Use of Biomarkers to Investigate Occupational and Environmental Lung Disorders*

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Biological markers are potentially useful to investigate occupational lung disorders because they allow more detail than classic approaches in assessing various aspects of exposure-disease associations. Historically, in epidemiologic research, the link between exposure and disease was often made without knowing the mechanism or intervening events. This is the strength of the epidemiologic approach, and it has led to much of the knowledge about lung disorders related to cigarette smoking and common occupational exposures such as silica, asbestos, and coal dust. However, such strong and specific associations are becoming less of a concern because for the most part, they have been identified and controlled. Attention now is to lower levels of exposures, mixtures, nonspecific respiratory disorders, and effective clinical and public health management of populations at high risk. To address many of the issues, it is important that mechanisms of action between exposures and diseases be identified. The relative inability to detect early signs of environmentally related adverse effects has prompted much interest in using biochemical, molecular, and pathologic changes as indicators for respiratory diseases.

Biological markers are not new to pulmonary scientists and clinicians. Pulmonary function tests are classic examples of biological markers that have been used in this field for years. The history of pulmonary function testing reveals the same type of factors that need attention with the current generation of molecular biological markers, namely the following: addressing issues of acceptability, simplicity, objectivity, reproducibility, accuracy, sensitivity, and specificity; and understanding the variability, confounding factors, and prevalence. Early pulmonary function tests were plagued by issues of lack of standardization, unreliability, and inaccuracy. Once tests and equipment such as spirometers had been developed in the laboratory, they still had to be validated for population use, that is, standardized by age, race, sex, smoking, and medical and physiologic condition. This took decades to occur. Thus, we should not be surprised that the current investigations of molecular biomarkers that are generally only in their first decade have yielded a lot of incomprehensible information on wide-ranging interpersonal variability. The most recent effort to use biological markers, dubbed “molecular epidemiology,” is only in the development stage and much of the same type of effort that went into validating pulmonary function tests must occur with molecular markers.

Despite the early stage of development of contemporary biomarkers, there are still informative examples of how they can contribute to the study of occupational and environmental lung disorders. The use of biomarkers to assess lung disorders has to be based on the realization that these are just another set of tools to be included in the array of tools available to researchers and clinicians, such as exposure monitoring, pulmonary function tests, exposure reconstructions, questionnaires, physical examinations, and diagnostic evaluations. Too often, the nonspecific question is asked, “Can you recommend any good biomarkers?” This is nonsensical because it fails to ask the specific questions: Good for what research question, what type of study design, in what populations?

A framework for assessing and describing biological markers has been recently developed. It is a two-dimensional matrix (Fig 1). It shows on one axis (abscissa) the types of biological markers and on the other axis (ordinate) the type of study designs and uses. The type of markers on the abscissa reflect the paradigm put forth in 1987 by the National Academy of Sciences (NAS) building on the work of Perera and Weinstein, which essentially categorized biomarkers into exposure, effect, and susceptibility. These have been widely discussed in general, and in 1989, specifically, with regard to pulmonary disease. The NAS publication Biological Markers in Pulmonary Toxicology presented an inventory of potential biomarkers under the following categories: exposure, physiologic effects in intact organisms, altered structure and function, inflammation, and immune response, and cellular and biochemical response. A further appraisal of biomarkers was presented in two other NAS publications Human Exposure Assessment for Airborne Pollutants and Environmental Epidemiology. Both of these publications stressed the need to utilize biomarkers as part of the array of tools available to assess environmental disease. More recently, work by Hulka and colleagues (1990), Biological Markers in Epidemiology, and by Schulte and Perera (1993), Molecular Epidemiology: Principles and Practices, extends the use of biomarkers to population studies. The issues that will confront investigators using the new generations of lung biomarkers have been identified previously with a more historic marker, the chest radiograph.

Wagner et al have provided a context for review of this and future markers.

The ordinate of the matrix shows the progression of study designs, including laboratory, transitional, etiologic, and public health applications. The laboratory studies involve both toxicologic and analytical methods to identify particular biological changes that can be represented by biological

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**Figure 1.** Matrix of biomarker combinations and study designs. From Journal of Toxicology and Environmental Health, 1993; 40:359-68; Schulte PA. Taylor & Francis, Inc., Washington, D.C. Reproduced with permission. All rights reserved.
markers and the assays to measure these. Transitional studies use biomarkers as outcome variables in studies of generally healthy people. This is where markers are taken from the laboratory and tested in field settings to determine correspondence with exposure, disease, or susceptibility. Through these studies, researchers also focus on assessing variability, practicality, reproducibility, and prevalence and identify confounding factors. Only when a marker is validated in terms of its characteristics in populations, as well as in the laboratory sense with regard to the validity of the assay, can it be ready for use as an independent or dependent variable in etiologic studies and public health applications. These validation efforts require interdisciplinary collaboration. The matrix of marker types and study designs is a useful device to foster communication between investigators from different disciplines. The validation of biomarkers includes addressing not only the extent to which the biomarker is related to the event it represents, but also the pharmacokinetics, temporal relevance, background variability, confounding factors, and prevalence in various population subgroups defined by age, race, sex, and other relevant factors.

Validation and ultimate use of biomarkers can be enhanced by taking advantage of stored biological specimens. Appropriately stored biological specimens with properly collected descriptive and covariate information are an efficient and powerful resource that can allow for validating markers and testing hypotheses. The issues pertaining to specimen banking have been clearly described in the proceedings from a recent conference (Lee, 1995) and by investigators experienced in banking and using biological specimens.

Although the matrix shows a progression of marker studies from laboratory to public health application, the process is iterative. Hence, an epidemiologic or clinical observation or need (such as for a better indicator of exposure or identification of important host factors) can trigger laboratory development, which can then be tested in the field, refined in the laboratory, and so forth. Similarly, a finding in the laboratory may be tested in the field to inform the laboratory of population characteristics of a biomarker.

SELECTIVE EXAMPLES OF THE USE OF BIOMARKERS IN THE STUDY OF MALIGNANT AND NONMALIGNANT LUNG DISEASES

Malignant Lung Diseases

Although there are still important unanswered questions (eg, such as the role of air pollution), many of the major causes of lung cancer have been identified. What role can biomarkers play now and in the future? The critical issue with lung cancer is that survival is low and detection occurs at a biologically "late" time. Thus, of the 150,000 new cases per year, about 70% have already metastasized to regional nodes or distant sites at first evidence of disease. Tockman and colleagues have addressed questions of how to identify biologically early lung cancer. This involves biomarker identification and selective validation against acknowledged disease end points, quantitative criteria for marker presence or absence, and biomarker confirmation in population specimens. With regard to markers of early neoplasms, the markers that capture initiation or transformation and progression are likely to be the most useful. The best studied series of markers for any cancer is in the progression of normal colonic epithelium to invasive metastatic colon carcinoma. The accumulation of events in this progression may be as important as the actual order. It is also likely that at least some of these events are not specific, but merely adaptive, and hence, are epiphenomena. An important priority is to distinguish these epiphenomena from those events that are critical to disease progression. To date and to my knowledge, not even a rough temporal sequence of events in the developmental process of lung cancer has been identified. However, there has been identification of possible components of a sequence. These include various changes in the following: growth factors and receptors (epidermal growth factor receptor, involucrin, retinoic acid receptors, gastric releasing peptide, and autocrine growth factors); tumor suppressor genes (3p14-23, 11p15, 11p13-11q13 [Wilms tumor gene]), retinoblastoma locus] 17p13 [p53 locus], 18q21.3); oncogenes (myc family, ras family, raf, myb, fos, Her-2/neu, jun, fms); cytogenetic changes; differentiation markers; and specific tumor products. The role of biomarkers of exposure in lung cancer can be their use to document generally recent exposure and also to integrate both exposure and host factors to indicate a biologically relevant or effective dose. Markers of biologically effective dose are more mechanistically relevant to carcinogenesis than markers of internal dose, but pose more challenging analytical problems. Consider carcinogen-DNA adducts. The biological basis for measuring DNA adducts derives from extensive experimental data supporting their role in the initiation and possibly progression of cancer. Still, questions remain about the quantitative relationship between adduct formation (as a necessary but insufficient event) and cancer risk. Other questions remain as to which of many adducts on DNA are critical for cancer and what accounts for the wide interindividual variability of adduct levels observed. Moreover, with an organ such as the lung, which is generally inaccessible for biomonitoring, can surrogate tissues, such as peripheral blood cells be used? Experimental studies with benzo[a]pyrene suggest that comparable levels of adducts are formed in WBCs and other tissues, and comparisons of these are now underway in lung cancer patients and controls. Polycyclic aromatic hydrocarbon-DNA adducts also have been found at greater levels in lymphocyte DNA of smokers vs nonsmokers and in various occupational groups at risk for lung cancer such as foundry workers, roofers, and coke oven workers. The ultimate question with DNA adducts has been the extent to which they predict cancer.

Tang et al (1995) evaluated the roles of PAH-DNA adducts and a polymorphism of glutathione-S-transferase (GSTM1), a susceptibility marker in a case-control study of non-small cell lung cancer. The GSTM1 null genotype (0/0) is associated both with decreased ability to detoxify PAHs and other carcinogens and with increased risk of lung cancer. Leukocytes from 119 patients with cancer (cases) and from 98 controls without cancer were analyzed for PAH-DNA adducts and GSTM1 genotype. After adjustment for potential confounders (age, smoking, etc), adduct concentrations were higher in cases (p<0.01) than in controls. Adduct levels also were increased in smokers and ex-smokers.
than in nonsmokers among both cases and controls (p<0.05). Fifty-seven percent of cases had the GSTM1 0/0 genotype compared with 41% of controls (p<0.05). After adjusting for confounders, odds ratios for adducts (dichotomized) and GSTM1 genotype were 6.8 (1.6 to 29.6, p<0.01) and 1.93 (1.1 to 3.4, p<0.05), respectively. When the subjects were classified by adduct levels (high/low) and GSTM1 genotype (0/0 vs 0/+ or +/+), the risk was 12-fold higher for those individuals with both high adduct levels and GSTM1 0/0, compared with those without either factor. This study shows that DNA adducts are not only markers of exposure but that they also may have the potential to be indicative of cancer risk, on a group basis, especially when combined with appropriate susceptibility markers (effect modifiers) such as GSTM1. Other susceptibility markers, such as CYP2D6 and DNA repair enzyme polymorphisms, also may be involved in lung carcinogenesis.1,2,4

Nonmalignant Lung Diseases

Born et al2,25 have described how the framework for biomarkers can be used in the study of nonmalignant respiratory disease. Lung disorders can be seen as a sequence of initiation, amplification, and resolution (or repair). The use of biomarkers in epidemiologic research of lung disorders can help to clarify the mechanism. Born and colleagues2,25 examined the role of antioxidant enzymes and tumor necrosis factor (TNF) and serum type III procollagen peptide (PIIIP) in studies of coal dust-induced lung disorders. These studies had to overcome the limitations of accessibility of lung material by using indirect disease confirmation and surrogate tissues. In a case-control study of antioxidant enzymes, cases of coal workers pneumoconiosis (CWP) were determined by clinical examination and radiographic analysis. Three groups of cases, based on the International Labor Organization radiographic classification, and a control group were selected. Because the pulmonary macrophages, the source of antioxidant enzymes, are not readily accessible, RBCs were selected as surrogate tissue for various reasons, including their ability to penetrate lung capillaries and play a role in lung damage due to reactive oxygen species. Glutathione levels were decreased in early-stage CWP but increased with progression to progressive massive fibrosis. The study showed that changes in antioxidant enzyme expression levels in blood reflect the elevated levels of reactive oxygen species in lungs of individuals with CWP. These levels in both tissues increased with the severity of CWP and highest in individuals with progressive massive fibrosis. Born2 concluded that antioxidant enzyme expression is related to early inflammatory response to mineral dust exposure and was not a predictive parameter for individual susceptibility.

In another case-control study, TNF was studied as the potential risk factor.25 Again the impracticability of measuring TNF release in lungs is addressed by looking at another inflammatory cell available from blood samples—the peripheral blood monocyte. To obtain release of TNF, blood monocytes were isolated and subjected to stimulation with different soluble and particle stimulators, such as an Escherichia coli lipopolysaccharide. silica, and coal mine dust. TNF release was highest in monocytes of subjects with CWP compared with dust-exposed and nonexposed controls. Born and colleagues2,25 suggested that TNF release was a marker of individual susceptibility to dust-induced lung fibrosis, but definitive proof must come from a prospective cohort study. A subsequent longitudinal study of coal miners is underway, and TNF appears to be a marker for progression of coal dust-induced lung fibrosis.

In a third study, Schins and Born26 examined whether PIIIP was a useful marker to predict development, outcome, or activity of fibrotic lung disease.26 This study was based on the finding that the formation and deposition of collagen fibrils was involved in pulmonary fibrosis. A 5-year prospective study was conducted, involving miners studied in 1987 and again in 1992. At each time, blood samples were collected and stored. A group of age-matched non-dust-exposed controls were also followed. Miners were categorized by chest radiographs and divided into six groups based on International Labor Organization scores. Mean PIIIP levels of miners without radiologic evidence of CWP and those of miners in groups 1 (1/0, 1/1, 1/2) and 2 (2/1, 2/2, 2/3) and for the control group were significantly lower in 1992 than in 1987. Since PIIIP levels were not significantly increased in coal miners vs non-dust-exposed controls, and because there was no downward trend of PIIIP levels vs pneumoconiotic stage, the investigators concluded that PIIIP is not a marker of early effect. From these three examples, Born2 cautioned: “There is a tendency to measure any biomarker and correlate it with another biomarker, or with some disease outcome, without full comprehension of the total system. Thus, although there may be many candidate biomarkers, carefully designed follow-up studies are a prerequisite to test the validity and use of events too often put forward as biomarkers.”

CONCLUSION

If biomarkers are to be a useful addition to the array of tools for investigating occupational and environmental lung disorders, they have to be shown to be valid and practical. This requires interdisciplinary collaboration and appropriate administrative and financial support. Interdisciplinary collaboration is often difficult owing to the failure to have common language and frame of reference for research. The matrix of biomarkers and study designs can overcome this problem. Too often, funding for validation studies, particularly epidemiologic validation, is not a high priority of funding agencies. Addressing this issue is more difficult and will require pressure or funding sources from clinicians, epidemiologists, industrial hygienists, and exposure assessors as well as workers and employers.

Finally, there are many ethical, legal and social issues that arise with the use of biomarkers. These issues need to be considered prior to conducting studies to avoid problems.10,11 The potential power of combining biological markers with other investigative tools is yet to be realized for occupational and environmental disorders, but the initial efforts appear promising. If an integrated approach is taken by investigators from various disciplines, further progress toward this goal should occur.
Inactivation of Neutral Endopeptidase in Lung Cancer*

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The enzyme neutral endopeptidase (NEP) hydrolyses small bioactive peptides, which are growth factors for normal airway epithelial cells and lung cancer. Therefore, low NEP expression could increase local concentrations of neuropeptides and promote lung tumor growth. We have found considerable (10-fold) interindividual variation in lung NEP levels. By immunohistochemistry, NEP expression is detectable in alveolar and airway epithelium and in fibroblasts of normal lung. We have previously shown that NEP protein expression is low or absent in small cell lung carcinoma (SCLC) and in some adenocarcinoma cell lines by fluorescent-activated cell sorter, enzyme-linked immunosorbent assay (ELISA), Western blot, and activity assay. Furthermore, NEP expression by immunohistochemistry, ELISA, Western blot, and activity assay is significantly lower in lung tumors than in adjacent uninvolved lung from the same patient. Finally, we have demonstrated that inhibition of NEP modulates calcium flux by bioactive peptides in lung cancer cell lines. Thus, NEP may act as a tumor suppressor. In order to investigate the currently unknown mechanisms of NEP inactivation, we assayed NEP messenger RNA (mRNA) from SCLC and non-SCLC cell lines as well as primary bronchial epithelial cells. Due to low mRNA abundance, a nested reverse transcription polymerase chain reaction (RT-PCR) strategy was developed resulting in 3 PCR products per cell line. The PCR products were graded as normal, abnormal (one or two bands) or an abnormal-sized band, or absent (all three products absent). Normalized PCR products were observed in 12 of 28 cell lines studied: 6 of 15 SCLC, 2 of 5 adenocarcinoma, 2 of 4 large cell, and in 1 of 1 mesothelium, 0 of 1 carcinoid, as well as in 1 of 2 primary bronchial epithelial cells. Of the remaining 16 cell lines, 8 had absent PCR products and 8 lines had abnormal bands. Undetectable mRNA was found in the majority of SCLC lines. Sequence analysis of four of these abnormal PCR products demonstrated amplification of a non-NEP transcript with partial homology to the primers used for PCR; this is presumably due to low or absent NEP mRNA. The remaining abnormal products are being se-

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