Protein Adduct Biomarkers: State of the Art

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Covalent protein adducts formed after exposure to xenobiotics may provide readily measurable indicators of these exposures. After adequate characterization of the dose-dependent formation of a specific adduct, the adduct can often be used as a quantitative marker for exposure, DNA adduct formation, or, possibly, risk of disease. By elucidating the structure of an adduct and studying the conditions under which it forms, information about the reactions that lead to its formation can be obtained. Continuing work in this area includes methods to expand the number, types, and levels of chemical exposures that can be studied by covalent adduct formation. In addition to the use of this technology in the field of occupational health, basic research in this area provides insights into metabolic pathways and biochemistry, as well. — Environ Health Perspect 104(Suppl 5):879–882 (1996)

Key words: protein adducts, biomarkers, dosimetry, hemoglobin, serum albumin

Introduction

A number of human diseases may result, at least in part, from exposure to chemicals. A dose–response relationship between disease and chemical exposure, however, can be difficult to establish. Although the occurrence of disease can be recorded, humans are typically exposed to low levels of chemicals present in complex mixtures over extended periods. Lacking objective measures of exposure, epidemiologists have been required to estimate exposure to chemicals using surrogate measures such as questionnaire data on lifestyle factors, past occupations, or medication.

Considerable research has been devoted to developing biomarkers that function as measures of dose, including urinary metabolites or blood levels of parent compounds or their metabolites. Recent innovations allow the detection of covalent interactions of xenobiotics with proteins and other macromolecules, providing a method to accurately measure exposure or acting as a surrogate marker for dose at the target tissue. Blood, for example, contains large amounts of hemoglobin (Hb) and serum albumin, which have reactive carboxyl, amino, and sulfhydryl groups that can interact with electrophilic compounds. Adducts formed with these proteins are often relatively long lived in the body (compared to the biological half-life of the exposure compound or its unbound metabolites). The lifespan of Hb in human red blood cells, for example, is approximately 4 months, allowing assessment of cumulative doses from exposures during this time. Because many of the reactive metabolites of organic compounds that form protein adducts also bind DNA, some protein adducts can serve as markers of DNA binding or damage as well.

In this review we discuss recently reported uses of Hb and serum albumin adducts for determining exposure, for estimating genetic damage and disease risk, and studying reactions and metabolic pathways. Some analytical methods that have been used in these studies are also included.

Estimation of Exposure, DNA Adduct Levels, and Risk of Disease

Traditionally, blood protein adduct analysis has been used to assess and estimate the dose associated with exposures to xenobiotics. The potential effects of these exposures have also been studied using adducts. Several recently reported examples of these applications are discussed below.

Occupational exposure measurements continue to be the focus of much of the latest protein adduct work. Autrup et al. (*1*), for example, measured the exposure of animal feed production workers to the liver carcinogen aflatoxin B_1 (AFB_1) using serum albumin adducts. This group demonstrated the formation of AFB_1 adducts with a detection limit of 5 pg/mg serum albumin using an enzyme-linked immunosorbent assay (ELISA). Other researchers have measured the exposure of workers to 2,4-difluoroaniline (*2*) and to ethylene oxide using Hb adducts (*3*). Goergens et al. (*4*) monitored fumigators for exposure to methyl bromide using methylated cysteine from Hb and serum albumin. This study detected exposure in workers despite their use of respiratory protection during fumigation operations.

Exposure to several dietary carcinogens has also been the subject of studies and monitoring method development. Many recent studies have focused on heterocyclic amines, some of which form adducts that may serve as acceptable biomarkers. In an assessment of human exposure to 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, Umemoto et al. (*5*) detected Hb adducts formed from this material in human blood samples. Other researchers, however, have reported analytical methods for measurement of heterocyclic amine adducts that appear to be unsuitable for exposure assessment. Lynch et al. (*6*) examined the use of the serum albumin adducts of the food-borne heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline and were unable to detect this adduct in the blood of human volunteers. They attributed this to low levels of formation of the compound as well as to limited recovery in the analytical process.

The measurement of protein adducts can be quantitative if dose-dependent formation has been demonstrated, as is the case for a variety of adduct-forming compounds, including 4,4′-methylenedianiline (*7*) in rats and 7*H*-dibenz[*c*,*g*]carbazole in mice (*8*). In humans, Vineis et al. (*9*) has shown that 4-aminobiphenyl–hemoglobin (4ABP–Hb) adduct levels in smokers reflect the number of cigarettes smoked. Walker et al. (*10*), however, found that high levels of ethylene oxide exposure caused disturbances in erythropoeisis and erythrocyte

This paper was presented at the Conference on Air Toxics: Biomarkers in Environmental Applications held 27–28 April 1995 in Houston, Texas. Manuscript received 24 May 1996; manuscript accepted 5 June 1996.

This review was prepared with the support of the Office of Health and Environmental Research, U.S. Department of Energy, under Contract No. DE-AC04-76EV01013.

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Abbreviations used: Hb, hemoglobin; AFB_1, aflatoxin B_1; ELISA, enzyme-linked immunosorbent assay; 4ABP–Hb, 4-aminobiphenyl–hemoglobin; BaP, benzo[*a*]pyrene; BaPDE, benzo[*a*]pyrene anti-diol epoxide; PAHs, polycyclic aromatic hydrocarbons; HPLC, high pressure liquid chromatography; MS, mass spectrometry.

lifespan in rodents. This limits the ability to relate exposure to Hb adduct levels at high concentrations of this material.

The use of blood protein adduct levels as indicators of DNA adduct levels has been reported since the 1970s (*11,12*) and continues to be an active area of research. Recent work has sought to establish quantitative relationships between protein adduct levels and DNA adduct or damage levels. Umemoto et al. (5) demonstrated a correlation between Hb and liver DNA adducts of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole in rats and suggested the use of this adduct for monitoring in humans (Figure 1). The relationships between protein and DNA adducts of 7*H*-dibenzo[*c,g*]carbazole in mice were studied by Meier and Warshawsky (*8*). Liver DNA, compared to other tissues studied, preferentially binds this compound and was shown to form adducts in a manner that was proportional to dibenzo[*c,g*]carbazole–Hb adduct formation. Anwar et al. (*13*) demonstrated a relationship between levels of serum albumin–AFB_1 adduct levels and indicators of cytogenetic damage (micronuclei per 500 polychromatic erythrocytes per animal and chromosomal aberrations per 50 metaphases per animal) in bone marrow cells of rats treated with this compound. In this study, protein adducts appeared to provide a much more sensitive marker of exposure than did cytogenetic changes.

Efforts have also been made to correlate protein adduct levels and risk of disease, assuming the existence of the adduct provides an indication of this risk. In a comparison across three ethnic groups, Yu et al. (*14*) demonstrated a correlation between acetylator phenotype, which could be determined by ABP–Hb adduct levels, and bladder cancer incidence. In a study of Hb adducts of acrylamide in exposed workers at a chemical factory and the signs and symptoms of the peripheral neuropathy that can result from exposure to this material, Calleman et al. (*15*) found a correlation between exposure and disease. In contrast, a comparison of lung cancer patients and controls by Weston et al. (*16*) demonstrated that 4ABP–Hb adduct levels do not correspond to lung cancer incidence.

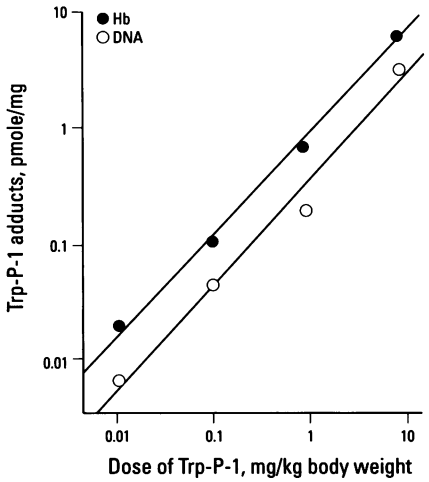

Figure 1. Correlation between hemoglobin and liver DNA adducts in rats exposed to 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole. From Umemoto et al. (*5*).

Studies of Metabolism

In addition to their use as markers of exposure, surrogate markers for DNA adduct formation, and indicators of potential disease risk, certain protein adducts may provide information about metabolism. The presence of an adduct may suggest the formation of a reactive intermediate. Bryant et al. (*17*), for example, demonstrated the formation of the *N*-hydroxy derivative of 2,6-dimethylaniline by measuring the Hb adduct of this compound from lidocaine-treated patients. This derivative may indicate the presence of the reactive intermediate responsible for the reported mutagenicity of 2,6-dimethylaniline in animals, a major metabolite of this anesthetic. Zwirner-Baier and Neumann (*18*) used the Hb adducts of Direct Red 46 and Pigment Yellow 17 to study the metabolic fate of azo dyes in Wistar rats. Because of the insolubility and low bioavailability of Pigment Yellow 17, it was thought that this material posed little risk as a carcinogen. Although found at low levels after chronic administration, hydrolyzable Hb adducts, which suggest the generation of reactive intermediates, were formed from both of these compounds.

Protein adduct measurement methods have been used to study specific reactions, as well. The P4501A2-catalyzed reaction responsible for the metabolically activating *N*-oxidation of 4ABP has been assessed using Hb adducts, which arise from the *N*-hydroxy form of this compound. In a study by Hammons et al. (*19*), the P4501A2 inhibitor 2-ethylnaphthalene caused a dose-dependent decrease in 4ABP–Hb adducts in rats. Methimidazole, an inhibitor of flavin monooxygenase, did not affect adduct levels. Vineis et al. (*9*) used levels of 4ABP–Hb adducts to identify genetically determined slow acetylators, which had higher levels of this adduct. This reflected the decreased levels of detoxifying *N*-acetylation in these individuals.

The structure of blood protein adducts may also provide structural information about the reactive species responsible for adduct formation. Day et al. (*20*) studied the chiral specificity of serum albumin-benzo[*a*]pyrene (B[*a*]P) adduct formation. B[*a*]P *anti*-diol epoxide (B[*a*]PDE) forms adducts through either carboxylic acid esters or through a histidine (imidazole) nitrogen in human serum albumin *in vitro* (*20,21*). This study demonstrated the binding of the less toxic (–) enantiomer to histidine(146) and the more carcinogenic and mutagenic (+) enantiomer to carboxyl groups of aspartate(187) or glutamate(188) on human serum albumin (*20*). Another study by Day et al. (*22*) confirmed the specific histidine nitrogen on human serum albumin through which these adducts form, as well as the B[*a*]PDE carbon involved.

Analytical Methods

Several analytical methods have been used for the detection and quantitation of covalent blood protein adducts. Adducts often must be adequately identified and characterized before their use in monitoring or in the course of studies of metabolism. Protein adducts may be analyzed intact, as peptide fragments, as modified amino acids, or as released parent compounds. The following examples of methods used to detect and measure adducts of AFB_1, polycyclic aromatic hydrocarbons (PAHs), and aromatic amines illustrate responses to differing requirements.

Analytical methods that are time and labor saving have often been used in studies that involve testing large numbers of individuals. Accordingly, immunoassays have been commonly used to measure AFB_1–serum albumin adducts in screening programs (*23*). Wild et al. (*24*) compared three methods for measuring these adducts. Enzymatic hydrolysis of serum albumin followed by ELISA was found to be better suited for large-scale screening than direct ELISA and high performance liquid chromatography (HPLC). They also found that HPLC/fluorescence resulted in fewer false positives and was useful if time and conditions allowed.

Many recently reported methods for the analysis of PAH adducts have been designed to study or characterize structure. This goal has been reflected in the analytical methods, which have often used relatively sophisticated analyses. Initial proteolytic cleavage of adducted proteins can

release adducts sequestered within hydrophobic regions of the protein or expose them to aqueous environments in which hydrolysis can take place. Shugart (*25*) used acid hydrolysis of B[*a*]P adducts to generate free tetrols, followed by HPLC/fluorescence. Day et al. (*20–22*) used a series of physicochemical methods to establish the amino acid and enantio-specificity of B[*a*]PDE binding. Included among these were fluorescence line narrowing spectroscopy and HPLC/fast atom bombardment mass spectrometry (MS).

Some aromatic amine adducts may be analyzed using similar methods. In recently reported studies of aromatic amine–Hb adducts, the adducting compound was removed before quantitation. Some aromatic amines form cysteine adducts with Hb that can be released with mild base or acid hydrolysis. Cheever et al. (*26*) used HPLC with a fluorescence detector to measure *o*-toluidine adducts isolated from both Hb and serum albumin in rats using base hydrolysis. Zwirner-Baier and Newmann (*18*) used alkaline conditions to isolate adducts followed by quantitation by HPLC with an electrochemical detector to study the bioavailabilty of the azo dye Pigment Yellow 17. Base hydrolysis was used in conjunction with GC–MS by Bailey et al. (*7*) to analyze rat Hb for 4,4-methylenedianiline and with GC/negative ion chemical ionization–MS in studies by Weston et al. (*16*) and Vineis et al. (*9*) to study 4ABP–Hb adducts in humans.

Conclusions

Work continues on the development of methods that allow for practical applications of protein adduct measurement, such as those designed to assess exposure to chemicals as well as the effects of these exposures. The ability to measure blood protein adducts also provides a method of studying complex metabolic processes. In addition to advancing technology associated with a variety of analytical methods, these studies generate valuable information about the exposure to and metabolism of xenobiotics. Only a sampling of recent protein adduct work has been discussed; numerous studies have been carried out to identify and quantify protein adducts, to improve analytical techniques, and to apply these improvements to new settings and chemicals.

The information obtained in these studies must, however, be interpreted carefully. As the measurement becomes farther removed from the event for which it serves as a surrogate, an understanding of the biological mechanisms responsible for the adduct formation becomes increasingly important. The qualities that make blood protein adducts useful as biomarkers, such as access and stability, also demonstrate the differences between the measured adducts and the events for which they serve as markers. Target tissue DNA, for example, may be somewhat less accessible by a chemical and is subject to repair mechanisms. A linear relationship between levels of DNA adducts and those formed with blood proteins may not exist. Assigning value to blood protein adducts for this purpose may be a highly empirical process.

Relationships between disease risk and blood protein adduct levels may be even more difficult to establish. In addition to factors such as distribution and DNA repair, the development of disease, especially cancer, is not a simple process. According to the multistage model of carcinogenesis, for example, several events must take place for the development of disease. As a marker of DNA adduct formation that may lead to mutation events, a protein adduct may only serve to provide a marker for one part of a complex process. Cautious interpretation of these data is often required.

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