The Utility of DNA Microarrays for Characterizing Genotoxicity

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Microarrays provide an unprecedented opportunity for comprehensive concurrent analysis of thousands of genes. The global analysis of the response of genes to a toxic insult (toxicogenomics), as opposed to the historical method of examining a few select genes, provides a more complete picture of toxicologically significant events. Here we examine the utility of microarrays for providing mechanistic insights into the response of cells to DNA damage. Our data indicate that the value of the technology is in its potential to provide mechanistic insight into the mode of action of a genotoxic compound. Array-based expression profiling may be useful for differentiating compounds that interact directly with DNA from those compounds that are genotoxic via a secondary mechanism. As such, genomic microarrays may serve as a valuable alternative methodology that helps discriminate between these two classes of compounds. *Key words:* biomarkers, gene expression profile, genetic toxicology, mechanism of action, toxicogenomics. *Environ Health Perspect* 112:420–422 (2004). doi:10.1289/txg.6709 available via *http://dx.doi.org/* [Online 15 January 2004]

Genetic damage elicits stress-related responses that may alter the expression of genes associated with numerous biological pathways (Hollander and Fornace 1995). It has been proposed that patterns of induced gene expression changes may be characteristic of specific classes of toxic compounds and that distinctive fingerprints may be identified that help classify agents with different mechanisms of action (Hamadeh et al. 2002a, 2002b; Waring et al. 2001a, 2001b). Historically, the expression of bacterial SOS response genes was used for detecting genotoxicity of chemicals. The bacterial DNA repair pathways have been well characterized, and various genes have been used as indicators of the SOS response. In these assays (Ben-Israel et al. 1998; Nunoshiba and Nishioka 1991; Oda et al. 1985; Ptitsyn et al. 1997; Quillardet et al. 1982), the transcriptional activities of SOS response–associated genes have been detected using promoter–reporter constructs (biosensors). Thus, each bacterial tester strain carried a construct capable of detecting induction of a single SOS-response pathway. An array of cell lines covering multiple genotoxic stress–associated pathways provided even more insight into bacterial genotoxic mechanisms. A similar approach also has been applied to human cells using an array of HepG2 cell lines carrying genotoxic stress–associated promoter or response element–reporter gene fusion constructs (Todd et al. 1995). The simultaneous treatment of these cell lines provided a genotoxic stress–associated gene expression profile in human cells exposed to various chemicals (Todd et al. 1995) and environmental pollutants (Vincent et al. 1997). In drug discovery, the array of cell lines has been used successfully in the study of the mechanism of genotoxicity of organometallic cytostatics (Aubrecht et al. 1999). However, recent development of oligonucleotide and cDNA microarrays for large-scale parallel gene expression profiling will make the cell-based promoter–reporter arrays obsolete. In contrast to biosensor cell lines that cover a limited number of biological pathways, gene expression microarrays allow measurement and characterization of genomewide gene expression changes in a single experiment. As such, toxicogenomic studies are expected to have a large impact on the field of genetic toxicologys as discussed by Aardema and MacGregor (2002).

In this overview we provide a summary of research activities of the Genotoxicity Working Group of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) Committee on the Application of Genomics to Mechanism-Based Risk Assessment. A description of the overall objectives and design of the committee is included in this mini-monograph (Pennie et al. 2004). The HESI Genotoxicity Working Group is an international collaborative effort that includes scientists from industry, academia, government, and regulatory agencies (Table 1). The major objective of the collaborative project was to evaluate the utility of gene expression profile analysis for risk assessment of genotoxicants. The group analyzed gene expression profiles of compounds with known mechanisms of genotoxicity to determine whether compounds from different mechanistic classes display distinct gene expression profiles. Particular focus was on identifying genes that discriminate between non–DNA–interactive compounds (initial target is not DNA) from DNA-interactive genotoxicants. By examining compounds with known mechanisms and well-characterized mutagenic activities, we attempted to address the issue of whether gene expression changes provide useful information for understanding how a compound exerts its genotoxic effects. Other important components of our evaluation were to ensure that one could distinguish a genotoxicity profile from a cytotoxicity profile and to evaluate the value of the technology for examining low-dose effects of genotoxic agents. The Genotoxicity Working Group also examined the correlation of measured gene expression changes via microarrays with traditional genetic toxicology end points. Similarly, a secondary question was whether microarray technologies could be a useful adjunct to the standard genotoxicity test battery.

Experimental Overview

To determine if gene expression patterns can be correlated with mechanisms of action of genotoxicants, we have generated gene expression profiles for several model genotoxic agents. Studies were conducted in the absence of S9 metabolic activation using two different cell lines employed in standard genotoxicity testing [L5178Y mouse lymphoma and human thymidine kinase (TK) 6 cells]. The mouse lymphoma cells used were *p53* deficient, whereas the human TK6 cells were *p53* proficient. The test agents, whose primary mechanisms are denoted, included a methylating agent [methyl ester methanesulfonic acid (MMS)]; DNA cross-linking agents (mitomycin C and cisplatin); DNA adduct inducers [benzo[*a*]pyrene diolepoxide (BPDE)] and 4-nitroquinoline oxide); free-radical generators [bleomycin and hydrogen peroxide (H_2O_2)]; a microtubule inhibitor (taxol); a topoisomerase inhibitor (etoposide); and a compound that perturbs

nucleotide pool balances (hydroxyurea). Most of the test chemicals listed above were evaluated in at least two separate laboratory sites. At least two independent replicate studies were conducted in each laboratory. The experimental parameters were designed to mimic a standard genetic toxicology assay. Two time points were examined; cells were treated for 4 hr and immediately harvested for RNA isolation, or cells were treated for 4 hr and allowed to recover for 20 hr (24-hr time point) before being harvested. At least two concentration levels of each test article were tested. A high concentration that induced 70–80% cytotoxicity (for a TK gene mutation assay) or approximately 50% cytotoxicity (for a micronucleus assay) with a robust induction of genotoxicity and a low concentration that produced limited cytotoxicity (10–30%) with a 1.5- to 4-fold increase in genotoxicity (gene mutations, chromosomal damage, or DNA adducts) were used. The relative reduction of cell growth compared with the negative control was used as a measure of cytotoxicity. The gene expression changes were detected using GeneChip oligonucleotide arrays (Affymetrix, Inc., Santa Clara, CA), PHASE-1 cDNA microarrays (PHASE-1 Molecular Toxicology, Inc., Santa Fe, NM), or Atlas cDNA microarrays (Clontech, Palo Alto, CA). Potential sources of variability such as the lot of test chemical, tissue culture reagents, or DNA microarrays were not rigorously controlled in order to assess the potential impact of these factors in a real-life situation where standardization of all reagents would not be possible.

Discussion and Conclusions

Although data analysis efforts continue, results to date have enabled us to make some initial conclusions. For most of the compounds evaluated in this study, few reproducible gene changes occurred at low

Table 1. List of participating organizations in the HESI Genotoxicity Working Group.

AstraZeneca Pharmaceuticals Inc.
Aventis Pharmaceuticals Inc.
Biogen, Inc.
The Dow Chemical Co.
E.I. duPont de Nemours & Co.
Eli Lilly and Co.
GlaxoSmithKline
Johnson & Johnson Pharmaceutical Research and
    Development, LLC
Monsanto Co.
National Institute of Health Sciences, Japan
Novartis Pharmaceuticals AG
Pfizer Inc, Global Research and Development
The Procter & Gamble Co.
Sankyo Company Ltd.
Sanofi-Synthélabo, Inc.
U.S. National Cancer Institute
U.S. National Center for Toxicological Research

concentrations and the early time point (4 hr), whereas a greater number of changes were observed at the higher dose levels and/or the 24-hr time point. Changes > 3-fold were not common even at highly genotoxic concentrations. These observations were made in all the participating laboratories. Nevertheless, gene expression changes of a few biologically relevant genes, including members of the *GADD45* family, showed robust upregulation (3- to 14-fold) at cytotoxic doses. Some of the participating laboratories identified significant gene changes on the basis of fold-change criteria (1.5-fold or more), whereas other laboratories used statistical methods for establishing whether the expression of a particular gene was changed significantly. On the basis of these results, it appears that early concerns that the technology might be overly sensitive (numerous, large-fold changes in gene expression changes at low or nongenotoxic concentrations) are unfounded.

The extent of gene expression changes resulting from treatment was not as sensitive an end point as those provided by more traditional genetic toxicology assays. For example, Aubrecht and colleagues (unpublished data) found that higher concentrations of cisplatin were required to see robust gene expression changes on a microarray platform compared with levels that induced micronuclei *in vitro*. Conversely, the gene expression changes of biologically relevant genes such as members of the *GADD45* family showed excellent correlation with the extent of DNA–platinum adducts and protein–DNA crosslinks. Morris and colleagues (unpublished data) found that induction of DNA adducts occurred at lower concentrations for BPDE than at those concentrations that elicited robust gene expression changes. These examples show that the induction of gene expression changes as measured by microarrays is not as sensitive an indicator of genotoxicity when compared with more traditional genotoxicity end points.

Observed changes in gene expression from the Genotoxicity Working Group studies were generally consistent with data in the literature for those chemicals studied previously. For example, with MMS many of the categories of the gene expression changes observed by the working group were consistent with those reported for yeast in the literature (Jelinsky et al. 2000; Jelinsky and Samson 1999). These studies suggested that in addition to inducing DNA-damage recovery genes, mammalian cells also induce genes to promote recovery from protein damage such as endoplasmic reticulum stress and/or unfolded protein response. Some of the genes identified in

our studies are concordant with genes from the same biological pathways affected in yeast. However, yeast studies on global transcriptional responses to environmental stresses including MMS revealed that 10–14% of all genes are induced or repressed in response to a wide range of stresses. We did not see such an encompassing gene expression response in the mammalian cell lines used for our studies. It is unclear whether this less-robust response is a by-product of high noise-to-signal ratios associated with the microarrays or to the biology of an *in vitro* gene expression stress response in mammalian cells.

Our data indicate that gene expression changes may enable us to distinguish classes of genotoxic compounds that operate via different modes of action. Prior to the HESI project, this concept had been explored by Fornace and co-workers using a panel of human cancer cell lines to categorize different stress agents by gene expression profiling using directed arrays (Amundson et al. 1999; Fornace et al. 1999). In an alternative approach, Aubrecht and colleagues (1999) used a panel of biosensor cell lines to investigate mechanism of genotoxicity of organometallic cytostatics. This latter concept was developed and demonstrated in the early 1990s by Spencer Farr and others using both bacterial and mammalian cell constructs to monitor stress gene responses (MacGregor et al. 1995). On the basis of these early studies, the HESI Genotoxicity Working Group hypothesized that gene expression changes could be identified that discriminate broad classes of genotoxicants, including DNA-reactive genotoxicants from non-DNA-reactive (or indirect) genotoxicants. Aardema and colleagues (unpublished data) compared gene expression patterns using a supervised statistical approach with DNA-interactive genotoxic agents (MMS, mitomycin C, and cisplatin) versus three compounds that act via a non-DNA–interactive mechanism (hydroxyurea, taxol, and etoposide). This comparison resulted in a fingerprint that discriminated the two classes of chemicals on the basis of a training set of genes for the two time points. When this training set of genes was applied to data from chemicals tested in other laboratories, this fingerprint led to correct identification of chemicals as DNA interactive or non–DNA interactive in approximately 80% of the cases. Aardema and colleagues are analyzing additional data to help validate the utility of this approach. In addition, Gollapudi and colleagues reported certain gene expression changes that could serve as biomarkers of exposure to free radical–generating agents on the

basis of their studies with bleomycin and H_2O_2 (Seidel et al. 2003). Some preliminary results indicate that even within a category, namely, indirect-acting genotoxicants (hydroxyurea and taxol), differences exist in the gene expression patterns generated by these two compounds. These differences are not surprising, as the primary cellular targets are quite distinct.

Aubrecht and colleagues (unpublished data) found that the gene expression profile of sodium chloride at micronucleus-producing concentrations was strikingly different from the profile of cisplatin. The most notable difference was the lack of upregulation of DNA damage–sensitive genes such as *GADD45* and *cFOS* in sodium chloride–treated cells. In an additional study, Gollapudi and co-workers (unpublished data) observed that the gene expression changes observed in sodium chloride–treated cells were qualitatively different from those observed in MMS-treated cells, with only one gene common to both the treatments. Testing of additional cytotoxic and genotoxic compounds is needed to confirm this ability to differentiate the two types of responses.

After their initial experience, the working group concluded that microarray technologies are not currently amenable for use as a high-throughput screening tool for genotoxicity. The data analysis is quite complicated; there are numerous sources of variability; the sensitivity of the technique appears to be less than standard genotoxicity measures; and the arrays are still fairly expensive. High-throughput approaches could be facilitated in the future with the development of more targeted arrays. These focused arrays might contain only a few highly biologically relevant genotoxic response genes as identified from previous experience with large global arrays. This approach could be used to examine a particular biological mechanism in a high-throughput screening paradigm.

For the Genotoxicity Working Group, as well as for other investigators in the field of toxicogenomics, a significant challenge has been the variation in approaches to data presentation and analysis across laboratories. Other sources of interlaboratory differences included variables such as the day RNA samples were processed, the photomultiplier tube adjustment, use of different lots of chips, use of different platforms (Affymetrix or PHASE-1 arrays), and different culture conditions for these *in vitro* studies. All these factors affect the outcomes of studies performed in different

laboratories. Further analysis of the interlaboratory variability issue is under way to determine how much each variable impacts the results of the studies.

The pending public release of the HESI Genomics Committee's experimental data as part of the European Bioinformatics Institute's ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) will allow the scientific community to evaluate the raw data of the consortium in alternative ways. Currently, an effort is under way within the Genotoxicity Working Group to evaluate various data analysis approaches including Affymetrix Microarray Suite 5.0, Rosetta Resolver, or other statistical approaches. To facilitate data analysis issues, more straightforward, easy-to-use bioinformatic tools need to be developed that provide a common format for data comparison between studies and across different laboratories. For investigators to fully utilize information from these studies, it is essential that user-friendly data-mining tools be developed that enable critical genes in relevant biological pathways (e.g., those involved in a genotoxic response) to be identified.

In summary, our data indicate that the value of the gene expression profiling technology is its potential to provide mechanistic insight into the mode of action of a genotoxic compound. The use of the technology to differentiate compounds that interact directly with DNA from those compounds that are genotoxic via a secondary mechanism is considered one of the valuable aspects of this tool and provides an alternative methodology to help discriminate agents that act by defined modes of action.

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