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA). The primers were designed on the basis of the sequences retrieved from the *C. elegans* database (www.wormbase.org) (Table 2). Actin mRNA was served for the normalization of the studied gene expression levels. The PCR products were separated through electrophoresis on 1.5% agarose gel (Promega, Madison, WI, USA) and were visualized with ethidium bromide (Bioneer Co., Seoul, Korea). All the tests were replicated at least 3 times, and the relative densities of each band were determined with the use of a Kodak EDAS 290 image analyzer (Kodak, Rochester, NY, USA), with a TFX-20.M UV transilluminator (Vilber Lourmat, Marne la Vallee, France).

Measurement of the Body Length and the Egg Mass Count

Following 24 h incubation with exposure to sublethal concentrations of metal, growth and reproduction were assessed. Growth was assessed by measuring the worms that had been killed by the heat through microscopy, with a scaled lens in each metal concentration and a control. The average length of the unexposed control worm was about 1.10 mm. Reproduction was preliminarily assessed by counting the eggs of each worm through the microscopic inspection of the transparent *C. elegans* body in each metal concentration and the control. Though this procedure differs from more commonly used reproduction tests of offspring counting from an age-synchronized single worm, this simple detection method seems appropriate for the rapid screening of the reproduction effect. The average number of eggs per worm in the unexposed controls was about 30. More than 100 worms were analyzed per treatment.

Data Analysis

LC50s were derived through Probits analysis. The statistical differences between the control and treated worms were determined with the aid of the parametric *t* test. All the statistical analyses were conducted using SPSS 12.0.1 (SPSS Inc., Chicago, Illinois, USA).

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