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DNA microarrays for detecting endocrine-disrupting compounds

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Abstract

It has recently been discovered that a number of synthetic chemicals and naturally occurring compounds released into the environment can influence endocrine activity. These endocrine-disrupting compounds (EDCs) are highly varied in structure, provenance, and mode of action (MOA). Many EDCs are anthropogenic, products of the chemical industry. Others are natural compounds. Although natural hormones exist at low levels in the environment, industrial sites such as pulp and paper mills and municipal sewage treatment plants can gather large amounts of natural EDCs and release them into the environment as part of their daily operations, thus increasing background environmental concentrations.

The potential consequences of endocrine disruption are serious; however, comparatively little is known about the phenomenon. There is considerable debate over the true concentrations, sources, identity, and effects of potential EDCs. Investigation of the problem is hampered by the diversity of potential EDCs, which may have synergistic as well as individual effects, and the complexity of the endocrine system itself. Effective and standardized tests to accurately detect the presence of such chemicals in the environment are not available. While many tests have been proposed, they are unsatisfactory because they have only one limited endpoint (e.g., they can detect only one of many potential hormonal responses) and do not provide any mechanistic information. In addition, results from rapid screening tests are difficult to correlate with whole organism response, while larger whole organism bioassays are very costly and time consuming to perform.

There is tremendous potential in the application of DNA microarray technology to screen for EDCs. DNA microarrays provide a “snapshot” of transcriptional activity in tissue samples showing

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which genes were actively expressed within the cells at one point in time. By combining this technology with human cell lines grown in vitro, it should be possible to conduct relatively rapid and straightforward assays to identify EDCs by observing the changes in gene expression patterns in response to exposure.
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1. Endocrine-disrupting compounds (EDCs) in the environment

The endocrine system regulates and coordinates cellular activities throughout the body, enabling the many cells and tissues of the body to work together as a single organism. Almost all actions undergone by animals are in some way regulated or influenced by the endocrine system, which also guides the organism's growth, development, and behavior. The endocrine system is as intricate as the processes it controls. In humans, the endocrine system consists of seven specialized glands that are found in human beings (pituitary, parathyroid, thyroid, thymus, adrenals, pancreas, and gonads) and are supplemented by additional endocrine tissues. These organs and tissues in turn produce several different types of endocrine signalers, each synthesized from separate metabolites. Endocrine signalers can be made from amino acids or peptide chains (e.g., insulin, growth hormone), from steroids (e.g., estrogen and testosterone), from iodinated tyrosine (e.g., the thyroid hormones), and from arachidonic acid (the eicosianoids, e.g., prostaglandin). Each of these categories differs in chemical structures and properties. Furthermore, each category comprises a variety of different hormones, all of which bind to different receptors (Hadley, 1996; Norris, 1997).

Endocrine receptors are found attached to the plasma membrane of their cells or floating freely in the cytosol. Upon binding signaling molecules, these receptors trigger a number of changes within the cell (Hadley, 1996; Norris, 1997). Endocrine receptors typically operate by altering the activity of proteins within the cell or by changing the rate at which specific genes are expressed. The estrogen receptor, for instance, exists in two different forms, one membrane-bound form and one cytosolic form. The membrane-bound receptor influences protein activity, while the cytosolic receptor, upon binding with estrogen, migrates to the nucleus and binds specific genes in the chromosomes to induce or repress the rate at which they are transcribed into RNA. Endocrine responses can be either activational or organizational in nature (Lister and Kraak, 2001). An activational change is a transient and reversible response to metabolic demands, which occurs continuously throughout development and normal functioning. On the other hand, organizational responses occur during critical stages of development and the effected changes last a lifetime, as was the case in the studies of Saal et al. (1992) of the behavioral tendencies of homozygous laboratory mice.

The endocrine system can be influenced by a wide range of chemical compounds. Bioactive compounds are found in most major classes of pollutants, including dioxins and furans, halogenated organic compounds, polychlorinated biphenyls, phthalate esters, pesticides (both banned substances, such as DDT, and others currently in use, such as

atrazine), and a number of other pollutants, such as polyaromatic hydrocarbons, tributyl tin, and heavy metals. Many of these compounds are highly persistent in the environment and are capable of bioaccumulation and biomagnification in living organisms. Human activities also introduce a number of natural endocrine signalers into the environment at abnormally high concentrations. For instance, municipal sewage and agricultural runoff following field application of manure can contain elevated levels of animal hormones (Hewitt and Servos, 2001). Many pharmaceutical products and medical wastes likewise influence the endocrine system, and disposal of these substances can introduce EDCs into the environment.

Of particular interest to the pulp and paper industry is the endocrine-disrupting activity detected in the effluent of numerous bleached Kraft mill effluents. It was originally believed that most of the endocrine-disrupting activity of pulp mill effluent was due to the production of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DF), which were known to be EDCs. However, while the introduction of elemental chlorine-free bleaching has greatly reduced or eliminated the production of these compounds, endocrine disruption effects can still be observed in wildlife exposed to effluent (Fox, 2001; McMaster, 2001). It is currently believed that a number of compounds present in wood and released during the pulping process possess endocrine-disrupting activity. A class of compounds, including several lignans, triterpene alcohols, resin acids, sterols, particularly β-sitosterol, and stilbenes, are known as phytoestrogens because they display estrogenic potential. Some mycotoxins produced by fungal parasites present in wood may also be potential EDCs (MacLatchy and Kraak, 1995; Mellanen et al., 1996). Additionally, research shows that there may be additional EDCs in pulp mill effluents that have yet to be characterized (Zacharewski et al., 1995).

EDCs can interfere with the endocrine system in several ways, by binding to hormone receptors, causing stimulation of the receptor in the absence of endocrine signalers or preventing normal activation of the receptor by endocrine signalers. EDCs can alter the expression of the receptor protein itself, increasing or decreasing the amount of receptor molecule in each cell, and thus altering cellular sensitivity to normal signalers. The organism's ability to synthesize various endocrine signalers can be impaired, resulting in reduced concentrations of signaler molecules. Likewise, the transport or metabolic elimination of normal endocrine signalers can be altered by EDCs, again upsetting hormone balance (Lister and Kraak, 2001; Phillips and Harrison, 1999).

These perturbations in the normal functioning of the endocrine system can have varied consequences. The endocrine system regulates and coordinates virtually all metabolic and developmental activities of the body. A perturbation to either response can seriously impact a living organism. Furthermore, there is a strong similarity in the chemical and protein structures of endocrine signalers and endocrine receptors across most vertebrate classes (Lister and Kraak, 2001). As such, EDCs are able to engender a great many responses in a wide range of organisms. As mentioned earlier, even plant compounds (i.e., the phytoestrogens) are able to influence animal life, and Fox et al. (2001) have found that estrogenic compounds can interfere with the flavonoid signaling that establishes symbiosis between alfalfa (*Medicago sativa*) and nitrogen-fixing rhizobial bacteria (*Sinorhizobium meliloti*). EDCs are suspected to have many subtle, complex, and adverse effects on

reproductive, neurological, and immunological systems throughout the animal kingdom (Colborn and Clement, 1992; Colborn et al., 1996; Naz, 1999).

2. Methods for detecting EDCs

A wide variety of testing methods have been developed to investigate EDCs (for a review, see Jobling, 1998). These include physical and chemical fractionation methods, the study of biomarkers in sentinel species, in vitro and single mode of action (MOA) oriented in vivo and in ovoassays, and life cycle or multigenerational in vivo tests.

Physical and chemical fractionation methods rely on analytical chemistry to separate and isolate suspected EDCs from environmental samples. Due to the wide variety of EDCs and other chemicals in the environment, these methods are most suited to looking for known compounds frequently associated with endocrine disruption, e.g., β-estradiol and its metabolites in sewage effluent (Huang and Sedlak, 2000; Smeds and Saukko, 2001; Solé et al., 2000).

Biomarkers for the purpose of EDC monitoring are measurable alterations within an organism indicative of exposure to or effects from environmental contaminants. These can range from repressed activity of metabolic enzymes to alterations in gross physiology or behavior. The impact of EDCs came to light when the scientific community noticed declines in health and reproductive functions in animal populations worldwide. Sensitive species living in natural environments can provide a warning of deteriorating conditions or an indication that a damaged environment is recovering from previous stresses (Fossi, 1998). Unfortunately, biomarkers in the wild are subject to many confounding factors. Environmental conditions such as age, health, habitat loss, or previous contaminant exposure, to name but a few, can affect the observations made. Even without such confounding variables, environments can be contaminated with multiple different EDCs and other pollutants, and the combined effects of these compounds are difficult to resolve based on physiological data. As a result, it is a difficult task to conclusively establish a causal link between effects observed in nature and the known molecular activity of specific EDCs (Fossi, 1998; Lister and Kraak, 2001).

A more precise form of environmental testing involves in vitro or single MOA-oriented in vivo and in ovo tests (Baker, 2001). Most in vitro tests are hormone receptor binding assays, which test a compound's affinity for a given receptor; cell proliferation tests, which measure a sample's ability to induce hormone-dependent proliferation; or reporter gene assays, which use cells (typically yeast or animal cell lines) transfected with a reporter gene induced by a specific cellular receptor, providing a quantitative measure of receptor-mediated gene induction (Villeneuve et al., 2000; Witters et al., 2001). Single MOA-oriented in vivo assays use specialized biomarkers in test animals to detect only one type of endocrine disruption, such as estrogenic, androgenic, or thyroid-stimulating functions. Similarly, the hormone-driven maturation of certain amphibians is suitable for use as an in ovo or in vivo bioassay for specific types of endocrine-disrupting activity. The in vitro bioassays are rapid, straightforward, and inexpensive. Combining them with chemical and physical fractionation could potentially allow for the isolation and identification of all contaminants in an environmental sample that shares the same endocrine-disrupting

activity. Reporter gene assays are likely superior to receptor binding assays, as an EDC may bind a receptor as an agonist or an antagonist, and this distinction can only be made with a reporter gene assay.

The in vivo bioassays are longer to perform (taking days or weeks) and more complex and work intensive. However, they verify that the suspected EDC have an influence on whole organisms. The in vitro bioassays detect an affinity for a receptor in cultured cells, but it is difficult to extrapolate from the cellular level to that of the whole organism. Finally, multigenerational or life cycle tests in vivo are the most exhaustive form of EDC assay. The test animals are observed through part or all of their life cycle (Tabata et al., 2001). A number of these assays expose the test animals to the suspected EDCs when in ovo or in utero, when they are most vulnerable to minute perturbations in endocrine functioning. Multigenerational tests follow the effect of exposure through more than one complete life cycle. These tests provide the most detailed information possible and are not limited to a single MOA. On the other hand, they are the most cumbersome, time consuming, and expensive to perform.

It must be stressed that there is no perfect screening method. The life cycle and multigenerational tests provide the most data but are lengthy and involved. The short-term in vivo tests are simpler but will only test one MOA at a time, so testing a sample for estrogenic potential when it is androgenic will be a waste of time. The in vitro assays are rapid but will also only test one MOA at a time and will be insensitive to endocrine-disrupting activities that are dependent on a full animal metabolism to operate. Furthermore, a compound's ability to stimulate a receptor in vitro does not always translate into observable endocrine disruption in an actual organism. Finally, EDCs are varied and most environmental samples are composed of complex mixtures of pollutants, which may exhibit different endocrine-disruption activities at once. The best testing approach combines a variety of tests to efficiently cover the broadest possible spectrum of endocrine-disrupting activity.

3. DNA microarray methodology

Genome sequencing projects have stimulated radical changes in experimental methods from those that focus on "one gene at a time" to those that aim to study thousands of genes or proteins at once. DNA microarray-based technology, a new genomics technology, is revolutionizing our ability to achieve rapid throughput of thousands of simultaneous hybridization reactions at a time. Significant developments in robotics, surface chemistry, and miniaturization have permitted rapid developments of this technology (Schena et al., 1995). In most configurations, a DNA microarray is a glass microscope slide onto which many thousands of DNA samples have been spotted in a grid. DNA or messenger RNA is extracted from cells or tissues, labeled with specific fluorescent molecules, and hybridized to the spotted DNA on the glass slide. The resulting image of fluorescent spots is visualized in a confocal scanner and digitized for quantitative analysis.

This study investigates the possibility of using DNA microarrays for EDC screening. Microarrays allow the messenger RNA (mRNA) transcripts of two different cell populations to be compared to each other. RNA is extracted from the two cell populations and

converted into complementary DNA (cDNA) in such a way as to incorporate fluorescent dye moieties into the nucleotide strand. Each messenger RNA population is labeled with a different dye; the two dyes used in this study were Cyanine 3 (Cy3) and Cyanine 5 (Cy5). Once one RNA sample has been labeled with Cy3 and the other with Cy5, both samples are combined and hybridized onto the microarray. The labeled samples hybridize to spots of matching sequence, labeling those spots with Cy3 or Cy5. The slide is then scanned with a confocal microscope, producing an image for both Cy3 and Cy5 fluorescence. Each image represents the mRNA transcripts present in the original cell population at the time of RNA extraction. By comparing these two profiles, each gene spot can be evaluated in terms of the ratio between the signal intensity of the two dye labels. A ratio value near unity indicates that the amounts of mRNA transcripts for that gene were similar in the two populations. A ratio value far from unity indicates that that particular gene transcript was more abundant in one population. As changes in mRNA levels for a given gene indicate changes in the rates of transcription for that gene, DNA microarrays reveal which genes were induced or repressed in each cell population with respect to the other (Duggan et al., 1999; Kao, 1999; Schena et al., 1998).

The microarrays used in this study are composed of copies of the expressed sequence tags (cDNA fragments) of over 19,000 separate human genes printed onto glass microscope slides by a robotic printer and immobilized onto the glass surface (University Health Network (UHN) Microarray Centre, Clinical Genomics Centre, Toronto, ON, Canada). We have chosen to investigate the use immortalized human cell lines, including the breast cancer cell lines T-47-D and MCF-7 (Kandouz et al., 1999), which express the estrogen receptor, as the test system from which to extract mRNA. These cell lines have been well studied, are easy to grow, and express a number of hormone receptors, including the estrogen and androgen receptors.

4. EDC screening by DNA microarray

The general procedure for determining gene expression patterns, following exposure to a particular challenge compound, are depicted in Fig. 1. Specific procedures for extracting RNA and conducting microarray experiments are described at www.microarray.ca (UHN Microarray Centre, Clinical Genomics Centre).

We have identified a core set of up- and down-regulated genes in response to exposure to the estrogen 17-β-estradiol (E2) in MCF-7 cells. Of particular interest are genes that are affected soon (i.e., 2–4 hours) after exposure to detect the primary effect of E2 on the cells. This early effect is expected to be more reproducible. About 55 genes showed early increased expression in MCF-7 after exposing to estrogen, and 38 genes showed decreased expression (Fig. 2). Several of these genes have been confirmed as estrogen-regulated either from other published data or by confirming the response using reverse transcriptase polymerase chain reaction (RT-PCR) assays with primers designed for the cDNA fragments detected by microarray analysis. This independent confirmation serves as an internal control for our microarray data. Work is ongoing to establish similar expression patterns for other hormones and suspected EDCs. EDCs in effluents will be assayed either by first extracting these compounds from an effluent sample and adding the extract to the cell

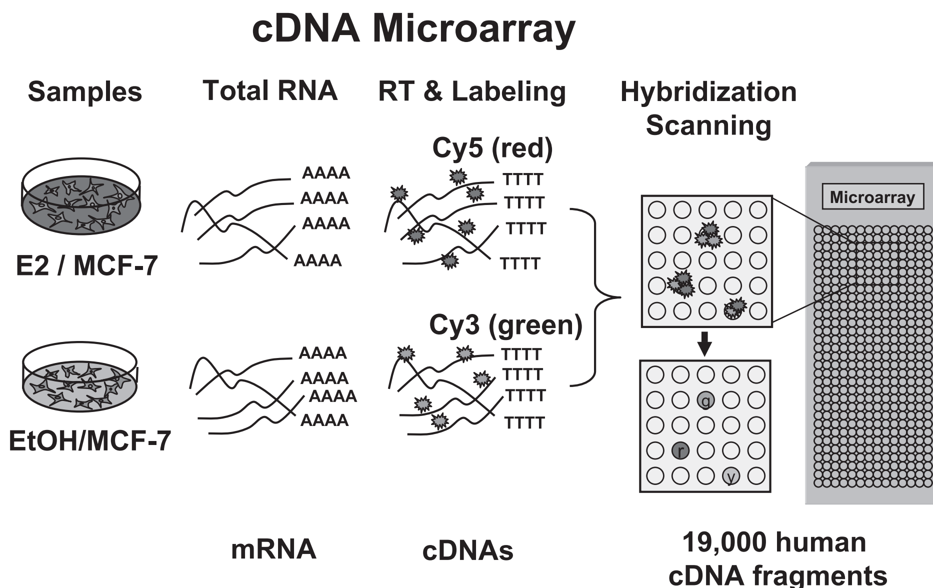

Fig. 1. Overview of DNA microarray methodology for detecting genes regulated by estrogen. Step 1: Grow cells in Fetal Bovine Serum that has been stripped of hormones for several days prior to exposure. Step 2: Expose half the cell population to estrogen (added as an ethanolic solution) and the other half simply to the carrier (ethanol). Step 3: Extract total RNA from exposed and unexposed cells. Step 4: Use reverse transcriptase to make cDNA from mRNA while incorporating fluorescent dye (Cy-3 or Cy-5). Step 5: Mix cDNA samples and hybridize on array. Step 6: Analyze data to determine normalized fluorescence intensity at each spot (i.e., gene) and for each fluor.

culture or by direct addition of portions of effluent to the cell culture. Once gene expression patterns for multiple hormones are established, a custom microarray comprised of identified hormone-responsive genes will be constructed to make data analysis and handling more manageable. This array is expected to comprise 500–1000 genes.

There are several advantages of this assay over other in vitro techniques. DNA microarrays provide a broad view of gene expression within the cell, as opposed to the single-gene responses measured in other assays, such as reporter gene assays. As a result, DNA microarrays may provide better insight into the regulatory mechanisms behind endocrine disruption. Furthermore, assay data may facilitate the discovery of new signal transduction links, as genes of unknown function are found to be regulated by specific endocrine signalers.

Finally, while other in vitro assays screen only for the ability to stimulate one specific hormone receptor, the microarray-based EDC assay should be able to screen for several different types of EDC-receptor interactions in a single run. Each cell expresses more than one hormone receptor, and each receptor has its own signal transduction pathways, controlling the expression of a unique suite of genes. As such, each type of receptor would be expected to produce a unique expression profile.

It should be possible to match the expression profile seen in the assay to the typical expression profile of a given receptor, and thus determine not only whether EDCs are

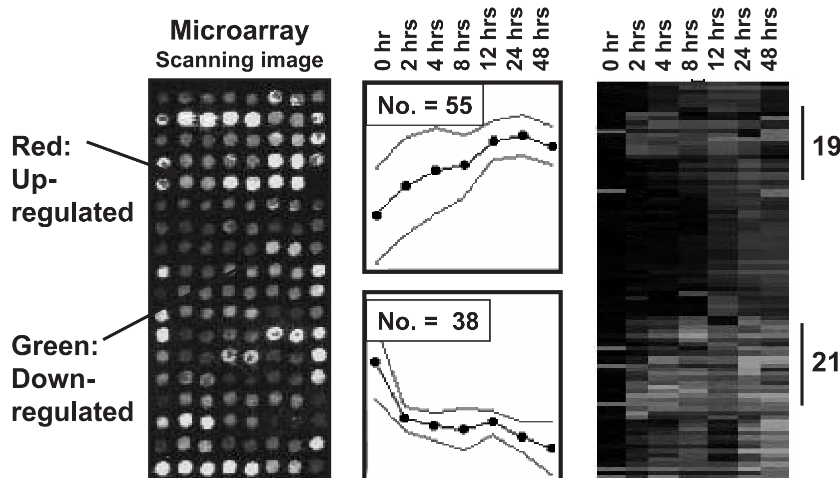

Fig. 2. Microarray scanning and image analysis. Left panel: image of a section of a scanned microarray revealing up- and down-regulated genes (as expressed by the ratio of normalized signal intensity from each fluor). Middle panel: average intensity of up- or down-regulated genes identified as responding early to the estrogen exposure (i.e., in the first 4 h). Right panel: image of the results of a cluster analysis of microarray data for several time points. Each row represents one gene or spot on the array and each column represents the data from one microarray experiment. Consistently up- and down-regulated genes are apparent from the color and intensity of the panels in the cluster diagram.

present but also what sort of activity they exhibit. The only theoretical limit to the breadth of the assay's spectrum is the number of different types of hormone receptor expressed in the cell lines used. The ability to rapidly screen for multiple types of endocrine disruption at once could accelerate investigations and assist in the selection of appropriate supplemental in vivo assays. It may even be possible to resolve the different types of endocrine-disrupting mechanisms present in a complex mixture of EDCs by deconvoluting the observed gene expression profile into receptor-specific subsets.

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