Incorporating Biomarkers into 21st Century Risk Assessments

(Received November 10, 2000; Revised February 10, 2001; In final form February 15, 2001)

In 1987, the National Research Council Board on Environmental Studies and Toxicology defined a biologic marker as any cellular or molecular indicator of toxic exposure, adverse health outcome or susceptibility. Since that time, significant advances in molecular biology and analytical chemistry bave lead to the use of these biomarkers in both molecular epidemiology and toxicology studies, providing the potential to integrate the results of two disciplines, which ordinarily evaluate health outcomes at population and organism levels respectively. Tempered success in molecular epidemiology studies has evoked the call for employing biomarkers in tbe risk assessment process. The identification, development and validation of biomarkers for use in environmental and human health assessments have tremendous potential to improve the quality and reduce uncertainty in risk estimates. However, application of biomarker methodologies across disciplines and incorporation within risk assessment processes is inherently complex and difficult. The potential benefits of such a merger are significant. We discuss a number of issues relevant to the successful incorporation of biomarkers into the risk assessment process.

Keywords: *biomarkers; risk assessment*

INTRODUCTION

Perera and Weinstein's[1] 1982 vision of molecular epidemiology provided a structure featuring four categories of biomarkers. These freshly minted terms for proposed molecular distinctions were categorized as biomarkers of internal dose, biologically effective dose, response and susceptibility. The focus initially was on cancer and cancer prevention

Comments On Toxicology
2001, Vol. 7, No. 5-6, pp. 519-539
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with a goal of incorporating molecular endpoints into epidemiological studies so that researchers "should be able to predict human risks more precisely than hitherto possible." In 2000, Perera[2] reported the success of a variety of efforts spanning the 17-year period from their initial proposal. While not voluminous, molecular epidemiology studies contributed to cancer prevention by: 1) providing new evidence that specific environmental agents pose human carcinogenic hazards; 2) helping to establish the causal role of such hazards; 3) identifying subsets of the population at special risk and 4) using the information gained to provide new and more effective strategies to reduce risk. Perera[2] concluded that what is currently needed is the timely translation of existing data into risk assessments and public policy as well as focussed research to fill gaps in scientific knowledge. If the incorporation of biomarkers into cancer risk assessments is on the near horizon, can the same be said of their use in non-cancer risk assessments?

The potential application of biomarkers in noncancer risk assessment has been discussed previously. For example, considerations for biomarker use have been described in the U.S. EPA guidance for deriving inhalation reference concentrations[3]. Risk assessment is the process of evaluating hazardous properties of a chemical(s), ascertaining potential exposures, and quantifying the resulting health risks from this exposure. The process was described by the National Academy of Sciences[4,5] within a four-part framework including hazard identification, dose-response assessment, exposure assessment, and risk characterization. Biomarkers have been applied to varying degrees in the distinct steps in the risk assessment process. Biological exposure monitoring has been widely used as a supplement to ambient exposure measurements in occupational and environmental health studies. Biomarkers of effect provide useful insight into mechanisms of toxicity and as such contribute to hazard identification and serve as a low-dose endpoint for the dose-response analysis. In this article, we focus primarily on the impact of biomarkers in the dose-response assessment phase of risk assessment.

Hattis and Silver[6] addressed the possibility of incorporating biomarkers in risk assessments as early as 1993 while answering the rhetorical question of why one would break open the presumed "black box" which exists between exposure and effect (adverse health outcome). They reported at least three reasons to use biologic markers in opening the "black box":

- This approach can lead to a more complete scientific understanding, incorporating more relevant information about causal mechanisms, than a simple input-output analysis.

- This approach offers the eventual prospect of better mechanism-based projection of risk beyond the range of possible direct observations, and better estimation of the magnitude and detailed source of uncertainties in these projections[7,8]
- This approach offers the possibility of greater sensitivity of detection and quantification of adverse effects and in some cases, progressing from the organism to the cell as a unit of analysis.

DEFINITIONS AND CHARACTERISTICS

There has been some extension and refinement in the definitions of biomarkers in the 17 years since Perera and Weinstein[1] first proposed molecular epidemiology as an "advanced laboratory method used in combination with analytical epidemiology to identify at the biochemical or molecular level specific exogenous and/or host factors that play a role in human cancer causation." In 1987, the National Research Council (NRC) Board on Environmental Studies and Toxicology[9] defined a biologic marker as any cellular or molecular indicator of toxic exposure, adverse health outcome or susceptibility. Three distinct types of biomarkers have emerged, which parallel and may be linked to the exposure-disease continuum. They are biomarkers of exposure, effects of exposure (and/or disease) and host susceptibility. It is easy to appreciate that these three have evolved from the four types of biomarkers originally proposed by Perera and Weinstien[1]. The refinement is that biomarkers of exposure include internal dose and biologically effective dose (BED) while biomarkers of effects of exposure include early biological effects (may be reversible) and altered structure and function (most often irreversible). Biomarkers of host susceptibility may distinguish subpopulations having significantly different risks than the general population as a result of a genetic or other predisposition to the occupational or environmental insult.

The relationship and overlap of the exposure-disease continuum is evident in the biomarkers described and is visually apparent in Figure 1; Schulte's[10] amplification of the NRC continuum[9] for use in toxicologic and epidemiologic research. The continuum includes exposure, internal dose, biologically effective dose, early biological effect, altered structure/function culminating in adverse health outcome (disease, death, etc.).

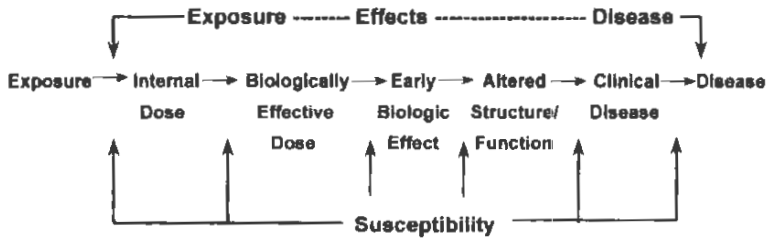

FIGURE 1 A model of the classes of biomarkers and the progression (left to right) in the exposure-disease continuum from one class to the next

Biomarkers of susceptibility may be expressed at the interface of each new step. Except for the extreme points of exposure and disease, the intermittent points on the continuum are included in the biomarkers described and it is not difficult to understand how the boundaries of some of these concepts have become blurred and the terms used interchangeably.

Biomarkers of exposure, effect, and susceptibility can improve the risk assessment process, primarily by reducing uncertainty. Biomarkers of exposure may reduce uncertainty associated with external dose measures from the dose-response analysis and expedite interspecies extrapolation by identifying measures more directly related to the response of interest (e.g., increased specificity). Biomarkers for early effect can increase the sensitivity of risk assessments, since such markers can often be observed at doses less than those that induce traditional default endpoints such as morbidity, mortality, tumors, body weight decreases, or tissue histopathology. Thus, the use of early effect markers reduces uncertainties surrounding low-dose extrapolation. This might be simply stated as looking at a new endpoint on the y-axis of a typical dose-response curve. Biomarkers of susceptibility provide information on the variability surrounding exposure or effect biomarkers, and provide an opportunity to replace default uncertainty factors in noncancer risk assessment with data-derived ones.

CONTINUUM AND RELATED ISSUES

Proposing a new molecular event for investigation as a potential biomarker should be driven by the prospective relationship, either docu-

mented or hypothesized to the current default or disease in question. This requirement describes the need to validate the biomarker. Ideally then, the molecular event proposed as a biomarker should be on the pathway from exposure to disease. Once a new dose-response curve is established between the insult in question and the potential biomarker, the relationship of the biomarker to the disease outcome must be confirmed. This is a type of validation and can be satisfied by the Hill[11] criteria established in 1965. The crucial components of the Hill criteria are 1) consistency of the association; 2) strength of the association; 3) specificity of the association; 4) temporal relationship of the association; 5) coherence of the association – inference of causality is greatly strengthened when it conforms to knowledge concerning the biologic behavior of a toxicant and its mechanism of action, and 6) a dose-response relationship – can we now plot the proposed biomarker versus the original risk assessment default? The cancer model offers some examples. One such example is the products of the covalent interaction of a chemical carcinogen with host cell deoxyribonucleic acid (DNA) which are appropriately termed DNA adducts. Mutti[12] has suggested that these adducts, at least in principle, are close to the ideal biomarker sought for risk assessment: they provide an integrated measure of exposure, they represent early and usually reversible biomarkers of effect and they are likely to reflect individual susceptibility to etiologic agents, so that they provide an integrated estimate of the individual risk. La and Swenberg[13] have recently reviewed the potential applications of DNA adducts in the risk assessment process. For all their potential, resistance to the use of DNA adducts in risk assessments remains, largely due to lingering questions surrounding adequate validation.

Prior to the application of biomarkers for risk assessment, their sensitivity and specificity should be evaluated. Sensitivity and specificity are two important concepts[14]. Specificity is when a biomarker can be uniquely associated with a specific exposure. Conversely, sensitivity is when a biological marker can he measured at low levels of exposure. Generally, as one moves along the exposure-response continuum, the sensitivity increases (i.e., increased relatedness to disease), but the specificity decreases (decreased relatedness to external dose). As an example, cotinine levels are commonly used to determine an active smoker from a passive or nonsmoker[15,16]. Those who smoke generally have higher levels of urinary cotinine than those exposed only to side stream smoke. If a cut-off level is made as to whether someone is a smoker or

nonsmoker based on urinary cotinine levels, setting this cut-off low would increase the sensitivity of this biomarker as you would be assured of identifying all smokers, but the specificity would be decreased. The opposite would occur if the cut-off level was set too high. As far as a biomarker being predictive in a health outcome of low prevalence, the specificity would have greater impact on the predictive value of the biomarker[17].

Biomarkers offer great potential in the information that they might generate with regard to a hazard or risk of a health outcome and subsequent use in intervention and prevention applications. Exposure biomarkers, such as metabolites in the urine may confirm an exposure occurrence, but provide little information on the biological significance of that exposure. On the other hand, an effect biomarker such as chromosomal aberrations may give an indication of the extent of risk or biological significance, but not be related back to a specific causative agent. Biomarkers can provide information to fill in gaps in the continuum[18].

For risk assessment, validation beyond sensitivity and specificity is necessary. Optimally, the proposed biomarker is related to the previously established dose or response metric used for the risk assessment. However, a biomarker need not always be so validated to provide useful information for risk assessment. Schulte[19] defines three types of studies; definitive, equivocal, and lacking, for which biological markers may be useful. In a definitive study, the relationship between exposure and disease is known. An example of this might be exposure to carbon monoxide and the extent of formation of carboxyhemoglobin resulting in symptoms of carbon monoxide poisoning. The biological response can then be modeled directly against exposure for the dose-response analysis and derivation of the critical effect level. In equivocal studies, epidemiological evidence may not be sufficient to definitively establish a relationship between an exposure and a disease. Biomarkers can be utilized to measure events that may occur between exposure and the disease to lend biological plausibility to that relationship. This might occur when several epidemiological studies have conflicting results in establishing an association between an exposure and a disease. An example might be the relationship of polycyclic aromatic amine exposure and lung cancer. Data of this kind are critical in the overall weight-of-evidence for determining the biological appropriateness of using a specific biomarker. The final type of study in which biomarker data may prove

useful is efforts in which there is no epidemiological evidence to determine an exposure to disease relationship. An example of this type of study is the exposure to endocrine disruptors and reproductive effects. Biomarkers may serve as early warning signals that can be used in intervention strategies to decrease exposures. These latter two study types, providing equivocal or no evidence for a relationship between an exposure and effect biomarker can play an important role in the hazard identification phase of the risk assessment. For example, information on exposure biomarkers can provide insight on important toxicokinetic parameters of the toxicant such as tissue distribution, metabolism, and excretion following exposure by different routes. Biological effect markers help to ascertain toxicodynamic aspects of an external exposure, such as target organ potency, factors that affect target organ toxicity, and interaction between components of mixtures.

After the biological relationship between the biomarker and the outcome has been validated, the variability of the biomarker must be considered in its application to risk assessment. Biomarkers of exposure and effect are subject to both biological variability and analytical variability. Biological variability arises from the dynamic nature of biological responses. Heritable (e.g., polymorphism of susceptibility genes) and nonheritable (age, diet, etc.) factors can affect qualitative and quantitative aspects of the biomarker in question. Considerations of analytical variability can also be important in assessing the utility of a biomarker for risk assessment. For example, it is important to establish assay characteristics such as sensitivity, specificity, accuracy, and precision, since assay measurements compound the inherent biological variability.

Temporal factors are also of significance in the selection and use of biomarkers since different markers peak at different times as demonstrated in Figure 2[20]. This is due to the natural progression of the toxic response or disease process and the pharmacokinetic and pharmacodynamic properties of the toxicant[20]. The optimum time to analyze for DNA adducts generally appears to he within a few hours to a few days. while hemoglobin adducts reflect an exposure of up to a 4-month time span. For chronic exposure, steady state levels can develop so that the biological response is balanced by fonnation resulting from current exposure and loss due to repair, and lifespan of the tissue (e.g., cell turnover). Chronic exposure favors adduct persistence[21]. In addition, the biological marker may not appear immediately upon exposure and

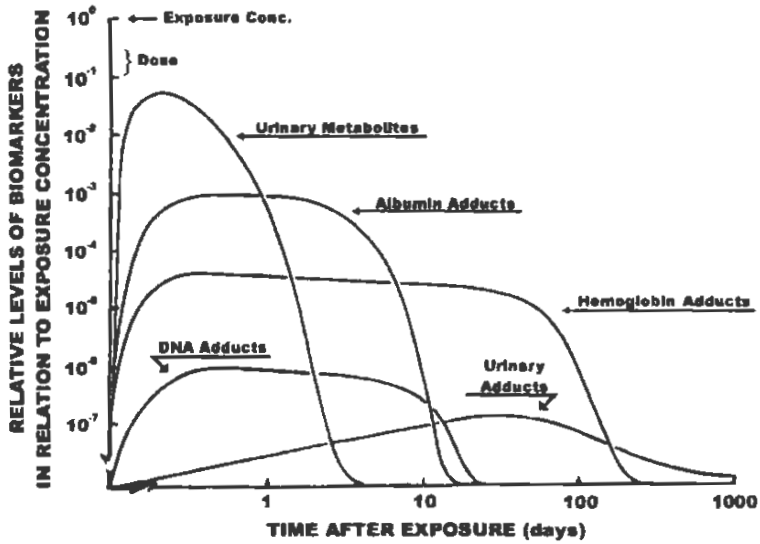

FIGURE 2 Hypothetical relationships among different biomarkers of exposure with respect to their relative levels and time of appearance after a single exposure

requires time to form as the toxic response progresses to disease. Non-specific biomarkers of effects such as sister chromatid exchange (SCEs) may be elevated for months or years.

WORKING MODELS

Without fanfare as such, certain biomarkers have already dramatically impacted the lives of the public. An ideal biomarker, but not necessarily so defined, routinely discussed between a patient and his/her physician is serum cholesterol. Many prospective epidemiological studies have substantiated the correlation between elevated serum cholesterol and increased risk of cardiovascular disease[22]. Since these and other studies have validated the link, the individual's biomarker information allows the patient to take action such as changing his/her diet, taking medication to reduce cholesterol levels, doing both or neither. It is important to

note that high blood cholesterol has been identified as a risk factor for cardiovascular disease and that risk assessments have not necessarily been conducted employing blood cholesterol levels.

As well as the link between increased cholesterol and increased risk has been established, the benefit of lowering elevated cholesterol and reducing risk – that which might seem intrinsically obvious has not been similarly validated. Thus, what one might consider as an ideal biomarker has shortcomings in its application. Cholesterol is an endogenous chemical that is necessary for life. Our endogenous levels are affected by a variety of exposures, but we are not exclusively dependent upon those exposures for our blood cholesterol levels. The concept of biomarkers as they relate to occupational or environmental exposures suggests that the biomarker measured is specific to the exposure. Groopman and Kensler[22] have refined the biomarker of exposure category to chemical-specific biomarker. One biomarker that approximates the strengths of blood lipid story, but encompasses the environmental exposure aspect, is blood lead. Unfortunately, the overwhelming majority of the chemical-specific biomarkers have yet to be validated for a person to be able to use this information in a health analysis[22]. There is growing consensus that employing multiple markers for the risk assessment process might be a more appropriate course of action. This expands the use of biomarkers into the realm of biomarkers of effects of exposure. As stated previously, ideally the latter are measurable biochemical or cellular endpoints mechanistically linked to more complex adverse biological endpoints such as disease. The combination then includes biomarkers of exposure, indicating the insult to which one is exposed to and the biomarkers of effects of exposure/disease. Several important applications featuring a multiple biomarker approach have recently emerged. These models have evolved from the belief that the increased use of more and different types of information will be required to improve the risk assessment process. Recent assessments of percholorate and vinyl acetate have been used to illustrate the positive direction of the field of risk science and the role of new methods and data in improving assessments[23]. In these assessments, key events have been identified and superimposed on the biomarker specific exposure-disease continuum. For perchlorate, the model features the following: Internal Dose – Perchlorate in Blood; Biologically Effective Dose – Uptake of Perchlorate versus Iodide in Thyroid Gland; Early Biological Effect – decreased T3 and T4 and increased TSH; Altered Structure and Func-

tion – Thyroid Hyperplasia (Cancer), Altered Development (Non-cancer) and continuing to include Clinical Disease – Tumors (Cancer), Birth Defects (Non-cancer) and lastly Prognostic Significance – Human Health Risk (Cancer), Children's Health Risk (Non-cancer). Once refined, such models could provide insights into the conditions necessary for response and the shape of the dose-response relationship for effects of concerns as one goes from high to low dose. Developing means for incorporating such data would allow the extension of the dose-response relationship developed by traditional toxicology studies to lower levels. This is based on an increased ability to detect responses in individual cells or by using more sensitive or molecular biological techniques. Incorporation of such information into risk assessments may also suggest targets for additional study in human populations by identifying potentially sensitive populations based on molecular or cellular characteristics or on sensitive stages in development and aging.

GAPS, CHALLENGES AND IMPEDIMENTS

Gaps

While progress on biomarkers in general has been made in the last two decades, much work remains, particularly as to their applicability in improving risk assessments. It is important to note that the field did not originate with risk assessment in mind, but has progressed to a position whereby application to the risk assessment process is an attractive possibility. Each discipline, which explores the use of biomarkers for their particular application, must establish pertinent criteria to support the use of biomarkers in their area of expertise. Non-invasive techniques might be significantly important to an industrial toxicologist concerned with occupational exposures to a proposed reproductive or neurological toxicant. However, for the purposes of deriving a risk value, this type of practical concern would not be immediately important. Another difference in the application of biomarkers of exposure to the workplace is the concern of individual risk versus the overall risk to the exposed population. Eventually concerns and criteria will mesh, but more immediate applications with criteria appropriate for each application can improve a particular discipline's use of biomarkers without attempting to construct

biomarkers which satisfy global conditions or multidisciplinary standards. Thus one gap is language and communication. Risk assessors must articulate their needs to their biomarker researcher counterparts. Risk assessors' requirements from biomarkers hinge on the two issues introduced earlier; improving on the selection of exposure and response metrics for the dose-response analysis and validating the proposed new biomarker. Ward and Henderson[24] have suggested that the greatest need for application of biomarkers to the risk assessment process is more mechanistic research on the early stages of disease development and offer that one cannot have a marker of biologically effective dose for a chemically induced disease if one does not know the mechanism of the induction. Intrinsic to this is the idea that more complete models must be developed in which the markers and mechanisms of chemical-induced disease are elaborated. With validated models, studies can be conducted to determine whether the proposed biomarker is a potential confounder or is causally linked to disease outcome. Once linked, the biomarker or suite of biomarkers can be used in improving the risk assessment process.

Biomarker validation seeks to establish links between the 21 proposed relationships[24] among markers along the continuum. The ability to characterize these relationships is dependent upon the degree of mechanistic knowledge, whereas the importance of assessing each relationship will vary depending upon the priorities and objectives and/or the application of the dose-response assessment. Exploring the link between any of 21 possibilities requires an experimental model that encompasses the continuum. Although laborious, time consuming and otherwise costly, the National Toxicology Program (NTP) chronic cancer bioassay, and variations thereof, offers possibilities along these lines. While the NTP chronic bioassay is primarily a tool for hazard identification, the potential exists to modify studies in order to track and validate biomarkers. However, because little is known about the mechanisms of toxicity of chemicals prior to testing, it is difficult to select appropriate biomarkers for evaluation. Nonetheless, NTP chronic bioassays do often include endpoints such as genotoxicity, sperm morphology/vaginal cytology, and clinical pathology and hematology, which can contribute to understanding of biomarkers. Interestingly, the NTP recently classified ethylene oxide as a *Known Human Carcinogen*[26] based largely on the consistency between the animal and human genotoxicity measurements. Since the NTP solicits nominations[27] for testing in addition to com-

ments on chemicals selected for testing, there is an opportunity for interested parties to propose additions or modifications to proposed testing. For example, 8-hydroxydeoxyguanosine, the commonly used biomarker of oxidative DNA damage, has been investigated in at least one NTP bioassay in an attempt to understand mechanisms[28]. More recently, the Occupational Safety and Health Administration (OSHA) and the National Institutes for Occupational Safety and Health (NIOSH) nominated 1-bromopropane for chronic testing and proposed that toxicological assessment include exposure and health-effects assessment in humans[29]. This presents a unique opportunity to coordinate biomarker evaluation into the experimental design of experimental animal and human field studies. In addition, the National Institute for Environmental Health Sciences (NIEHS), an NTP participating organization, has developed the ToxChip[30] and it is anticipated that future NTP testing strategies will incorporate this new DNA array technology to some degree.

Occupational studies offer an additional means for validation. However, legitimate ethical and legal issues inhibit employers from participating in important studies. This is unfortunate since occupational settings provide prime opportunities to most easily validate markers. Pertinent issues include how to explain the results to participants and the legal ramifications that may result for the employers[24]. Several committees have been formed in recent years, such as the National Bioethics Advisory Committee, to provide guidance in the areas of human subject research and may help to solve some of the ethical, legal and social issues around human research. This guidance may make it more palatable for companies to participate in occupational health studies.

In vitro studies can often provide insight, but not definitive conclusions on a compounds mode of action and potential for human toxicity. However, it is possible that the development of reliable and relevant *in vitro*[31,32] tests/assays may increase the usefulness of such data for assessing human risk in the future and minimize the need for live animal testing[33].

Challenges

Perhaps one of the greatest challenges will be the incorporation of host susceptibility factors that result in increased or decreased response in risk assessments. Non-heritable factors may be related to diet, health

status, pregnancy or other exposures. Molecular epidemiological studies have made great strides in elucidating the gene-environment interactions that might exist with regards to inherited susceptibility. Heritable or genetic susceptibility markers that have been widely studied are those that deal with metabolism of xenobiotics. One example is the role of N-acetyltransferase activity and bladder cancer. Extensive literature is available on the association of slow acetylator phenotype and increased risk of bladder cancer particularly after occupational exposure to specific aromatic amines[34–36]. How might susceptibility markers be used in risk assessment? Identification of a subset of the population that might have greater risk is possible. In 1995, Bois *et al.*[37] developed a model to evaluate human interindividual variability in metabolism and risk using 4-aminobiphenyl (4-ABP). The model demonstrated that the formation of 4-ABP-DNA adducts in the most sensitive individual would be approximately ten thousand times greater than the least sensitive individual. A current challenge is to further utilize biomarkers of susceptibility for risk assessments. This is particularly true for consideration of multiple gene-gene interactions that impact on toxicant responses. Incorporation of susceptibility markers can be exceedingly complex, since in many cases it is not one gene that affects the progress of the disease, but rather a cascade of genes often times with competing effects that alter the progress of the disease.

Determination of whether a susceptibility gene contributes to the disease outcome is necessary so that risk can be put in context. In risk calculations, the actual impact that genetic factors have is minor compared to environmental exposure[38,39]. The attributable proportion of risk for a genetic factor, such as slow acetylator phenotype is only 25% if looking at a two-fold association compared to 50% attributable proportion due to the environmental exposure to arylamines. Nevertheless, genetic susceptibility factors may be particularly valuable for their role in explaining or accounting for the large variability seen with human data and thus will aid in the refinement of the risk assessments – for example by serving as the basis for data-derived uncertainty factors[40].

Impediments

Some general, programmatic obstacles are also present that inhibit the progress of employment of biomarkers in risk assessment. Groopman

and Kensler[22] and Perera[2] have identified several infrastuctural impediments that will impact upon the use of biomarkers in risk assessment. They are: a lack of interdisciplinary research training, a lack of infrastructural foundation and support for large-scale collaborative studies and a lack of infrastructure to aid in the translation of biomarker and risk assessment findings to populations and communities. These are all necessary to develop partnerships between scientists and policymakers and other stakeholders to interpret and apply biological marker data to existing and emerging public health problems. Lastly, addressing the ethical, legal, and social issues surrounding biomarker research in particular genetic marker research used in risk assessments is of particular importance.

BIOMARKERS FOR THE 21ST CENTURY – THE IMMEDIATE FUTURE

Advances in both genomics and proteomics are likely to have an important impact in biomarker research. These tools will perhaps be most important in the identification of novel biomarkers of effect and susceptibility. The potential applications of genomics in the field of toxicology have been described in recent reviews[41–43]. The search for novel cellular responses to toxicants at the gene expression level is not new. For example, mRNA differential display techniques have been used to identify gene expression targets of environmental toxicants that induce noncancer toxicity through mechanisms such as endocrine disruption[44–46] and oxidative stress[47,48].

Differential display yields important information on gene expression, particularly in combination with the polymerase chain reaction. However, cDNA microarray technology has greatly expanded the potential for identifying gene expression changes induced by noncancer toxicants, and microarray data have been published for several environmentally important toxicants[49–51]. While this technology has the sensitivity to detect individual gene changes that might serve as novel biomarkers of effect, to date gene expression changes have not served as the critical endpoint in noncancer risk assessments. This is largely due to uncertainty surrounding the correlation between early gene expression changes and disease outcome. In short, gene expression changes typically have not been well validated. The use of microarrays may help to

resolve this shortcoming. The ability to look at the expression of thousands of genes at once opens the possibility for more fully characterizing clusters of genes that are activated (or inactivated) with exposure. By evaluating gene clusters, rather than single genes, the correlation of a novel effect biomarker with gene families known to be related to disease progression provides a level of validation not possible in single-gene experiments. Analysis of complex patterns of gene expression can also serve to identify molecular signatures representing effect biomarkers that maintain a high degree of specificity, consistent with the idea of using biomarker suites for risk assessment. Application of this technology to identify effect biomarkers for liver toxicants and xenoestrogens has been described recently[52]. Microarrays are also envisioned to identify later biological events in noncancer toxicity. Microarray analysis has been suggested as a tool to characterize "molecular fingerprints of disease processes" as has been suggested for renal disease[53] and the same logic could be applied for other noncancer endpoints.

Recent advances in proteomics will enhance the application of protein level changes as effect biomarkers for noncancer risk assessment. The potential capabilities of proteomics for toxicology applications have been discussed in recent reviews[43,54,55]. This approach has been used for an array of diverse toxicants associated with environmental exposure. Identification of changes in protein expression through two-dimensional gel electrophoresis (2D-PAGE) has been conducted for biological responses for metals[56–59], TCDD[60], male reproductive toxicants[61,62], oxidative stresses[63,64], and respiratory sensitizers[65]. Comparison of results against 2D-PAGE databases[55,66,67] provides a means to identify novel protein level changes following toxicant exposure.

Similarly to gene expression, changes in protein expression have not seen widespread application in risk assessment, due to the absence of clear linkage to adverse effects. Advances in proteomics are overcoming this problem by identifying the functional consequences of altered gene expression[55]. The increased use of screening methods for altered protein function provide the link between altered gene expression, protein levels, and resultant functional changes in cellular activity of the impacted proteins[68]. For example, coupling protein separation with detection of post-translational modifications such as phosphorylation[69] or alkylation or oxidation of cysteine groups[70] has been reported. Screening for functional enzyme activity has also been reported. One example is the detec-

tion of proteins with serine hydrolase activity[71]. As an example of the recent use of proteomics approaches, the identification of early biomarkers of effect for urinary biomarkers of cadmium toxicity[72], and markers of kidney and liver toxicity[73-75] has been reported. Strategies to assess the relatedness of protein changes and cancer endpoints have been explored extensively in the search for targets for therapy, and this approach is applicable for noncancer endpoints as well. For example, the relatedness of protein changes and disease can be established by comparing protein expression in normal versus diseased tissue and can be further validated by gain or loss of function analysis[54].

While microarrays have applications for identification of biomarkers of effect, this technology is also being used to identify novel biomarkers of susceptibility. cDNA microarray technology is sufficiently sensitive to detect single nucleotide differences, and thus has been useful for identifying genetic polymorphisms[76]. As described above, a current major area of emphasis is on identification of genetic polymorphisms that alter susceptibility to environmental pollutants and this idea has served as the basis for the Environmental Genome Project coordinated by the NIEHS[77]. Thus, microarray techniques will play an important role in the identification of putative biomarkers of susceptibility[41,42]. Application of proteomics for the identification of susceptibility biomarkers is also likely to increase. For example, cytochrome p450 protein profiling has recently been described[54]. In the case of genetic susceptibility markers, microarrays can identify, but not validate, biomarkers of susceptibility. These markers can only be used in the risk assessment process after the functional consequences of the genetic change are quantified through other techniques, such as molecular epidemiology or mathematical modeling approaches as described below.

The continued evolution of molecular biology techniques coupled with quantitative modeling approaches including physiologically based pharmacokinetic modeling (PBPK) and biologically based dose-response modeling (BBDR) will likely catalyze the use of cellular and molecular biomarkers of exposure, effect, and susceptibility into the risk assessment process.

PBPK modeling provides a mathematical description of the disposition of a toxicant in the body. As such, PBPK modeling facilitates the use of alternative biomarkers for external dose in the risk assessment process by relating measures of external exposure to measures of biologically effective dose. Several recent examples highlight this point. In

a risk assessment for methyl mercury, a PBPK model was used to estimate oral daily intakes associated with mercury levels in hair, which served as the dose metric in the dose-response analysis of neurological endpoints in children exposed *in utero*[78,79]. The application of a PBPK model to estimate daily intakes of cadmium that result in urinary cadmium levels associated with increased kidney toxicity (proteinuria) has also been discussed[80]. Once the biological basis for an alternative biomarker of exposure is determined, PBPK models can be used to estimate the value of appropriate internal dose metrics. For example, a PBPK model was used to estimate potential dose metrics for trichloroethylene relevant to its noncancer toxicity[81].

PBPK modeling is just beginning to be applied as a tool for quantifying the impact of biomarkers of susceptibility on toxic endpoints. This application has been of particular interest for polymorphism of genes that encode toxicant metabolizing enzymes, and would thus he expected to impact the target dose. Renwick and Lazarus discussed the potential importance of genetic polymorphism as modifiers of toxicokinetic variability in risk assessment[40]. An advantage of the application of PBPK modeling for identification of exposure markers, is the ability to conduct sensitivity analyses to determine the impact of selected parameter values on the tissue dose of the active moiety. Thus, the degree of modification engendered hy a given biomarker of susceptibility can be quantified. The importance of polymorphisms of genes that metabolize ethanol[82] and dichloromethane has been evaluated through this approach[83].

While PBPK modeling identifies alternative biomarkers of exposure, BBDR modeling is useful for interpreting the significance of changes in early biological effect markers. As discussed above, genomics and proteomics are useful tools for identification of early biological effect markers following toxicant exposure. BBDR models provide mathematical predictions of the biological events that lead to the critical toxicological end point, and as such provides a quantitative link between early events and more clearly adverse changes. Thus, BBDR models provide a tool for incorporation of early effect markers in the risk assessment. Recent examples include modeling of cell proliferation events in response to vinyl acetate[84], formaldehyde[85] and chloroform[86]. In addition to cell proliferation responses, efforts to develop BBDR models for developmental toxicity[87] and neurotoxicity[88] are underway.

SUMMARY

The application of biomarkers of exposure, effect, and susceptibility has the potential to greatly reduce uncertainty in the risk assessment process, leading to an increasing call to incorporate biomarkers into noncancer risk assessments. However, to date biomarkers have seen limited application, due largely to the lack of adequate validation. Recent advances in molecular biology and application of mathematical modeling techniques provide the opportunity to resolve some of these impediments for the use of biomarkers. The fields of genomics and proteomics will continue to play an important part in the identification of novel effect and susceptibility biomarkers and provide better linkage between early biological effects and disease. Mathematical modeling approaches are likely to be a bridge serving to integrate new biomarker identification with the quantitation of its impact on the dose-response analyses. PBPK modeling identifies alternative markers for external dose and is beginning to be used to explore the impact of genetic polymorphisms on tissue dose. BBDR modeling will be increasingly used to integrate information on the relationship between early biomarkers of effect and later stages in the continuum to clinical disease.

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