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Discriminating the molecular basis of hepatotoxicity using the large-scale characteristic molecular signatures of toxicants by expression profiling analysis

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ABSTRACT

Predicting the potential human health risk posed by chemical stressors has long been a major challenge for toxicologists, and the use of microarrays to measure responses to toxicologically relevant genes, and to identify selective, sensitive biomarkers of toxicity is a major application of predictive and discovery toxicology. To investigate this possibility, we investigated whether carcinogens (at doses known to induce liver tumors in chronic exposure bioassays) deregulate characteristic sets of genes in mice. Male C3H/He mice were dosed with two hepatocarcinogens (vinyl chloride (VC, 50–25 mg/kg), aldrin (AD, 0.8–0.4 mg/kg)), or two non-hepatocarcinogens (copper sulfate (CS, 150–60 mg/kg), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, 150–60 mg/kg)). Large-scale molecular changes elicited by these four hepatotoxicants in liver tissues were analyzed using DNA microarray. Three days after administration, no significant phenotypic changes were induced by these four different hepatotoxicants in terms of histological examination or blood biochemical assay. However, unsupervised hierarchical analysis of gene expressional changes induced by hepatotoxicants resulted in two major gene subclusters on dendrogram, i.e., a carcinogen (VN, AD) and non-carcinogen group (CS, 2,4,5-T), and also revealed that distinct molecular signatures exist. These signatures were founded on well-defined functional gene categories and may differentiate genotoxic and non-genotoxic carcinogens. Furthermore, Venn diagram analysis allowed us to identify carcinogen and non-carcinogen-associated molecular signatures. Using statistical methods, we analyzed outlier genes for four different classes (genotoxic-, non-genotoxic-carcinogen, genotoxic-, non-genotoxic non-carcinogen) in terms of their potential to predict different modes-of-action. In conclusion, the identification of large-scale molecular changes in different hepatocarcinogen exposure models revealed that different types of hepatotoxicants are associated with different epigenetic changes and molecular pathways and that these large-scale characteristic molecular changes could be used as predictable toxicity markers.

1. Introduction

The past few years has witnessed a flood of new bioactive compounds that are being developed to treat a wide range of diseases. However, development on the majority of such compounds is halted due to unforeseen toxicity profiles and side effects encountered during clinical studies. Moreover, although major advances have been made over the past 25 years in terms of predicting and understanding the potential adverse effects of compounds in man, there exists a need for faster, more sensitive, more predictable methods for evaluating the safeties of new drug candidates (Ulrich and Friend, 2002). Recently, powerful functional genomic-based methods have been devised to provide new, mechanism-based, assays for predicting toxic risks in humans. It has also been suggested that enormous benefit could be derived from the application of DNA microarray technologies to the analysis of chemically induced gene expression alterations (Waring and

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Halbert, 2002; Waters and Fostel, 2004). In terms of gene expression analysis, DNA microarray technology has had a major impact on many different subject areas, including toxicogenomics, e.g., for predicting the adverse effects of new drug candidates and by improving the risk assessment and safety evaluation processes. Ultimately, research in this area may lead to rapid, high throughput screening systems, and to more acceptable animal testing regimes than are capable of predicting human safety with a high measure of safety.

The rapid accumulation of genomic-sequence data and associated gene and protein annotations have further accelerated the application of gene expression analysis to determine the modes-of-action of chemicals and other environmental stressors in biological systems. The establishment of DNA microarray technology enables us to perform genome-wide analysis of gene expression profiles, and has already provided an unprecedented understanding of the nature of toxicant-related diseases (Waters and Fostel, 2004). Currently, the applications of these technologies in toxicological research are drastically expanding and the major focus is on the unraveling of the modes-of-action of toxic compounds and on the identification of the gene expression profiles that can be utilized as biomarkers of specific toxic endpoints (Ellinger-Ziegelbauer et al., 2005; Thukral et al., 2005; van Delft et al., 2005).

Chemicals causing tumors in animals are generally classified into two broad categories, i.e., as genotoxic or non-genotoxic, based on their primary tumorigenic mechanisms in animals (Kim et al., 1998; Ramel, 1992; van Delft et al., 2005; Yoshikawa, 1996). The mechanisms whereby genotoxic chemicals generate tumors have been relatively well studied as compared with those of non-genotoxic chemicals. Genotoxic chemicals can cause DNA damage in a variety of ways. Subsequently, some damage escapes repair or is repaired incorrectly, leading to mutations that can accumulate with time, resulting in anaplastic transformation of cells and ultimately formation of tumors. In contrast, non-genotoxic chemicals may cause tumor formation via a number of relatively diverse modes-of-action. These may include chronic cytotoxicity, immunosuppression, decreased tumor suppressor gene function, decreased cell–cell communication, activation of a receptor, decreased apoptosis, and increased secretion of tropic hormones or various combination of these factors. Moreover, carcinogenesis induced by these compounds have some features that distinguish them from those induced by genotoxic carcinogens (Gonzalez et al., 1998; Kitchin et al., 1994; van Delft et al., 2005). In fact, attempts to estimate risk potential have given rise to contradictions between Salmonella genotoxicity and rodent carcinogenicity due to the existences of non-genotoxic (Ames-negative) carcinogens and genotoxic (Ames-positive) non-carcinogens (Yoshikawa, 1996). Therefore, the identification and characterization of genome-wide molecular changes early after liver exposure to non-genotoxic or genotoxic carcinogens are crucial to the understanding of the different mechanisms of chemical carcinogenesis.

The applications of gene expression techniques using microarrays in toxicological studies facilitate an interpretation of the mode-of-action of toxic compound and may allow the prediction of selected toxic effects based on gene expression changes. Thus, here, we investigated whether the possibility of relationships between the hepatotoxic phenotypes and gene expression profiles of hepatotoxic chemicals as determined by DNA microarray analyses, and sought to determine whether gene expression profiling can classify chemical carcinogens and non-carcinogens on a mechanistic basis. Whole-genome mouse DNA microarrays were used to compare the global transcriptional profiles elicited by four representative hepatotoxicants, i.e., a genotoxic carcinogen, a non-genotoxic-carcinogen, a genotoxic non-carcinogen, and a non-genotoxic non-carcinogen, to investigate relations between their large-scale molecular signatures and the modes-of-action of their hepatotoxicities.

2. Materials and methods

2.1. Animals and treatments

The four hepatotoxicants were obtained from Sigma–Aldrich (St. Louis, MO). Single, high doses of the compounds were administered to male, 6-week-old C3H/He mice (Charles River Laboratories, Inc.). Vinyl chloride (VC) (Sigma, St. Louis, MO; CAS Number: 75-01-4), aldrin (AD) (Sigma, St. Louis, MO; CAS Number: 309-00-2), Copper sulfate (CS) (Sigma, St. Louis, MO; CAS No.: 7758-98-7), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma, St. Louis, MO; 93-76-5 5892) were prepared following manufacturer's protocol. High toxic doses were 50% of the published LD_{50}s and low toxic dose were 25% of the published LD_{50}s for each hepatotoxicant. Mice were sacrificed and livers were removed at 72 h after oral administration of VC (50, 25 mg/kg), AD (44, 22 mg/kg), CS (150, 80 mg/kg) and 2,4,5-T (150, 80 mg kg) with vehicle (corn oil), respectively. All hepatotoxicants were dosed for 3 days. Animals were observed at least once daily for signs of overt toxicity and readily apparent clinical symptoms. In all instances, animals were humanely handled in accordance with IACUC guidelines. Following treatment, mice were sacrificed, and serum samples were preserved to determine hematological parameters. Livers were processed for histopathological examination and RNA extraction. RNA was isolated using TRIzol (Invitrogen, MO), and quality control was performed using RNA 6000 Nano chips on an Agilent 2001 Bioanalyzer (Agilent Technologies, Germany), as previously reported (Nam et al., 2006, 2005).

2.2. Clinical chemistry and histology

Blood for clinical chemistry measurements was collected at 3 days after administration. ALT, AST, albumin, and total bilirubin were determined using standard clinical chemistry techniques. For histology, formalin-fixed tissues were embedded in paraffin blocks, sectioned at *ca.* 5 μm, and stained with hematoxylin and eosin. Histology slides were evaluated in-house by a board-certified veterinary pathologist (J.J. Jang)

2.3. Whole-genome mouse oligoarray and formulation

High-density mouse whole-genome oligonucleotide microarrays were manufactured at the array core facility of the Microdissection Genomics Research Center at The Catholic University of Korea. The Mouse Exonic Evidence Based Oligonucleotide (MEEBO) sets were purchased from Illumina (USA). This set contains a total of 35,302 probes targeting mouse genes. Seventy mers of synthesized oligos were robotically printed and processed (Park et al., 2004).

2.4. RNA extraction, hybridization, and data acquisition

Total RNA was extracted from frozen tissues using TRIzol, according to the manufacturer's instructions (Life Technology, Rockville, MD, USA). Universal mouse reference RNA was prepared, as previously described (He et al., 2004), and used as reference RNA. Twenty micrograms of total RNA was used to prepare the DNA targets, as previously described (Nam et al., 2005). The reference RNA was labeled with Cyanine-3 (Cy-3), and test samples were labeled with Cyanine-5 (Cy-5). In brief, cDNA targets were generated by reverse-transcription from total RNA in the presence of Cy-3 or Cy-5 deoxyuridine 5′-triphosphate using SuperScipt II enzyme (Invitrogen, Carlsbad, CA). Residual dye was removed using a Microcon YM-30 column (Millipore, Billerica MA). Cy-5-labeled cDNA targets were hybridized with Cy-3-labeled reference in 40 μl of hybridization solution [50% DIG EasyHyb (ROCHE, Basel, Switzerland) and 30 μg of herring sperm DNA] for 18 h at 42 °C in a humidified conventional hybridization chamber. After hybridization, washing was carried out as follows: (1) 2× SSC, 0.1% SDS at room temperature (RT) for 2 min, (2) 1× SSC at RT for 2 min, (3) 0.2× SSC at RT for 2 min, and (4) 0.05× SSC at room temperature for 2 min. Washed slides were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA), and Cy-3/Cy-5-signals were measured using GenePix Pro 4.1 microarray analysis software (Axon Instruments, Union City, CA).

2.5. Scanning and data analysis

Arrays with hybridized targets were scanned using an Axon scanner and scanned images were analyzed using GenePix® Pro 4.1 software (Axon Instruments). Spots of poor quality, as determined by visual inspection, were excluded from further analysis. Data collected from each array was submitted to the BioArray Software Environment (BASE) database at the microarray core facility of the Department of Pathology, College of Medicine, Catholic University of Korea (http://genomics.catholic.ac.kr/). Data were normalized using the Linear Models for Microarray Data (LIMMA) and the Statistics for Microarray Analysis (SMA) R-package. Spots of size <50 μm were excluded from the analysis, unless otherwise specified. Pearson's correlation coefficients were calculated using the S-PLUS pro-