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THE IMPACT OF GENOMICS-BASED TECHNOLOGIES ON DRUG SAFETY EVALUATION

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■ **Abstract** Determining the potential toxicity of compounds early in the drug discovery process can be extremely beneficial in terms of both time and money conservation. Because of the speed of modern chemical synthesis and screening, to accurately evaluate the large number of compounds being produced, toxicology assays must have both high-fidelity and high-throughput capabilities. In addition, assays must be performed using limited amounts of compound. In the past decade, several new and innovative techniques have been developed that not only allow for high-throughput screening but can also provide detailed information concerning the molecular mechanisms behind toxic effects. Techniques such as hybridization microarrays, real-time polymerase chain reaction, and large-scale sequencing are some of the methods that have been or are starting to be used routinely in pharmaceutical companies. This review examines the contributions of these and related techniques toward toxicity evaluation of potential drug candidates and their future role in the discovery of new therapeutics.

INTRODUCTION

In the pharmaceutical industry, drug safety evaluation laboratories are currently charged with two distinct functions: aiding in discovery of lead selection and conducting human risk assessment. As part of the drug discovery process, assessment of toxic potential is used to select compounds that are more likely to succeed during preclinical development (lead selection and enhancement). Determining the potential liabilities of compounds early in the drug discovery process can save development time and money by focusing resources on compounds that are more likely to succeed. The success of toxicity screening in drug discovery depends both on the speed or turnaround time of evaluation and on the reliability of results. In order to keep up with high-throughput screening hits or chemical syntheses (chemists usually outnumber biologists several times over), toxicology assays must have sufficient throughput to be able to handle relatively high volumes of compounds and, hence, must be of relatively low fidelity, usually measuring a

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single parameter focused on a single toxicity issue. The reliability of an assay depends on the relatedness of the parameter measured to the actual mechanism of toxicity for the lead or template compound; nonspecific cell viability or lysis assays are of limited value because all chemicals will kill cells at some concentration. As with any screen for high throughput, toxicity assays must also be economical to conduct. Screening results from mechanism-based assays are generally used to prioritize compounds for subsequent examination rather than eliminate compounds from further consideration.

During drug development, toxicology is charged with determining or predicting potential adverse effects in humans (risk or safety assessment). Risk assessment is used both to establish a dose that is safe to administer to humans during clinical development and to evaluate risk due to prolonged exposure. Drug toxicity is conventionally determined by conducting animal studies and examining for changes in serum chemistry parameters and histopathology; human risk is determined by extrapolation from animal study results. Toxicity evaluation from animal studies is thorough, though fidelity decreases when comparing animal species or extrapolating results to human risk assessment. For a detailed discussion of risk assessment, see the recent review by Brecher (1). Here, too, the reliability of risk assessment depends on the relationship between the parameters measured and the actual mechanism of toxicity of the drug candidate, particularly when extrapolating to humans from animal species used in the laboratory.

Determining the mechanism of toxicity for a xenobiotic requires a diversity of investigative techniques, sufficient time, and a degree of serendipity [for a review, see Ulrich & Slatter (2)]. Although some problems can be readily solved, others may require the dedication of more resources than a company is prepared to risk. Investigative techniques can yield quite useful results, however; structure-toxicity relationships can be used to guide chemical synthesis toward less toxic analogues or problem structures can be eliminated from development. Mechanistic data can also aid in the interpretation of animal toxicology findings and help clarify their significance in human risk assessment. However, most mechanistic toxicology is conducted retrospectively in an attempt to salvage a discovery program or clinical candidate. A more useful approach would be to generate mechanistic data early so as to predict animal and human toxicities prior to conducting expensive developmental studies. This is the challenge currently faced by toxicologists and it is formidable. In order to meet the demands of drug discovery teams, toxicity assessment must be rapid, accurate, and modest in terms of drug requirements (usually $<$100 mg, though for particularly interesting compounds a chemist can generally synthesize gram quantities). For high-throughput toxicology screening, assays must also be simple in design and even more compound sparing ($<$1 mg). The application of genomics-associated tools to toxicity or safety assessment holds the promise of meeting these demands.

The past decade has seen an explosion in the number and variety of techniques available for molecular analysis of toxicological effects, and the emphasis has begun to sway toward molecular toxicology as an early assessment of chemical

effects. These techniques focus on determining changes in gene expression at the level of transcription. This article reviews several of these techniques and their current application in toxicity assessment, including differential display, subtractive hybridization, serial analysis of gene expression (SAGE), hybridization microarrays, real-time polymerase chain reaction (PCR), scintillation proximity, and branched-DNA (bDNA) signal amplification. These techniques can be roughly divided into two categories: high-fidelity, low-throughput techniques and low-fidelity, high-throughput techniques (see Figure 1). Large-scale hybridization techniques such as microarrays and sequencing techniques such as SAGE produce a detailed picture of gene responses that can bridge the entire expressed genome.

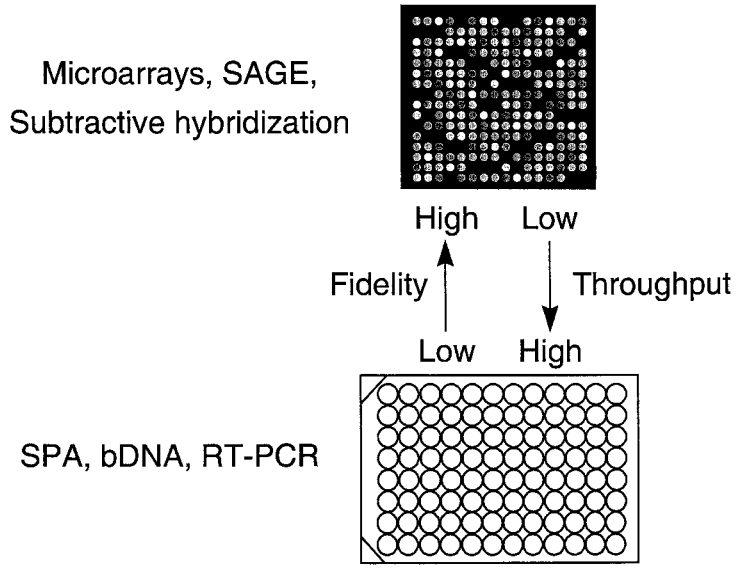

Figure 1 Genomics-based technologies that have an application in toxicity assessment. Microarrays, serial analysis of gene expression (SAGE), or subtractive hybridization can be used to comprehensively characterize or ''fingerprint'' responses. These techniques are of high fidelity in that they generate a considerably detailed image of a response to a particular compound. Because of the amount of information generated, the amount of mRNA required, the number of steps involved, and the costs, these techniques are not high throughput but can be used to identify specific markers to be used in other assay formats. Scintillation proximity (SPA), branched-DNA (bDNA), and real-time polymerase chain reaction (RT-PCR) assays are used to follow responses in one or a few genes and can be constructed as high-throughput screens using cultured cells. A toxicology screening assay using one of these techniques is low in fidelity, however, because only a single parameter is evaluated compared with the large number of possible responses. Hence, a degree of caution must be used when extrapolating from results because the accuracy and reliability of these assays decreases even further when they are applied outside of a chemical analogue series or to a different species.

Microarrays in particular have generated a lot of excitement in toxicology because it is now possible to generate a comprehensive image of cell and tissue responses to a compound without the time and labor investment or subjectivity of traditional analyses such as histopathology. This new capability has been the subject of several recent reviews and perspectives (3–7). However, these techniques are expensive and require a relatively large amount of mRNA (much more than can be generated from a 96-well plate) and, hence, are most appropriate for individual compound studies rather than for screening of chemical libraries. Real-time PCR, scintillation proximity, and branched-DNA technologies are designed to follow single endpoints (changes in expression levels for an individual gene), or small sets of endpoints, and require small amounts of mRNA. These assays can be automated for high-throughput screening of compound sets.

DIFFERENTIAL DISPLAY

Differential display was developed by Liang & Pardee (8) and has been modified several times since its inception. RNA is isolated from two different cell populations and then subjected to reverse transcription using four different sets of oligo(dT) primers. The oligo(dT) primers have the sequence T_NAB, where *N* represents the number of thymidines in the primer, usually 10 or 12; *A* can be guanine, adenine, or cytosine and *B* can be guanine, adenine, cytosine, or thymidine. The resulting cDNA is then amplified using the same oligo(dT) primer and a random primer at the 5′ end. The amplified PCR products represent different subpopulations, which are defined by the oligo(dT) primer, and they should represent most of the mRNA species in a cell. The PCR products from two or more different cell populations are then run on a denaturing polyacrylamide gel, and differentially expressed RNA samples can be identified, isolated, and amplified.

For analyzing gene responses during toxic reactions, differential display has several advantages over other high-throughput techniques. One strength is that mRNA samples from several cells can be analyzed at the same time; this is not possible with other techniques, such as subtractive hybridization. In addition, differential display has the ability to identify previously unknown genes that may be regulated during a toxic response, which is not possible using hybridization microarray assays.

Differential display has been used by several researchers to identify genes whose expression is regulated by certain toxins. One toxin that has been studied fairly extensively using differential display is TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). For example, Wang et al (9) treated Hep G2 cells with TCDD for 24 h and then analyzed gene expression changes using differential display. Using this method, four clones were isolated that were shown to be regulated by TCDD treatment. Two of the clones were fibrinogen and plastin, both of which were

down-regulated with TCDD treatment. The other two clones were induced with TCDD treatment and their sequences did not match with any known genes in the database (9). Fibrinogen is important for the formation of fibrin clots, which suggests that TCDD may play a role in hemostasis, whereas plastin may indicate a role for TCDD in tumorigenesis. Significantly, neither fibrinogen or plastin was previously known to be regulated by TCDD, demonstrating how techniques such as differential display can reveal dimensions that could not be predicted by other standard methods. In 1996, Selmin et al (10) used differential display with total liver mRNA from rats that had been treated with acute or chronic levels of TCDD. Approximately 30 potential responsive genes were isolated. Of these 30 genes, 13 were shown to be not regulated by TCDD, indicating that the method can have a high rate of false positives. However, Selmin et al (10) did isolate a novel gene that was consistently up-regulated in response to both chronic and acute TCDD treatment and that was shown to be related by amino acid sequence to the interleukin (IL)-6 receptor. Other researchers have also used differential display to examine TCDD-induced gene expression changes in rat prostate (11) and in mouse lung (12). In both of these cases, genes were identified that were not previously known to be regulated by TCDD.

Differential display has been used to identify genes regulated by other known toxins as well. The results from Muhlenkamp & Gill (13) show how methods such as differential display can identify different molecular mechanisms of compounds that seem to have the same phenotypic effect. Both clofibrate and diethylhexylphthalate (DEHP) are known peroxisomal proliferators (14). Differential display showed that the gene GRP58, a carnitine palmitoyl transferase, is down-regulated by DEHP. Subsequent work showed that clofibrate also can down-regulate GRP58, but to a much smaller extent than DEHP, indicating that although these two compounds both cause peroxisomal proliferation, they may do so by different mechanisms (13). In a different study, Ye et al (15) used differential display to identify another gene regulated by DEHP, cytochrome 450 *Cyp2f2*, a naphthalene hydroxylase (15). Other investigators have used differential display to study the genetic changes induced by chloroform (16), phenobarbitol (6), and liver regeneration (17). In all these cases, genes were identified that were not previously known to be regulated by these agents.

SUBTRACTIVE HYBRIDIZATION

Subtractive hybridization is another high-throughput technique that allows one to isolate and clone mRNA species unique to a cell population. Its main advantage over differential display is that generally it yields a lower number of false positives. However, it can only compare two populations at a time. There are different methods of subtractive hybridization; one of the most common was pioneered by Sive & St. John (18) in 1988 and modified by Wang & Brown (19) in 1991. For this method, mRNA from two different cell populations is harvested and reverse transcribed to make cDNA using oligo(dT) primers. Following this, the cDNA is

digested with a restriction enzyme to make short fragments, and the two sets of cDNA samples have different adapters ligated to their ends. The samples are then amplified by PCR. One set of cDNA is designated ''driver'' and the other set is designated ''tracer.'' The tracer population is radioactively labeled, and the driver population is labeled with biotin. The two populations are then denatured and hybridized to each other at a ratio of 20:1 driver to tracer. This ensures that any cDNA present in the tracer population will anneal to its complement in the driver population, if it is present. Following this, biotin-labeled cDNA species are removed by streptavidin. Any cDNA in the tracer population that is unique will not hybridize to the driver population and will not be removed by streptavidin. The hybridization steps need to be repeated several times to fully enrich for unique mRNA species (18, 19).

A study was conducted whereby primary hepatocyte cultures from male Fischer rats were treated with aflatoxin B_1, a known hepatotoxin in both rats and humans (20, 21). In order to identify genes that are regulated by aflatoxin, three different methods were used: differential display and two variations on subtractive hybridization, one called representational difference analysis (22) and the other suppression subtractive hybridization (23). It is interesting to note that the three methods all identified different genes, and no gene was identified in more than one assay. In this study, differential display had the highest rate of false positives identified, 93%, followed by representational difference analysis at 30% and lastly by suppression subtractive hybridization, which did not identify any false positives. The three methods identified several genes, such as cytochrome P450 4F1 and 3A1, transferrin, and serum amyloid A, that may shed light on molecular mechanisms underlying aflatoxin B_1 toxicity (24).

Subtractive hybridization has also been successfully used to identify genes that are involved in the molecular mechanism of toxicity caused by the neurotoxin trimethyltin (TMT). Toggas et al (25) used subtractive hybridization to isolate genes that cause some neural cells to be sensitive to TMT. They identified a gene they called *stannin*, which was expressed only in neurons sensitive to TMT (25). Subsequent work with antisense oligonucleotides directed against *stannin* strongly suggests that the expression of *stannin* is necessary for TMT toxicity (26). Chen & Safe also used subtractive hybridization to look for genes regulated by TCDD. They identified several genes, among them estradiol-induced genes that were down-regulated by TCDD (27).

HYBRIDIZATION MICROARRAYS

By far, the greatest excitement in genomics-based techniques in the past few years has been the development of microarrays. Hybridization on microarrays is a relatively new technique and has seen widespread application only in the past 2–3 years. Because of this, there are relatively few papers in peer-reviewed journals utilizing hybridization microarray techniques in toxicology. Thus, much of the

work reviewed in this section has only recently been submitted for publication or is available only in abstract form. Nonetheless, the potential benefits of hybridization microarrays for the field of toxicology are enormous, and in a very short time it has become a standard method for studying the molecular mechanisms of toxicology.

Hybridization microarrays are basically an extension of techniques that have been available to molecular biologists for decades, specifically northern blotting and dot blotting assays. There are different types of hybridization microarrays, but they fall essentially into two catagories: cDNA spotted onto a solid surface, such as glass slides or nylon membranes, and oligonucleotides synthesized onto a solid surface (an example is the Affymetrix chip).

Membrane-based microarray assays are the forerunner of current hybridization microarrays. An advantage of membrane-based over other methods of hybridization microarrays is that they are reasonably affordable. Membrane filters containing numerous genes that encode for proteins involved in various aspects of cell regulation are available from several companies, including Clontech (http://www.clontech.com/), Research Genetics (http://www.resgen.com/), and Genome Systems (http://www.genomesystems.com/). The disadvantage of doing membrane-based microarray analysis is that to compare two mRNA species, one has to use duplicate filters. In addition, the cDNA is labeled with radioactivity as opposed to fluorescence, which is not as sensitive (28).

The mechanism of how the filters are made and used in microarray analysis is summarized by Cheung et al (29). A high-precision robot is used to spot hundreds to several thousands of cDNA sequences into set quadrants on two identical nylon membranes. mRNA from two different cell populations is then reverse transcribed and radioactively labeled. The two sets of radioactively labeled cDNA are then hybridized to the two filters overnight and washed, in much the same way as a southern blot is done. The blots are developed against X-ray film or a phosphoimager screen, and the two filters are compared for intensity of hybridization.

Membrane-based hybridization microarrays have been used to study the molecular mechanisms of toxicity caused by carbon tetrachloride. The human hepatoma cell line HepG2 was treated with carbon tetrachloride (CCl_4) or with dimethyl formamide (DMF), a chemical that does not cause liver damage (PR Holden, personal communication). RNA from the two populations was harvested, reverse transcribed, labeled, and hybridized to a microarray (Atlas Human cDNA expression array). The results showed that 47 genes appeared to be either up- or down-regulated by CCl_4 compared with DMF. These genes included genes involved in apoptosis, cell cycle regulation, and gene expression. Two genes were selected for further investigation. These were IL-8, which appeared to be up-regulated 7.5-fold by CCl_4, and prohibitin, an antiproliferative gene that appeared to be down-regulated 5.3-fold by CCl_4. Further analysis of the gene expression by northern blot analysis could not confirm the prohibitin down-regulation; however, both northern blotting assays and ELISA assays showed that CCl_4 does up-

regulate IL-8 at both the mRNA and protein levels (PR Holden, personal communication). The finding that CCl_4 regulates IL-8 expression is a novel discovery and has many implications for molecular mechanisms of CCl_4 toxicity.

Although microarray analysis using filter membranes is a useful tool, the next generation of microarray technology has the potential to provide even faster and more efficient methods of studying molecular toxicology. Microarray analysis on glass slides is similar to analysis using membrane filters, but it has several key improvements. First, the cDNA is spotted onto a glass slide coated with polylysine or amino silanes instead of a nitrocellulose or nylon membrane (30). This surface is less porous than nitrocellulose or nylon membranes, which enhances hybridization, washing, and visualization (31). Another advantage of hybridization microarray on a glass slide over microarray analysis on membrane filters is that the cDNA from two cell populations is labeled with fluorescent probes Cye3 or Cye5-dUTP instead of with radioactivity. This allows one to hybridize the cDNA from both populations to the same slide, which makes the comparison of the two cell populations more accurate. The glass slide is then scanned by a fluorescent scanner, which quantitates the ratio of the Cye3 to Cye5 signal (30). Companies that provide custom-made or commercially available glass slides are listed elsewhere (28).

In our laboratory, we have used hybridization microarray on glass slides to study the effects of various drugs or chemicals on mRNA regulation in cultured human hepatocytes; two examples are illustrated here. The first example is with the drug efivirenz, a reverse transcriptase inhibitor with minimal hepatic toxicity. The drug in the second example (designated CpdR) produced hepatotoxicity in rats; the mechanism is unknown. Studies in rats showed CpdR to produce significant increases in liver weight and severalfold induction in serum levels of alkaline phosphatase, alanine amino transferase, and aspartate amino transferase following 3 days of administration. Human hepatocytes from organ donor tissue were isolated by using a two-step liver perfusion method (32). Cultured cells were either vehicle treated (DMSO) or treated with noncytolytic concentrations of efivirenz or CpdR for 24 h, followed by RNA isolation. cDNA was prepared, labeled, and hybridized to the Human UniGem V chip from Incyte Pharmaceuticals. This chip contains approximately 7000 genes from the human database. Treating human hepatocytes with efivirenz caused a low number of genes to have a greater than twofold change in expression. The results are shown in Figure 2. Two of the genes that were up-regulated were cyp3A7 and aminolevulinate delta-synthase 1. Cytochrome P450 3A7 is a major fetal cytochrome P450 that is normally not expressed in adults. However, the gene sequences of cyp3A7 and cyp3A4 (the major cytochrome P450 in adults) are very similar so it is possible that cross hybridization occurred and efivirenz actually up-regulated cyp3A4. Aminolevulinate is an intermediary in heme synthesis, and up-regulation of aminolevulinate delta-synthase 1 could be associated with the increased cytochrome P450 expression.

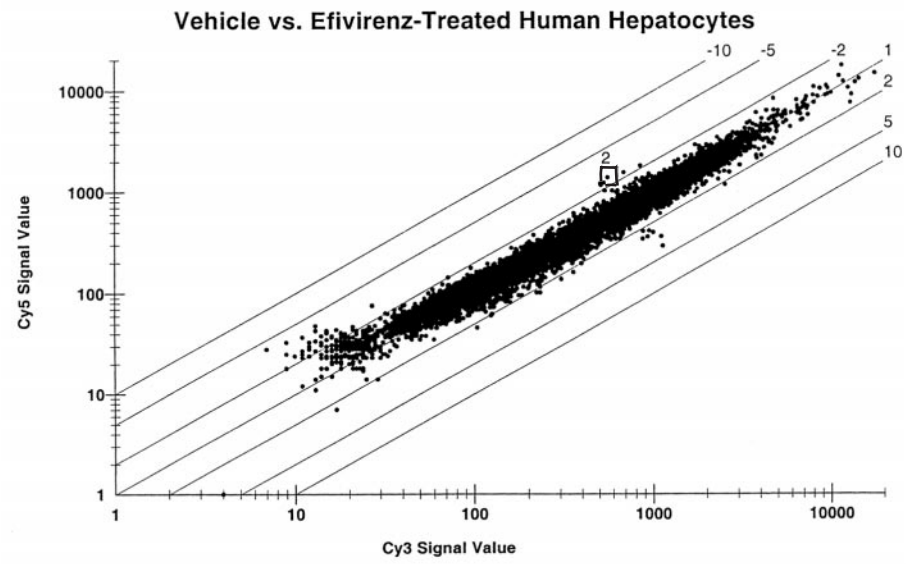

Figure 2 Changes in gene expression levels in cultured human hepatocytes treated with reverse transcriptase inhibitor, efivirenz. Hepatocytes were treated with the vehicle DMSO or with efivirenz. The mRNA was harvested, reverse transcribed, and labeled with Cy3 (vehicle-treated cells) or with Cy5 (efivirenz-treated cells). The labeled cDNA was hybridized to the UniGemV microarray chip (Incyte Pharmaceuticals). The results are displayed on the graph as a ratio of Cy5 versus Cy3 signal. (*Square*) The location of the genes cyp3A7 and aminolevulinate delta synthase 1.

In contrast to the results with efivirenz, several genes were shown to be changed in expression in cultured human hepatocytes when treated with the hepatotoxic CpdR. The results are shown in Figure 3. The gene that showed the greatest change in expression level was phospholipase A2, which showed an increase in mRNA levels of over eightfold. Phospholipase A2 (PLA2) is an enzyme that hydrolyzes the acyl bond of phospholipids, resulting in the release of arachidonic acid and lysophospholipid. PLA2 is also present in many different types of snake venom, including that of elapids, vipers, crotalids, and colubrids (33). PLA2 has also been shown to be up-regulated with CCl_4 treatment of rat hepatocytes and has been directly linked to the mechanism of toxicity of CCl_4 (34–36). Up-regulation of PLA2 in human hepatocytes by treatment with CpdR suggests a role for increased arachidonic acid in the mechanism of hepatic toxicity for this compound. Additonal useful observations were made from these and other studies. First, DMSO produced no changes in gene expression observable after 24 h of exposure. This is important because this is the most commonly used solvent in drug discovery research. Second, attempts to hybridize liver cDNA libraries from rats treated with CpdR to human cDNA chips were not successful;

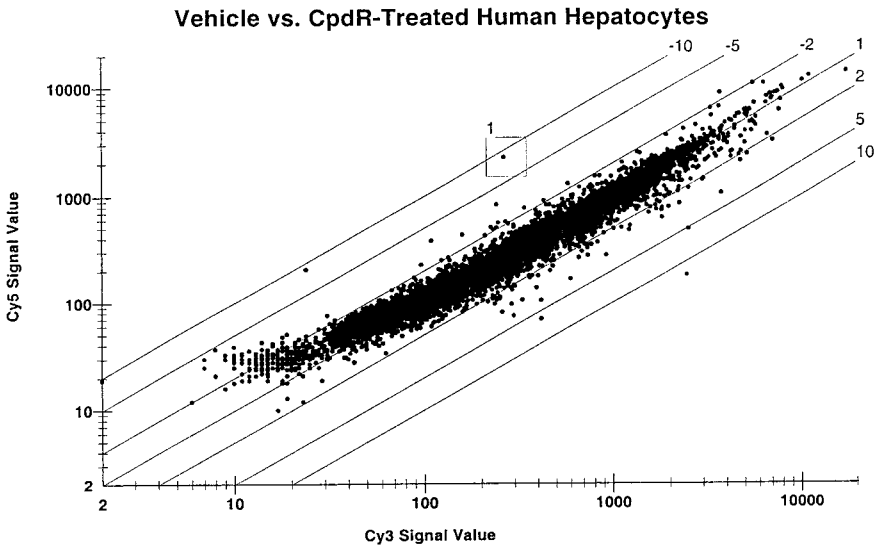

Figure 3 Changes in gene expression levels in cultured human hepatocytes treated with DMSO (Cy3) or CpdR (Cy5). (*Square*) The location of the phospholipase A2 gene.

no significant changes in gene expression were observed. This stresses the need to conduct analyses using same-species arrays.

Studies were done on the genetic expression changes that occur in HT29 colon tumor cells when treated with the DNA methylation inhibitor 5-aza-2′deoxy-cytidine (5-Aza-CdR) (DA Jones, personal communication). The microarray slide contained 4608 randomly selected cDNAs from the Unigene set. HT29 cells were exposed to 5-Aza-CdR for 9 days, whereupon RNA from the cells was harvested and hybridized to the microarray slide. It is interesting to note that all 19 genes induced by 5-Aza-CdR were also inducible with interferon. This result led to the further observation that STAT (signal transducers and activators of transcription) factors 1, 2, and 3 were transcriptionally activated by 5-Aza-CdR. These results are potentially significant because STAT 1 expression is often depressed in certain metastatic melonama cell lines, and these tumor cell lines often respond poorly to interferon treatment (DA Jones, personal communication). These results again demonstrate how techniques such as hybridization microarray can direct research down pathways that had not been previously anticipated.

Schena et al (37) used hybridization microarray analysis to identify genes that are regulated during heat shock or by phorbol ester in human T-cells. Jurkat cells were incubated at 37°C for control and then were either incubated at 43°C for 4 h or were treated with phorbol ester. The mRNA from the cell populations was harvested, reverse transcribed, labeled, and hybridized to a microarray glass slide containing a total of 1056 cDNAs. The microarray results showed 17 genes that

were regulated during heat shock, all of which were confirmed by dot blotting assays. Of the 17 genes identified, many encoded for proteins involved in protein degradation or factors that function as molecular chaperones, which is consistent with mechanisms of heat shock induction. Six genes were identified as being regulated by phorbol ester, one of which, NF-κB1, is a known target gene of phorbol ester regulation. It is interesting to note that several unknown genes were identified that may be important for understanding the molecular mechanisms of phorbol ester and heat shock cellular regulation (37).

High-density synthetic oligonucleotide slides are another powerful tool that has greatly transformed molecular analysis of toxicology and will continue to do so. With this method, oligonucleotides are synthesized directly onto a glass substrate. The major company that markets synthetic oligonucleotide microarray slides is Affymetrix. However, other companies will no doubt start to market other microarray chips. (For updates on microarray technology, consult the following website: http://www.mpiz-koeln.mpg.de/%7Eweisshaa/Adis/DNA-array-links.html.)

Affymetrix chips are constructed using a photolithographic method. A glass substrate is coated with covalent linker molecules that terminate with a photolabile protecting group. Light is directed through a mask that exposes selected portions of the probe array to ultraviolet light. This removes the photolabile protecting group, which allows nucleotides to couple to the unprotected sites. This process is repeated using different filters until a complete set of oligonucleotides is synthesized on the glass slide. The Affymetrix chips contain hundreds of thousands of genes on an area 1.28 $\times$ 1.28 cm on each array. The oligonucleotides are generally 20 bp long, and every gene is represented by multiple oligonucleotides of different sequences that will hybridize to the same mRNA. In addition, mutated oligonucleotides, containing a single base pair change, are present for every perfect oligonucleotide sequence. By using multiple oligonucleotides for the same gene, and by having the mutated sequences present, the number of false positive signals is vastly reduced (38). Another difference between Affymetrix chips and microarray chips with cDNA spotted onto glass slides is that mRNA from individual cell populations is hybridized to an individual chip. This is done because the Affymetrix chips give a quantitative level of expression of mRNA from each cell; the data are not displayed as a ratio of the expression of one mRNA population over the other. This has certain advantages because once a cell population has been hybridized to an Affymetrix chip, the gene expression results can be compared to those from other cell populations without the need for multiple hybridizations. (More information on the Affymetrix chip is available at the following website: http://www.affymetrix.com/.)

The genetic changes induced in *Saccharomyces cerevisiae* when treated with the alkylating agent methyl methanesulfonate (MMS) were studied. MMS has been shown to cause DNA damage and to activate DNA repair genes (39). Yeast cells were treated with a low dose of MMS, which induces DNA-repair genes while causing minimal cell death. The mRNA was harvested from the cells and

hybridized to Affymetrix chips containing 6218 open reading frame (ORF) yeast sequences. The results showed that of the 6218 genes present on the chip, 325 showed a more than fourfold induction in transcript level compared with the control group, and 75 of the ORF sequences were down-regulated more than threefold. To confirm these results, 50 of the ORF sequences that were either up- or down-regulated were chosen for further confirmation by northern blot analysis. The northern blotting results showed that 48 out of the 50 ORF sequences chosen were indeed changed in expression by MMS, and the amount of induction or repression as shown by Affymetrix or northern blotting analysis was very similar. Of the genes that were shown to be regulated by MMS, many fell into the category of genes that would be expected to be activated in case of DNA damage, such as DNA repair, cell wall biogenesis, membrane transport, and signal transduction genes. In addition, 91 of the 143 known protein degradation genes were induced, which is interesting considering the dosage level of MMS was relatively nontoxic. Overall, Jelinsky & Samson (40) showed 15-fold more genes than had previously been thought to be induced by a DNA-damaging agent. In addition, the study suggested several new mechanisms that cells may utilize to protect against chemicals that induce DNA damage (40).

Oligonucleotide microarrays have been and are being used in many different areas of biological research as well, such as polymorphism analysis and genotyping (41), disease management (42), and cell signaling (43). Because oligonucleotide microarray analysis is a relatively new technique, there is not a great deal of literature on its use in toxicology. This will undoubtedly change as more pharmaceutical companies begin exploiting its vast potential.

SERIAL ANALYSIS OF GENE EXPRESSION

SAGE analysis was first described in 1995 by Velculescu et al (44). Similar to differential display or subtractive hybridization, this method allows one to compare the expression profiles of genes between different cell populations. Unlike the aforementioned techniques, however, SAGE actually quantitates the level of RNA in each individual cell population. In this regard, SAGE is similar to oligonucleotide microarrays. Unlike microarrays, however, SAGE allows for the identification of unknown gene sequences. SAGE is based on the principle that a short gene sequence has enough information to identify a transcript. RNA is isolated from a cell population and reverse transcribed using a poly(dT) primer. Following this, the cDNA is cleaved with a frequent restriction enzyme cutter, termed the anchoring enzyme; the 3′ end of the cDNA is captured and isolated using streptavidin-coated magnetic beads. The cDNA is then split into two populations, and each population has ligated onto it via the anchoring restriction site and a linker (A or B) containing a site for a type IIS restriction enzyme (tagging enzyme). The tagging enzyme is then used to release a 9-bp fragment of the cDNA. The two populations are then ligated together and amplified with primers

specific for linkers A and B. The linkers are then released using the anchoring enzyme, and the resulting cDNA fragments are ligated together, forming concatemers of many different 9-bp fragments. The concatemers are then cloned and sequenced to identify each fragment. The number of times a given fragment appears is a direct measure of the quantity of that RNA species in the original cell population (44, 45).

SAGE has been used to study the expression levels in a variety of systems, such as the transcriptional changes induced by p53 expression in human colorectal cancer cells (46, 47), to study gene expression profiles in normal and cancer cells (48), and to characterize the yeast transcriptome (49). (For more information concerning SAGE, consult the following website: http://www.genzyme.com/sage.) A similar system has been developed by Perkin Elmer GenScope, called GeneTag™ (http://www.genscope.com). Though no published reports utilizing SAGE or GeneTag™ specifically for toxicology studies could be found, this will likely change as these techniques becomes more widely used.

REAL-TIME PCR, BRANCHED-DNA, AND SCINTILLATION PROXIMITY ASSAYS

Real-time PCR, branched-DNA (bDNA), and scintillation proximity assays (SPA) are examples of high-throughput, low-fidelity techniques available to toxicology. These techniques are similar in that unlike the previous techniques reviewed in this article, which give in one experiment the expression changes for thousands of genes regulated by one compound, real-time PCR, bDNA, and SPA can potentially give information on expression changes for one gene using many different compounds in an experiment. These three methods are extremely useful for confirming and expanding upon information gained from other high-throughput techniques, such as hybridization microarrays. Thus, far from being exclusive, real-time PCR, bDNA, and SPA are complementary to other high-throughput methods.

Real-time PCR kinetics was pioneered in 1993, when Higuchi et al (50) constructed a system that detected the accumulating levels of double-stranded DNA by monitoring the increase in the fluorescence of ethidium bromide that binds to duplex DNA. By calculating the number of cycles necessary to detect a signal, it is possible to determine the starting levels of a certain gene present in the cell population. The method has been subsequently improved on to allow detection using a specific probe rather than ethidium bromide. The current method for real-time PCR detection is as follows. A probe is designed that hybridizes specifically to the gene of interest. This probe hybridizes between the forward and reverse primers and contains both a reporter fluorescent dye and a quencher dye attached to it. If the probe is intact, the proximity of the quencher strongly reduces the fluorescence emitted by the reporter. When the probe is cleaved by the 5′ nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher,

and a signal is generated. This signal is monitored by a fluorescence reader. The starting copy number of the gene of interest can then be determined based on the cycle at which the PCR signal is first detected (51). The PCR reaction is done in a 96-well plate, thus making it possible to test numerous compounds or conditions in the same experiment.

The principal company offering a real-time PCR system is Perkin Elmer Applied Biosystems, which markets the ABI Prism 7700 PCR® technology. Bio-Rad also markets a real-time PCR system, the iCycler. (For more information on these systems, see the following websites: http://www.pebio.com/ab/about/pcr/sds and http://www.discover.bio-rad.com.)

SPA is similar to real-time PCR in that many different conditions or compounds can be tested in the same experiment for the regulation of a target gene. Cells are plated directly onto Cytostar-T™ 96-well scintillating microplates, into which solid scintillants have been incorporated. Cultured cells are treated, then fixed in the wells. Radiolabeled probes are added, and the cells are RNase treated and washed. Bound radioactive probes are then quantitated on a scintillation counter (7, 52). Harris et al (52) used this method to detect the levels of *c-fos* in quiesced rat smooth muscle cells with or without induction of platelet-derived growth factor. The results were compared against those from a northern blotting assay. Both assays gave the same results, but SPA was found to be 20-fold more sensitive and easier to perform (52). (More information can be obtained at the website for Amersham Pharmacia Biotech: http://www.apbiotech.com.)

Branched DNA is similar to real-time PCR in that it is extremely sensitive. However, unlike real-time PCR, which amplifies the starting material, bDNA amplifies the signal. This is accomplished by the use of probes specific for the gene, which are attached to alkaline phosphatase conjugated labels. Cells are plated in 96-well plates and treated. Following this, the medium is aspirated off, and cell lysis buffer containing the alkaline phosphatase labeled probes are added. The cell lysate is then transferred to a bDNA assay plate, which captures the mRNA of interest. The level of expression is assayed by adding a chemiluminescent substrate, and the light emission is measured using a luminometer (7). An example from our own laboratory is shown in Figure 4. In this example, cultured rat hepatocytes were treated with either the peroxisome proliferator activated recepter alpha (PPARα) agonist bezafibrate or the PPARγ agonist pioglitazone. The endpoint for the assay was quantitation of mRNA expression for acyl-coenzyme A oxidase, a marker gene for peroxisomal proliferation, as compared with the ''housekeeping'' gene glyceraldehyde phosphate dehydrogenase. The bDNA technique clearly differentiates between the responses generated by these two compounds: a robust response for the peroxisome proliferator bezafibrate and a mild response for the relatively weak PPARα agonist pioglitazone. In our hands, results from bDNA evaluation for this gene response are similar or identical to those from SPA.

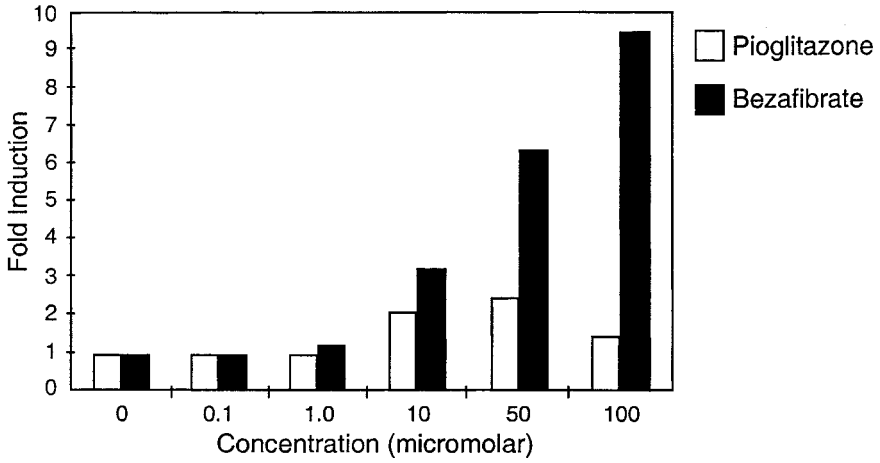

Figure 4 Changes in acyl-coenzyme A oxidase gene expression in cultured rat hepatocytes treated with either the peroxisome proliferating drug bezafibrate or the thiazolidinedione pioglitazone. Hepatocytes were treated overnight with the compounds, and specific mRNA was quantified by using the branched-DNA signal amplification method. Glyceraldehyde phosphate dehydrogenase gene expression was used as a control (not shown).

DISCUSSION

In this review we discussed several genomics-based tools that are currently available for use in toxicity evaluation. The advent of these technologies and their application in drug safety research represents a change in approach, if not in paradigm, from histopathology to molecular pathology/toxicology. Microarrays offer the promise of identifying liabilities and mechanisms in a short amount of time. In addition to evaluation responses due to single-compound exposure, these technologies may be used to assess complications due to interactions between drugs, assessing the contribution of each compound as well as the combined effects. Enthusiasm for microarray technologies is certainly justifiable but needs to be tempered; microarrays are too new for their full value or potential to be understood (in fact, no reports showing the use of microarrays specifically for safety or risk assessment appear in the peer-reviewed literature).

Of more practical current value is the application of these tools to toxicology applied at the early drug discovery level. Gene response targets, identified by microarray or sequencing analysis, can be used to develop toxicology screens with techniques such as real-time PCR or bDNA. As with any single endpoint assay, however, a degree of caution must be used when extrapolating from results because the fidelity of these assays decreases even further when applied outside of a chemical analogue series or to a different species.

Application of genomic-based technologies in day-to-day safety evaluation, and particularly in regulated studies and other studies required for product registration, will likely require the development of large databases. It remains a challenge for the pharmaceutical and vendor industries to cooperate in developing shared databases that can be queried by other companies and by regulatory agencies; this is important for reducing product liability and ensuring consumer safety. Clearly, the new tools available to the toxicologist have opened a new age of discovery.

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