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TOXICOLOGICAL HIGHLIGHT

Toxicoproteomics—The Next Step in the Evolution of Environmental Biomarkers?

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The scientific community has become increasingly concerned about the potential adverse health effects to humans and wildlife resulting from environmental exposure to persistent industrial, pharmaceutical, and natural chemicals with estrogenic, androgenic, or thyroid-disrupting properties (Colborn *et al.*, 1993; Waring and Harris, 2005). To assess exposure to these endocrine-disrupting chemicals (EDCs), researchers have employed a variety of molecular biomarkers. A high-quality biomarker of a specific chemical class or specific mechanism of action should have the following attributes: (1) the biomarker should be inducible or repressible, (2) the measured response should be specific to chemicals within that class, (3) the response should have sufficient sensitivity for routine detection, (4) the biomarker should be highly accurate and reproducible among experiments within a laboratory and among different laboratories and animal models, and (5) the biomarker should be quantifiable so that degree of risk can be estimated. One of the most utilized biomarkers of EDC exposure is the egg yolk precursor protein vitellogenin (Vtg), which is highly expressed in the liver and plasma of oviparous animals in response to 17β-estradiol. Induction of plasma Vtg in fish has been routinely used as a biomarker of xenoestrogen exposure, both in controlled laboratory settings and in field studies (Sumpter and Jobling, 1995), and is currently being incorporated as a standard tier 1 bioassay within the U.S. Environmental Protection Agency Endocrine Disruptor Screening Program. Other examples of biomarkers of estrogenicity include *in vitro* assessment of MCF-7 cell proliferation, estrogen receptor binding and transactivation, and *in vivo* assessment of endometrial thickness in mammals.

Recent technological advances in the areas of genomics, proteomics, and metabolomics have provided researchers with new tools for developing biomarkers, specifically indicators that reflect both chemical exposure and the subsequent biological effect. This paradigm shift is particularly relevant in that traditional, single end point bioassays for xenoestrogens, such as the Vtg assay, provide little insight about the physiological consequences of exposure, particularly when attempting to estimate potential adverse human health effects. These new ''omics'' disciplines apply high-throughput methodologies in which changes in expression of hundreds to thousands of genes, proteins, or metabolites are assessed simultaneously. A direct comparison of expression values obtained for a control versus an altered condition reveals a set of biomarkers indicative of that altered state. This exposure ''fingerprint'' can then be used as a tool for classifying chemical exposures and predicting mode of action (Hamadeh *et al.*, 2002). The use of toxicogenomic and toxicoproteomic approaches to biomarker discovery can be widely applied to both environmental and clinical exposure scenarios, such as environmental exposure to xenoestrogens in wastewater effluent or altered gene or metabolite expression in response to disease.

The greatest advances to date in the application of ''omics'' technologies to EDC biomarker development have been in the field of toxicogenomics. Both commercial and custom DNA microarray platforms have been utilized to evaluate changes in gene expression in response to a number of known toxicants, including xenoestrogens, in both mammalian and aquatic animal models (Benninghoff and Williams, 2006; Brown *et al.*, 2004; Larkin *et al.*, 2003; Moens *et al.*, 2006; Naciff *et al.*, 2002; Wang *et al.*, 2004). In these studies, gene sets that were responsive to estrogen exposure were identified, and the biological processes associated with these transcriptional profiles were evaluated to gain insight into the network of biological processes responding to the chemicals in question. While gene expression profiling has proven to be useful in the development of biomarkers of EDC exposure, one must recognize that changes in rates of gene transcription do not necessarily correlate with protein expression or protein activity

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(Anderson and Seilhamer, 1997) and that proteins are primarily responsible for cellular responses to physiological stimuli.

In the past few years, researchers have successfully employed proteomic approaches in the development of diagnostic biomarkers of human disease such as ovarian cancer (Petricoin *et al.*, 2002), lung cancer (Yang *et al.*, 2005), and rheumatoid arthritis (de Seny *et al.*, 2005). However, to date, efforts to establish protein expression profiles indicative of xenoestrogen exposure have been limited. Indeed, a single study by Shrader *et al.* (2003) has employed a large-scale proteomics approach using two-dimensional gel electrophoresis (2DE) to identify unique protein expression profiles in zebrafish embryos exposed to 17β-estradiol and the weak xenoestrogen, 4-nonylphenol. While 2DE coupled with mass spectrometry for protein identification has been successfully utilized in a number of toxicology studies and has been scaled up for high-throughput industrial applications, there are continuing concerns regarding the standardization of electrophoresis protocols, the reproducibility of the data, and the subjective nature of 2DE gel image analysis (Ong and Pandey, 2001). Thus, alternative proteomics approaches, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI, also known as MALDI-TOF-MS) and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI, also known as SELDI-TOF-MS), are becoming more popular in clinical medicine and very recently in environmental toxicology.

The article selected as the Toxicological Highlight for this issue describes the first application of a SELDI-based proteomics approach to identify a set of protein biomarkers of xenoestrogen exposure and represents a major advance in the area of environmental biomarker development (Walker *et al.*, 2006). Dr Calvin Walker and his colleagues at the U.S. EPA have utilized a high-throughput proteomic approach to examine estrogen-induced changes in low-molecular weight plasma proteins in sheepshead minnows. SELDI utilizes protein chips with various chemical (e.g., hydrophobic, hydrophilic, ion-exchange, metal binding) or biological (e.g., antibody, DNA, receptor) capture surfaces. A fraction of proteins solubilized from tissues or body fluids binds to the selected capture surface, the appropriate matrix solution is added, and the proteins are then laser desorbed and ionized for MS analysis. The resulting mass spectra reflect specific protein mass patterns with variable protein expression intensities (mass peak height); spectra for normal and altered experimental conditions may be compared to identify differentially expressed proteins. (See review by Merchant and Weinberger [2000] for details regarding SELDI technology and applications in proteomics.)

The objective of the highlighted study was to determine whether exposure to estrogen agonists altered plasma protein expression in male sheepshead minnows. Male fish were exposed to estradiol and several known weak xenoestrogens including bisphenol-A (BPA), 4-*tert*-pentylphenol (TPP) and methoxychlor (MXC). Importantly, this elegant study design also included sufficient experimental controls, including an unexposed male ''normal'' control, a mature female positive control, a solvent vehicle control, and two other nonestrogenic chemical stressor treatments, endosulfan and chlorpyriphos, so that a protein expression pattern specific to xenoestrogen exposure could be confidently identified. A second experiment was performed to evaluate the sensitivity of this protein expression profile at low levels of estradiol exposure. Following SELDI analysis of plasma proteins, biomarkers of xenoestrogen exposure were discovered by identification of discriminator peaks that represent proteins uniquely expressed in plasma of estrogen-exposed males. The authors found that 13 proteins were differentially regulated in estradiol-exposed males and that these discriminator peaks were also observed with 100% specificity in males exposed to the xenoestrogens TPP, BPA, and MXC. One of these proteins was identified as a zona radiata protein fragment. Interestingly, several toxicogenomics studies have shown that the hepatic gene encoding this protein is highly inducible by estrogen exposure (e.g., Benninghoff and Williams, 2006; Knoebl *et al.*, 2006), thus suggesting that proteomic and genomic data may share at least a modest degree of correlation.

Observations from the highlighted study as well as recent experiments in other aquatic animal models (Bjørnstad *et al.*, 2006; Provan *et al.*, 2006) have shown that the application of SELDI-based toxicoproteomics to the identification of biomarkers for specific environmental exposures or stressors has the potential to significantly advance risk assessment efforts. Indeed, one can envision this proteomics approach applied to wide-scale biomonitoring of numerous wildlife species in which contaminant concentration and the presence (or absence) of low molecular weight biomarkers in blood plasma are assessed concurrently. As pointed out by Dr Walker and colleagues, a proteomic approach to biomarker development presents several advantages over a genomics methodology, including the important issue of improved sample accessibility. Blood plasma is readily available, and the very small amount of sample required can be acquired by nonlethal methods. Moreover, compared to a toxicogenomics approach, a SELDI proteomics methodology is more useful for biomarker identification in alternative animal models and wildlife since platform development (i.e., design and manufacture of custom or commercial microarrays) is not required. However, limitations in the ability to identify proteins of interest, particularly for nontraditional animal models such as the sheepshead minnow, present a significant drawback to a proteomics approach in biomarker development. Because the genome for a number of species, including mammalian and some fish models, has been sequenced and at least partially annotated, a toxicogenomics approach to EDC screening may seem to be a more attractive option. Although the ability to rapidly, if only tentatively, identify genes or gene homologs in microarray studies exceeds the current capacity for rapid protein or peptide identification, it should be acknowledged that protein expression more

accurately represents the physiological response to a given environmental condition.

While the application of toxicoproteomics to the development of biomarkers for EDCs has significant promise, many issues remain to be resolved. Indeed, a number of these concerns are shared among all the ''omics'' disciplines. First, are protein (or gene) expression profiles for a particular chemical class similar among species? As mass spectra libraries for numerous chemical exposures in multiple species are obtained, it may become apparent that there is not a consensus set of specific plasma biomarker proteins for a particular chemical class. Secondly, are structurally diverse chemicals with a similar mode of action intrinsically similar? Results of the highlighted article suggest that the protein expression profile in response to four structurally diverse estrogen agonists is highly similar. However, given the surprisingly diverse assortment of chemicals that are known to bind the estrogen receptor, one cannot conclude from this limited data set that all xenoestrogens will elicit the same protein expression profile. Additionally, one must consider that an expression profile is merely a snapshot of a highly dynamic system, and temporal changes in gene and protein expression should be anticipated. Finally, as most researchers in the field of environmental toxicology are acutely aware, the issue of mixtures continues to be a significant problem that has not been adequately addressed. No exposure occurs as a single event. Aside from the important issue of chemical mixtures, other habitat and physiological parameters can influence a biological response, including diet, disease state, water quality, diurnal cycles, etc. It is not known whether genomic or proteomic expression signatures for a specific chemical exposure, such as xenoestrogens, can be manifest above background responses to these other stimuli.

What is the best biomarker of xenoestrogen exposure? I concur with the authors of the highlighted article that highly efficient and scalable methodologies are needed for biomarker development so that regulatory agencies and industry can rapidly screen chemicals for potential endocrine-disrupting properties. Moreover, I would like to emphasize that selection of biomarkers that reflect not only exposure but also provide information about potential physiological effects will generate data that are more valuable. Single end point bioassays such as Vtg induction and MCF-7 cell proliferation seem inadequate to this purpose. An EDC-monitoring program that uses genomic and proteomic biomarker profiles as complementary screening tools will likely provide the most complete, robust, and informative data set for risk assessment.

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REFERENCES

Anderson, L., and Seilhamer, J. (1997). A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* **18,** 533–537.

Benninghoff, A. D., and Williams, D. E. (2006). Identification of an estrogen receptor mediated transcriptional fingerprint in rainbow trout liver. *Drug Metab. Rev.* **38**(Suppl. 2), 140–141 (Abstract).

Bjørnstad, A., Larsen, B. K., Skadsheim, A., Jones, M. B., and Andersen, O. K. (2006). The potential of ecotoxicoproteomics in environmental monitoring: Biomarker profiling in mussel plasma using ProteinChip array technology. *J. Toxicol. Environ. Health A* **69,** 77–96.

Brown, M., Robinson, C., Davies, I. M., Moffat, C. F., Redshaw, J., and Craft, J. A. (2004). Temporal changes in gene expression in the liver of male plaice (*Pleuronectes platessa*) in response to exposure to ethynyl oestradiol analysed by macroarray and real-time PCR. *Mutat. Res.* **552,** 35–49.

Colborn, T., vom Saal, F. S., and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101,** 378–384.

de Seny, D., Fillet, M., Meuwis, M. A., Geurts, P., Lutteri, L., Ribbens, C., Bours, V., Wehenkel, L., Piette, J., Malaise, M., *et al.* (2005). Discovery of new rheumatoid arthritis biomarkers using the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry ProteinChip approach. *Arthritis Rheum.* **52,** 3801–3812.

Hamadeh, H. K., Bushel, P. R., Jayadev, S., DiSorbo, O., Bennett, L., Li, L., Tennant, R., Stoll, R., Barrett, J. C., Paules, R. S., *et al.* (2002). Prediction of compound signature using high density gene expression profiling. *Toxicol. Sci.* **67,** 232–240.

Knoebl, I., Blum, J. L., Hemmer, M. J., and Denslow, N. D. (2006). Temporal gene induction patterns in sheepshead minnows exposed to 17beta-estradiol. *J. Exp. Zool. A Comp. Exp. Biol.* **305,** 707–719.

Larkin, P., Sabo-Attwood, T., Kelso, J., and Denslow, N. D. (2003). Analysis of gene expression profiles in largemouth bass exposed to 17-beta-estradiol and to anthropogenic contaminants that behave as estrogens. *Ecotoxicology* **12,** 463–468.

Merchant, M., and Weinberger, S. R. (2000). Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis* **21,** 1164–1177.

Moens, L. N., van der Ven, K., Van Remortel, P., Del-Favero, J., and De Coen, W. M. (2006). Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. *Toxicol. Sci.* **93,** 298–310.

Naciff, J. M., Jump, M. L., Torontali, S. M., Carr, G. J., Tiesman, J. P., Overmann, G. J., and Daston, G. P. (2002). Gene expression profile induced by 17alpha-ethynyl estradiol, bisphenol A, and genistein in the developing female reproductive system of the rat. *Toxicol. Sci.* **68,** 184–199.

Ong, S. E., and Pandey, A. (2001). An evaluation of the use of two-dimensional gel electrophoresis in proteomics. *Biomol. Eng.* **18,** 195–205.

Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., *et al.* (2002). Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* **359,** 572–577.

Provan, F., Bjørnstad, A., Pampanin, D. M., Lyng, E., Fontanillas, R., Andersen, O. K., Koppe, W., and Bamber, S. (2006). Mass spectrometric profiling—A diagnostic tool in fish? *Mar. Environ. Res.* **62**(Suppl.), S105–S108.

Shrader, E. A., Henry, T. R., Greeley, M. S., Jr, and Bradley, B. P. (2003). Proteomics in zebrafish exposed to endocrine disrupting chemicals. *Ecotoxicology* **12,** 485–488.

Sumpter, J. P., and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **103**(Suppl. 7), 173–178.

Walker, C. C., Salinas, K. A., Harris, P. S., Wilkinson, S. S., Watts, J. D., and Hemmer, M. J. (2006). A proteomic (SELDI-TOF-MS) approach to estrogen agonist screening. *Toxicol. Sci*. **95**(1), 74–81.

Wang, D. Y., McKague, B., Liss, S. N., and Edwards, E. A. (2004). Gene expression profiles for detecting and distinguishing potential endocrine-disrupting compounds in environmental samples. *Environ. Sci. Technol.* **38,** 6396–6406.

Waring, R. H., and Harris, R. M. (2005). Endocrine disrupters: A human risk? *Mol. Cell. Endocrinol.* **244,** 2–9.

Yang, S. Y., Xiao, X. Y., Zhang, W. G., Zhang, L. J., Zhang, W., Zhou, B., Chen, G., and He, D. C. (2005). Application of serum SELDI proteomic patterns in diagnosis of lung cancer. *BMC Cancer* **5,** 83.