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Genomic Response in *Daphnia* to Chemical Pollutants

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Abstract—Chemicals released into the environment have a potential to affect on various species and evaluation of the impacts on ecosystems including aquatic organisms is urgent issue. In order to evaluate chemical effects on various species, it is important to understand their mode of action. Recent advances in toxicogenomics, the integration of genomics into toxicology is promising for understanding mode of action in responding to chemical pollutants. In this study, we applied the toxicogenomics to an aquatic organism, *Daphnia magna*. Among aquatic organisms, *Daphnia magna* has been used extensively in acute toxicity or reproductive toxicity tests. Although these types of tests can provide information on hazardous concentrations of chemicals, they provide no information about their mode of action. Thus we developed an oligonucleotide-based DNA microarray based on a *Daphnia* expressed sequence tag (EST) database and applied for ecotoxicogenomic assessment of *Daphnia magna*. The DNA microarray was used to examine gene expression profiles of neonate daphnids exposed to several different chemicals. Exposure to these chemicals resulted in characteristic gene expression patterns that were chemical-specific. This result indicates that our newly developed DNA microarray can be useful for a mechanistic understanding of chemical toxicity on a common freshwater organism, *Daphnia magna*, and that the *Daphnia* genomics is useful for aquatic ecotoxicology.

Keywords: DNA microarray, omics, *Daphnia*, toxicology, toxicogenomics, ecotoxicogenomics

INTRODUCTION

Some of the chemicals released into the environment are suspected to have toxic effects not only on humans, but also on various species. Recently the putatively toxic effects of chemicals on different species are becoming a great concern, in terms of the impact of pollutants on ecosystems. However, the number of species that have been examined for the effects of chemical exposure is limited to date. This is partly because the difficulty of estimation of chemical effects on various organisms. As there are large variations in morphology, physiology and life cycle

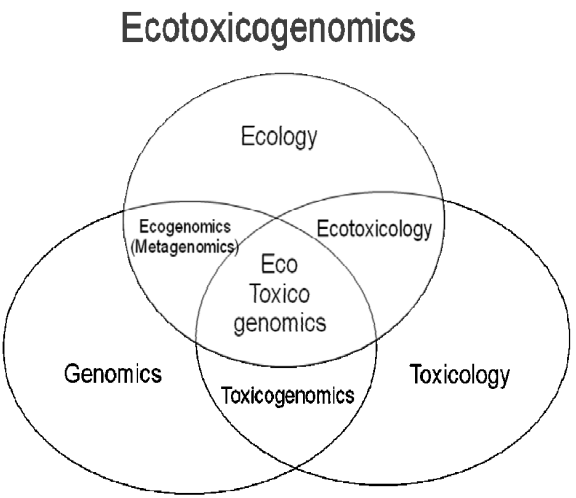

Fig. 1. Schema of ecotoxicogenomics.

among species, it is difficult to understand the mode of action of the chemical pollutants.

Recent advances in molecular biology have provided a technique for better understanding the responses of organisms to chemicals, which was emerged as toxicogenomics. Toxicogenomics is defined as an integration of genomics (transcriptomics, proteomics, metabolomics) into toxicology. Toxicogenomics has at least three major goals: 1) an understanding of the relationship between environmental exposure and adverse effects; 2) identification of useful biomarkers of exposure to toxic substances; and 3) elucidation of the molecular mechanisms of toxicity (Waters *et al.*, 2003; Waters and Fostel, 2004). Generally cells respond at the level of gene expression before a phenotype emerges in response to toxic chemicals, thus genomic approach can be sensitive tool. Changes in gene expression in respond to chemical exposure can be detected by DNA microarray, and multiple endpoints can be analyzed. Since chemicals do not always affect the same pathways, DNA microarray analysis can potentially provide multiple clues for understanding the molecular pathways that result in phenotypic changes in response to chemicals.

Although this approach, named toxicogenomics, has been applied to the study of model mammals, and characteristic changes in gene expression profiles in response to certain chemicals have been reported, it can be applied to the study of non-model animals in the environment.

In this context, the term "ecotoxicogenomics" has emerged (Bartosiewicz *et al.*, 2001; Snape *et al.*, 2004; Miracle and Ankley, 2005; Iguchi *et al.*, 2006; Watanabe and Iguchi, 2006) to describe the toxicogenomic approach to ecotoxicology (Fig. 1). The application of a toxicogenomic approach to *Daphnia*

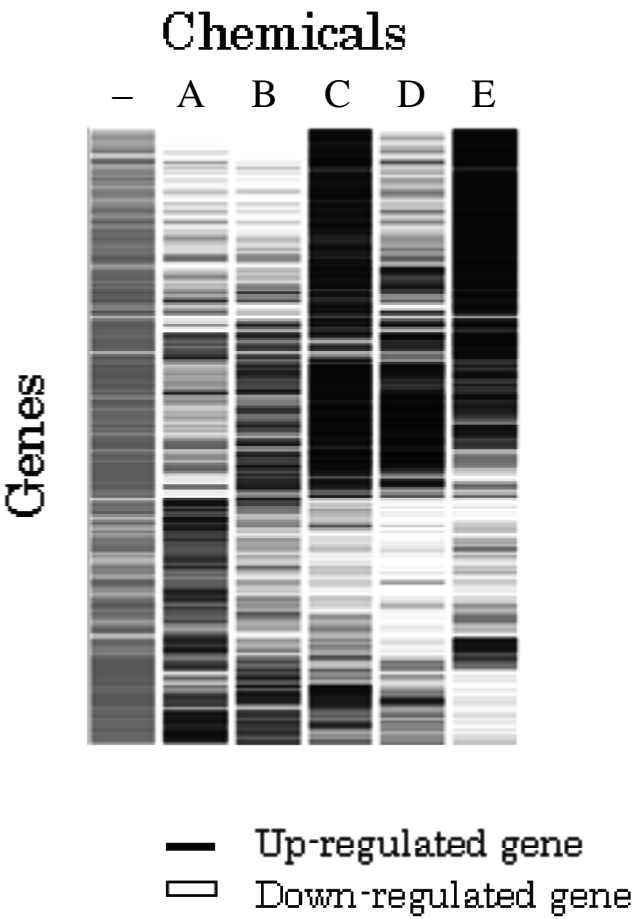

Fig. 2. Gene expression changes after chemical exposure Up-regulated genes are indicated in dark and down-regulated genes are indicated in light. Each column corresponds to gene expression change after a chemical exposure and left column corresponds to control.

magna has the potential to increase our knowledge and understanding of ecotoxicity, in particular because a mechanistic understanding of chemical toxicity in invertebrates is rather limited, and useful biomarkers have not been identified.

Among aquatic organisms, *Daphnia magna* has been used extensively to evaluate organism and population-based responses of invertebrates to pollutants. In the field, daphnids are widely distributed, they play a central role in the food web, and, like other aquatic organisms, are constitutively exposed to multiple chemicals. In the laboratory, *Daphnia magna* is easy to maintain and manipulate because of its short generation time, and thus, has been used as a model organism for aquatic toxicity testing (OECD, 1981; OECD, 1998). Based on standard and

other related toxicological tests, data on the effect of a large number of chemicals on daphnids has accumulated (Agency, 2002; von der Ohe *et al.*, 2005). Thus genomic information in respond to chemical exposure can be helpful for the understanding of ecotoxicity.

We recently analyzed ESTs for *D. magna* and created a database (Watanabe *et al.*, 2005). Based on this sequence information, we constructed an oligonucleotide-based DNA microarray, and explored the acute toxicogenomic response of *D. magna* to several different types of chemicals.

MATERIALS AND METHODS

Daphnid strain and culture conditions

The *D. magna* NIES clone used in these studies was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan (Tatarazako *et al.*, 2003). The clone was originally from the Environmental Protection Agency, USA, and has been maintained for more than ten years at the National Institute for Environmental Studies in Japan. Culture medium was prepared by filtering tap water through charcoal and maintaining it at room temperature overnight before use. Cultures of 20 individuals/L were incubated at 24 ± 1°C with a photoperiod of 14 h light–10 h dark. *Chlorella* was added daily to each culture as a 0.01 ml suspension of 4.3×10^8 cells/ml. The water quality (pH and dissolved oxygen concentration) was measured every two days by Environmental Research Center (Tsukuba, Japan). Water hardness, pH, and dissolved oxygen concentrations were 72 to 83 mg/L, 7.0 to 7.5, and 80 to 99%, respectively.

Chemical exposure of neonates

For acute toxicity testing, all offspring were removed from a culture of 20 adult females (two-three weeks of age) one day before testing. Neonates (age <24 h) were exposed to chemicals for 48 h in a static exposure regimen. Each concentration of chemicals was prepared by diluting a stock solution with fresh culture medium. For the acute toxicity test, eight to ten replicates were prepared for each concentration to estimate EC50. Immobilization, determined according to organization for economic co-operation and development (OECD) test guidelines, was used as the criteria for EC50 (OECD, 1981).

For DNA microarray analysis, the same protocol was used except that neonates were exposed to chemicals for 24 h, and three biological replicates were performed.

Preparation of the DNA microarray

Based on an EST database of *D. magna* (Daphniabase, http://daphnia.nibb.ac.jp), genes were selected for the DNA microarray. The ribosomal L8 protein gene were specifically selected; the other genes were randomly selected from the database.

An oligonucleotide corresponding to each gene was designed so that it did

not have similarity to other sequences in the database and the oligonucleotides (60 mers) were synthesized in situ (Nimblegen).

Microarray experiments

Neonates (age <24 h) were obtained as for the acute toxicity test. Generally, 20 adult daphnids (two-three week old) were cultured in a 2 L flask and neonates delivered from the culture were used for analysis. Neonates were exposed to chemicals for 24 h in a static exposure regimen and then harvested. Harvested daphnids were briefly washed and homogenized using the physcotron NS-310E (Nichion, Tokyo, Japan). Total RNA was purified with TRIZOL reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. In order to amplify signals from limited sample material, RNA was converted to cDNA, and cRNA was then amplified (Van Gelder *et al*., 1990; Baugh *et al*., 2001) using the Low RNA Input Linear Amp Kit (Agilent Technologies Japan, Tokyo, Japan). Briefly, 500 ng of total RNA was converted to cDNA using a T7-oligo dT primer. cRNA was then synthesized using T7 RNA polymerase in the presence of either Cy3-UTP. Cy3 labeled cRNAs were combined and hybridized to the microarray overnight at 42°C in hybridization buffer (5x SSC, 0.5% SDS, 4x Denhaldt's solution, 10% formamide, 0.1 mg/ml denaturated sermon sperm DNA). After hybridization, arrays were washed in buffer (5x SSC, 0.1% SDS) for 10 min at 30°C and scanned by GenePix4000B (Axon Instruments, Union City, CA, USA). The number of biological replicates was three.

Data analysis

The scanned microarray was analyzed by GenePixPro 5.1 software (Axon Instruments, Union City, CA, USA) to obtain signal intensities. The signal intensity of each spot was calculated by subtracting the local median background. Signal intensities were normalized to the 50th percentile per array and used for further analysis. Fluorescent intensities of each spot on the microarray were measured directly (Peixoto *et al*., 2006).

For cluster analysis, the Student's *t*-test was performed using the results of three independent experiments, and genes were identified whose expression was significantly changed ($t < 0.05$) by chemical exposure. The average signal intensity from three independent experiments was obtained, and fold-changes were calculated by dividing the average signal intensity by the average signal intensity of the control sample. For hierarchical clustering, genes showing more than a 2-fold change in gene expression were selected and analyzed. Similarity was estimated using Pearson's correlation coefficients, and average linkage was used for the clustering algorithm. Principal component analysis was performed using the same set of genes used for cluster analysis.

Quantitative polymerase chain reaction (PCR)

Total RNA was purified as described above. cDNA was synthesized from purified total RNA using Superscript II RT(-) (Invitrogen, Tokyo, Japan) and

random primers at 42°C for 60 min. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green PCR core reagents (PE Biosystems, Tokyo, Japan) in the presence of appropriate primers, according to the manufacturer's instructions. Each PCR amplification was performed in triplicate using the following amplification protocol: 2 min at 50°C and 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C).

Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. Primers were chosen to amplify short PCR products of less than 100 base pairs. Primer sequences are available upon request.

RESULTS AND DISCUSSION

Chemical exposure and changes in gene expression profiles

In order to examine gene expression changes due to chemical exposure, we examined the effects of different chemicals. Firstly, EC50 values were determined by exposing neonatal daphnids to a range of chemical concentrations. Based on the EC50 of each chemical, we chose two concentrations of chemical for DNA microarray analysis. One was a half concentration of the EC50 and the other was one fourth concentration of the EC50. Following chemical exposure of neonatal daphnids, microarray signal intensities were measured and averaged, and evaluated for evidence of characteristic changes in gene expression profiles.

Gene expression profiles of neonates exposed to the chemical were characteristic to each chemical. These results indicated that different types and doses of chemicals lead to different gene expression profiles.

Among the various genes whose expression changed, it was also possible to identify those that displayed chemical-specific responses. Changes in the expression of these genes were confirmed by quantitative PCR, as were changes in several other genes in response to specific chemicals. Evaluation of the characteristic gene expression profiles for a specific chemical could potentially provide valuable information about the mechanisms of toxicity in *Daphnia magna*.

DNA microarray platform

While conventional DNA microarrays use a cDNA library printed onto a glass slide, the use of oligonucleotide DNA microarrays has several advantages (Petersen *et al.*, 2005). For example, larger amounts of high-quality oligonucleotides can be reproducibly generated for printing onto glass slides because oligonucleotide synthesis is an established technology. In addition, oligonucleotide arrays synthesized in situ is also available. In contrast, when cDNAs are used for a DNA microarray, reproduction and quality control of cDNA synthesis become time consuming steps in the process (Attoor *et al.*, 2004). The use of cDNA microarrays also presents a higher risk of cross-

hybridization, because the DNA sequence is longer, thus increasing the probability of detecting the expression of similar genes. Furthermore, selecting shorter sequences (50–80 bp) that represent specific genes is easier than selecting longer sequences (>500 bp) for cDNAs. Although sequence information should be obtained before the fabrication of a DNA microarray, the DNA microarray based on oligonucleotides represents an ideal platform for ecotoxicogenomics as well as toxicogenomics.

Our results also indicate that it is possible to obtain gene expression profiles using one-color evaluation, based on direct measurement of fluorescent intensities. The advantages of single-channel measurement have also been reported by other groups (Peixoto *et al.*, 2006). From a toxicogenomic point of view, one-color evaluation of DNA microarray data is an ideal method, potentially allowing one to easily obtain multiple data points, such as different chemicals, doses and times of exposure. Two-color DNA microarray analysis would require the same number of control samples as data points, meaning that the scale of the experiment would double. In contrast, using a one-color DNA microarray approach, a single control sample is required, and variations among control samples can readily be estimated.

Feasibility

For evaluating our DNA microarray, we examined performance from both a technical and biological point of view. From a technical point of view, the data generated by a DNA microarray platform should be highly reproducible. In this sense, oligonucleotide based array yields higher reproducibility.

From a biological point of view, gene expression profile obtained from neonates showed higher reproducibility than that obtained from adults.

Gene expression changes

Daphnids were exposed to several chemicals whose effects have previously been studied in model vertebrate and other organisms. By increasing the number of genes on the *Daphnia* DNA microarray, it may be possible to compare toxic effects between vertebrate model organisms and daphnids in more detail, which may help facilitate an evaluation of the effects of different chemicals on various species. In addition, taking advantage of common features between traditional vertebrate models and daphnids may help in the development of alternative toxicological tests to animal model testing.

Detailed DNA microarray analysis at different doses and time points may help in our understanding of the relationship between gene expression and toxicity.

Although our result show common aspects in the response to chemical exposure between vertebrates and invertebrates, the DNA microarray may also be useful for detecting effects that are specific to daphnids. For example, we and others have previously shown that some chemicals that mimic juvenile hormone activities can induce the production of more male phenotypes (Olmstead and

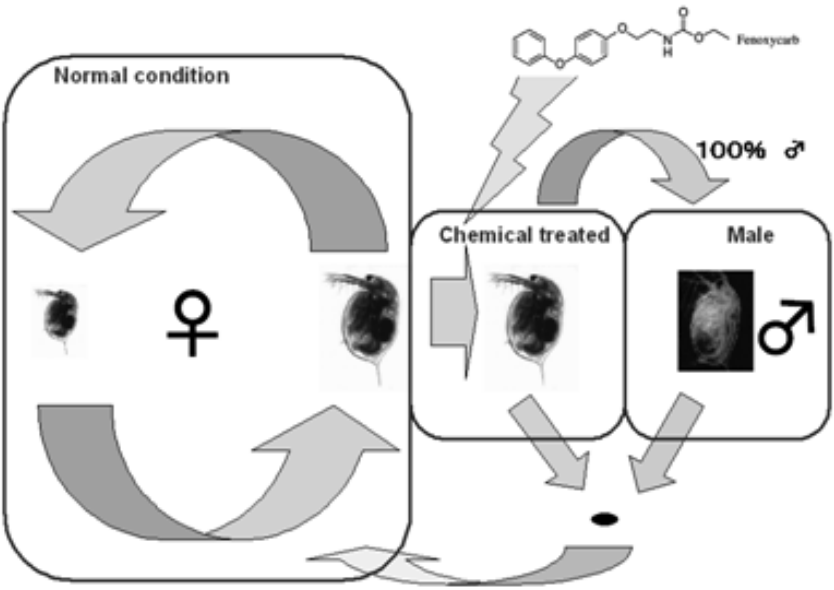

Fig. 3. Schema of *Daphnia* reproduction.

LeBlanc, 2001a, b; Tatarazako *et al*., 2003; Oda *et al*., 2005) (Fig. 3). Effects of such chemicals cannot simply be interpreted as the activation of genes related to detoxification. By applying DNA microarray analysis to these and other types of chemicals, a mechanistic understanding of how these chemicals mediate their effects may be possible.

It is also notable that DNA microarray analysis took less time compared to reproductive toxicological tests. Although we did not directly compare reproductive toxicity and changes in gene expression profiles in this study, we expect that the DNA microarray would also detect reproductive toxicity. The general protocol for reproductive toxicity takes 21 d for one set of tests (OECD, 1998), and one obtains only the effective dose of the chemicals of interest. DNA microarray analysis takes only a few days, and characteristic profiles of chemical effects could be obtained, which may in turn provide additional information on the mechanisms of reproductive toxicity.

We have demonstrated the feasibility of a molecular genomic approach to studying toxicity in daphnids, using DNA microarrays to analyze changes in gene expression profiles of neonates in response to chemical exposure. Although acute toxicity or reproductive toxicity tests can provide information on the hazardous concentrations of chemicals, they provide no information about their mode of action. Our study represents a breakthrough for the evaluation of chemical toxicity on environmental organisms, and our results suggest that chemical effects can be classified according to changes in gene expression profiles.

Classification of chemicals according to gene expression profiles or pathways may contribute to the estimation of relative risk of various chemicals. By increasing the number of genes on a DNA microarray, detailed gene expression profiles in response to chemicals can be obtained, and new biomarkers and/or new pathways characteristic of environmental chemicals can be identified.

An advance in our basic physiology, comparative molecular endocrinology, genomics and toxicology is necessary to apply omic technologies in wild life species.

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