DNA Damage as an Intermediate Biomarker in Intervention Studies (44166)

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Abstract. **The development of sensitive assays for measurement of DNA damage in humans has great potential for enhancing intervention studies. Methods for DNA adduct measurement include immunoassays, [^{32}P] postlabeling, high-performance liquid chromatography with fluorescence or electrochemical detection, and gas chromatography/mass spectroscopy. It is now well established that DNA adducts are a marker of exposure to various environmental, lifestyle, or occupational chemical carcinogens. Our own studies concentrate on immunologic detection of adducts by enzyme-linked immunosorbent assay (ELISA) of isolated DNA or quantitative immunohistochemical analysis of intact cells. Polycyclic aromatic hydrocarbon (PAH)–DNA adducts are elevated in blood cells of foundry and coke oven workers, individuals with high levels of exposure to environmental air pollution, and smokers. The study in smokers also found an inverse relationship between serum antioxidants and PAH-DNA, and is the basis for an ongoing antioxidant intervention. DNA adducts of PAH and 4-aminobiphenyl and oxidative DNA damage (8-oxo-deoxyguanosine) are being measured in blood mononuclear cells and exfoliated oral and bladder cells from subjects on antioxidants or placebo. Data on published intervention studies investigating oxidative damage and general aromatic DNA adducts measured by postlabeling are also summarized. These studies have already demonstrated that DNA adducts can be modulated by interventions and suggest that they can provide important mechanistic information in support of larger scale studies.** [P.S.E.B.M. 1997, Vol 216]

The use of biologic markers of exposure in cancer epidemiology has expanded dramatically over the past few years with the development of sensitive methods for measurement of carcinogens and their DNA and protein adducts in human samples. The various stages in the process of tumor development in which biomarkers can be used has been reviewed previously (1). This report will specifically discuss biomarkers measuring the biologically effective dose defined as the amount of carcinogen bound to DNA in the target tissue or a surrogate. This measurement takes into account individual differences in absorption of the chemical, its metabolism into DNA reactive forms, detoxification of reactive intermediates, as well as repair of DNA damage. Measurement of carcinogen adduct levels, as a marker of exposure to the compounds of interest, was carried out for occupational exposure to polycyclic aromatic hydrocarbons (PAH), styrene, and diesel exhaust, for dietary exposure to PAH and aflatoxin, for clinical exposure to PAH, psoralen, procarbazine, and cisplatin, and for lifestyle factors such as smoking (PAH, tobacco specific nitrosamines, and 4-aminobiphenyl) (reviewed in Refs. 2–5). Mean levels of adducts are generally elevated in subjects with exposure, but background levels are frequently observed in some apparently unexposed individuals. For PAH, the ubiquitous exposure of the general population though diet and air pollution as well as passive smoking is probably responsible. However, for other types of damage, such as malondialdehyde-DNA, alkylation adducts, and oxidative damage, endogenous production of reactive intermediates is probably responsible for the levels observed. Large interindividual variability in adduct levels in those with the same external exposure was also found. However, while it is now generally accepted that measurement of DNA adducts indicates exposure to the compound of interest, a lack of detectable adducts does not prove an individual was unexposed.

Another goal of biomarker measurements in humans is the determination of individual risk for cancer development.

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The demonstration that adducts are markers of risk requires an alternate type of study design than was used for the determination that adducts are markers of exposure. A case-control study nested in a prospective cohort using banked samples from individuals who are followed for cancer development is necessary to ensure that the marker is not influenced by the disease. The first two studies using this type of approach with adduct biomarkers are now published. Both investigated the relationship between liver cancer and exposure to hepatitis B virus and aflatoxin B_1, a dietary mold contaminant. One study, carried out in Shanghai, demonstrated elevated risk in subjects with detectable urinary levels of aflatoxin-guanine and/or aflatoxin metabolites (6, 7). A synergistic effect was observed in subjects with exposure to both the virus and aflatoxin. Our own studies, carried out in Taiwan, measured aflatoxin-albumin as a surrogate for DNA adducts and total aflatoxin metabolites in urine (8). Levels of aflatoxin were higher in banked samples from subjects who went on to develop liver cancer than in those who were disease free at the time of selection. A synergistic increase in liver cancer risk was again observed in those with exposure to both virus and carcinogen.

DNA adducts also have tremendous potential as intermediate biomarkers in intervention studies. DNA damage is well established as the initiating event in the process of carcinogenesis leading to mutations, translocations, amplifications, etc. While DNA adducts have not yet been proven to be indicators of individual cancer risk, they are elevated in populations at high risk for cancer development such as coke oven and foundry workers. Animal studies also suggest a good correlation between adducts and cancer (9, 10). Thus, DNA adducts meet the criteria suggested for biomarkers as end points in intervention studies: a strong association with the specific disease, an easily measured end point, and a high prevalence of the lesion in the study population (11, 12). DNA adducts have a well-established role in the carcinogenic process, are highly prevalent in the general population, and, as demonstrated below, can be relatively easily measured in humans.

Several different analytical methods have been developed for measuring the low levels of DNA damage found in humans and include immunoassays, [^{32}P] postlabeling, high-performance liquid chromatography with electrochemical or fluorescence detection, and gas chromatography/mass spectroscopy (GC/MS) (reviewed in Refs. 2 and 13–15). Each method has its specific advantages and limitations. Immunoassays require the development of antibodies to the particular adducts of interest. These antibodies frequently show cross-reactivity with structurally similar adducts, which limits their ability to give precise quantitative DNA adduct levels. However, once an antibody has been developed and characterized, large numbers of samples can be readily analyzed. Antibodies are frequently used in highly sensitive competitive enzyme-linked immunosorbent assays (ELISA) which have femtomole (10^{-15}) sensitivity. This allows the quantitation of adducts in isolated DNA with a sensitivity of about 1 adduct/10^8 nucleotides. As described below, the antibodies can also be used for quantitative immunohistochemical detection of damage in intact cells or tissue biopsies. The advantages of the immunohistochemical method include the requirement for small numbers of cells making it applicable to small blood samples, exfoliated oral and bladder cells, tissue biopsies, and stored paraffin sections. The assay is relatively easy to perform, and semiquantitative data can be easily obtained. Since the assay has the same cross-reactivity problems as the ELISA, it only provides information on relative levels of damage of a class of adducts and is not adduct specific. However, since the multiple adducts are likely to be important in the carcinogenic process, this type of information is useful.

Postlabeling is also frequently used for measuring adducts in humans and has the advantage of high sensitivity allowing detection of 1 adduct/10^{8-10} nucleotides (reviewed in Ref. 3). It is a nonspecific assay and can be used to look for general levels of aromatic carcinogen-DNA adducts. Initially DNA is enzymatically digested to a mixture of normal and adducted nucleotides. To enrich the sample in adducts, hydrophobic adducted nucleotides are extracted with butanol or the normal nucleotides digested with nuclease P1. This is followed by labeling with [^{32}P] using T4 kinase and γ[^{32}P]ATP then thin-layer chromatography and autoradiography. While adducts can be visualized as spots on the film, they cannot be identified without appropriate standards. In most cases, the lack of an appropriate standard makes absolute quantitation difficult since the efficiency of digestion and labeling of specific adducts can vary. Thus, DNA can be screened for the presence of modified nucleotides, but adduct identification is frequently not possible. In some cases adduct spots have been removed from the chromatography plate and further characterized by HPLC. A major advantage of the postlabeling method is its requirement for small amounts of sample making it ideal for biopsy samples. However, a major limitation is the high amounts of radioactivity required.

There are numerous studies using DNA adduct detection as a marker of exposure (reviewed in Ref. 2–5). The use of adducts as intermediate biomarkers in intervention studies is just beginning. The limited studies published to date are summarized below. Also described are our studies using antibodies to carcinogen-DNA adducts as markers of exposure, and a brief description is given of their application to an ongoing antioxidant vitamin intervention in smokers.

Intervention Studies Using Biomarkers of DNA Damage

Oxidative DNA Damage. 8-Hydroxy- or 8-oxodeoxyguanosine (8-OHdG) is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals generated endogenously or exogenously. Although numerous oxidative lesions occur in DNA, oxidation of the

C8 of guanine is one of the more abundant types and is also a major mutagenic lesion producing predominately G→T transversion mutations. The most frequently used method for measurement of 8-OHdG in humans is quantitation of the damaged nucleoside by HPLC with electrochemical detection in urine or enzymatically digested DNA. Kiyosawa *et al.* found higher levels of 8-OHdG in white blood cells of smokers ($5.1 \pm 2.5/10^6$ dG) than nonsmokers ($3.3 \pm 0.8/10^6$ dG) (16), although another study was unable to confirm these results (17). Excretion of 8-OHdG is also higher in smokers than nonsmokers (18) and increases after exercise (19).

The first studies using DNA damage as a marker of the efficacy of an intervention measured oxidative DNA damage. Ascorbic acid was found to prevent endogenous oxidative DNA damage (8-OHdG) in sperm (20). Increasing dietary vitamin C from 5 to 250 mg/day caused a doubling of seminal fluid levels and reduced 8-OHdG in sperm DNA by 36%. Several investigators measured urinary excretion of oxidized bases as an intermediate biomarker in interventions although the difficulties of inferring DNA damage from levels of damaged bases in urine has been discussed (5, 21). Urine can be collected much more readily than blood or tissue. However, the origin of the adducts (DNA, RNA, or diet) is unknown. Oxidative damage, measured as excretion of 8-OHdG in urine, decreased 28% in nonsmoking volunteers consuming 300 g/day brussels sprouts (22) but a similar study administering 20 mg/day β-carotene to heavy smokers saw no change in urinary 8-OHdG (23).

DNA Strand Breaks. An alternate assay for DNA damage, termed the "comet" assay, measures DNA strand breaks in single cells by electrophoresis under alkaline conditions. The length of the "comet" of DNA fragments released from the nucleus is a measure of the level of strand breaks. In a modification of the comet assay, before alkaline treatment, cells are treated with endonuclease III, which introduces breaks in the DNA at sites of oxidized pyrimidines. This assay, in conjunction with the standard comet assay, was used on lymphocytes of smokers and nonsmokers to determine the efficacy of a 20-week antioxidant intervention (100 mg of vitamin C, 280 mg α-tocopherol, and 25 mg of β-carotene/day) (24). With the standard comet assay, no difference in DNA strand breaks was observed in lymphocytes of smokers or nonsmokers receiving placebo or supplement. However, using the assay that detects oxidation of pyrimidines, a significant effect ($P < 0.002$) of supplementation was observed in both smokers and nonsmokers, with a mean value of 60.5 ± 7.0 (arbitrary units of damage) for the combined smokers and nonsmokers on placebo compared with 38.0 ± 5.9 in the supplemented group. Lymphocytes from supplemented subjects were also resistant to *in vitro* induction of strand breakage induced by H_2O_2.

In another study, consumption of 500 mg of vitamin C with breakfast decreased DNA strand breaks, measured by the comet assay, in lymphocytes collected 1 hr later compared with lymphocytes collected before breakfast (25). The comet assay was also used to demonstrate that vitamin E prevents exercise-induced DNA damage in lymphocytes (26). Bloods collected 24 hr after subjects ran on a treadmill until exhaustion had higher levels of DNA strand breaks than observed in bloods collected before exercise. Short-term consumption of multivitamin pills or vitamin E (3 × 800 mg) resulted in smaller increases in DNA damage in some subjects. Vitamin E (1200 mg/day) consumption for 14 days prior to exercise prevented the induction of DNA damage after exhaustive exercise. Finally, *in vitro* effects of ionizing radiation on lymphocytes in relation to diet consumed just prior to blood collection were examined with the comet assay (25). A significant protective effect of vitamin C ingestion was found both in the unirradiated control samples and in the dose response to ionizing radiation.

Postlabeling of Carcinogen-DNA Adducts. Because of its ability to detect a wide range of DNA damage, [^{32}P] postlabeling was also used as an intermediate biomarker in two intervention studies (27, 28). Gastric mucosal aromatic DNA damage decreased in 28 of 43 patients after vitamin C supplementation from 7.9 to 5.4 adducts/10^6 nucleotides ($P = 0.01$) (27). In the other study, a combination of vitamin A, riboflavin, zinc, and selenium or placebo was administered to reverse smokers of chutta (rolled tobacco) from four villages in India (28). At the end of 1 year, total aromatic DNA adducts, detected by postlabeling, in the supplemented group decreased by 95%.

Cytogenetic Assays. Cytogenetic assays are not generally considered biomarkers of exposure but rather markers of an effect. However, because they have been used extensively in intervention studies, they are briefly summarized here. Consumption for 4 months of a vitamin-antioxidant mixture containing 15 mg of β-carotene, 75 mg of rutin, 3 mg of retinol acetate, 30 mg of α-tocopherol, 150 mg of ascorbic acid, and 0.2 mg of folic acid decreased spontaneous and *in vitro* γ-ray–induced micronuclei in lymphocytes of young (23–30 years) and old (56–83 years) donors (29). Aged donors showed a significant decrease in spontaneous micronuclei in lymphocytes after a 4-month period of micronutrient consumption, while for *in vitro*–induced micronuclei both age groups demonstrated a significant effect.

Micronuclei in buccal cells were used to monitor the efficacy of β-carotene and vitamin A interventions (30–32). Betel nut chewers with leukoplakia were assigned to 180 mg/week of β-carotene, β-carotene plus 100,000 IU vitamin A, or placebo for 6 months (30). The two treatment groups had a significant remission of leukoplakias and an inhibition in the development of new leukoplakias at 3 months, but only the combined treatment was statistically significant at 6 months. At 3 months, the frequency of micronucleated cells was significantly reduced with β-carotene treatment ($P < 0.001$). A β-carotene intervention (180 mg/week) in Inuits using smokeless tobacco also demonstrated a significant

reduction in micronuclei ($P < 0.001$) (31). The combination of vitamin A and β-carotene also reduced micronuclei (32).

Immunoassays for Carcinogen-DNA Adducts

The work carried out in our laboratory is concentrated on the development, validation, and use of immunoassays for measurement of DNA adducts. We use both polyclonal antisera and monoclonal antibodies recognizing specific carcinogen-DNA adducts in highly sensitive competitive ELISA for quantitation of damage in isolated DNA or for immunohistochemical detection of damage in intact cells. Summarized below are data demonstrating that assays developed to quantitate three types of DNA damage, PAH, 4-aminobiphenyl, and oxidative, are sufficiently sensitive to detect exposure to cigarette smoke. These assays are currently being tested as intermediate biomarkers in a vitamin intervention as also outlined below.

Polycyclic Aromatic Hydrocarbon–DNA. We developed antisera recognizing PAH-DNA adducts from animals immunized with 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene–modified DNA (33, 34). These antisera cross-react with structurally related PAH diol epoxide adducts but with slightly different affinity (35). Humans are normally exposed to benzo[*a*]pyrene (BP) as part of a complex mixture of PAH. Thus, as mentioned earlier, the antisera will detect a class of carcinogen-DNA adducts but do not provide precise quantitative data on specific adducts such as those of BP. Initial studies used these antisera to measure damage in total white blood cell DNA, but more recent studies have used DNA from the longer lived mononuclear cell fraction. Elevated levels of damage were found in smokers compared with nonsmokers (36), lung cancer cases compared with controls (37), foundry and coke oven workers (38–40), and subjects exposed to high levels of air pollution in the Silesean region of Poland (41). Large interindividual differences in damage levels (10- to 100-fold) given similar exposures (e.g., number of cigarettes smoked, workplace air pollution level) were found in all studies, suggesting the importance of individual differences in absorption and metabolism of carcinogens and DNA repair. Genetic or lifestyle factors may also influence adduct formation. For example, an inverse relationship was found between lymphocyte PAH-DNA and serum levels of smoking-adjusted vitamin C ($r = -0.22$; $P < 0.09$) and cholesterol-adjusted vitamin E ($r = -0.25$;$P < 0.05$) in smokers, suggesting a mechanism by which these micronutrients may protect against cancer (42). This result was the impetus for our ongoing antioxidant intervention trial using several DNA damage biomarkers as intermediate end points. In the same population, we also genotyped for polymorphisms in glutathione-*S*-transferase M1, a detoxification enzyme which is responsible for conjugation of the reactive intermediates of a number of carcinogens including BP and aflatoxin B_1. This gene is deleted in about 50% of the population, and the null genotype is associated in epidemiologic studies with increased risk for lung cancer (43). The protective effect of the serum antioxidants was observed entirely in subjects who were *GSTM1* null.

A major limitation of the ELISA is the requirement for large amounts of blood (approximately 30–50 ml) in order to isolate sufficient DNA for analysis. To overcome this limitation, we adapted the PAH-DNA antiserum to immunofluorescence and immunoperoxidase detection of damage. The formation of PAH-DNA adducts was studied in peripheral blood lymphocytes obtained from men with occupational and environmental exposure in Poland. Subjects included coke factory workers, residents from the vicinity of the cokery, and rural region inhabitants. We had previously demonstrated that total white blood cell DNA adducts, measured by ELISA, were elevated in cokery workers and those with environmental exposure compared with rural controls (15.2, 13.0, and 2.3 adducts/10^8 nucleotides, respectively [39]). Using a quantitative immunofluorescence method on isolated lymphocytes with the same antiserum, we found that PAH-DNA levels were significantly elevated ($P < 0.001$) in both occupational and environmental groups, compared with the rural control group (44). Thus, similar results were found with the ELISA on isolated DNA and the immunohistochemical method on intact cells, but the latter requires only 1–2 ml of blood, making it more convenient for biomonitoring studies.

When the immunofluorescence method was first applied to exfoliated oral cells, we discovered that these cells had high levels of autofluorescence. Thus, a quantitative immunoperoxidase method using the same primary antiserum but with peroxidase-labeled secondary antisera and a diaminobenzidine detection system was developed for analysis of this cell type (45). Quantitation of staining in 50 randomly selected cells indicated that levels of PAH-DNA damage were elevated in each of 16 smokers (mean relative staining: 503 ± 104) compared with 16 age-, race-, and sex-matched nonsmokers (251 ± 82; $P < 0.0001$). In a later study, both oral cells and exfoliated bladder cells were collected from 20 smokers and 20 matched nonsmokers (46). Smokers had higher levels of PAH-DNA damage in oral mucosa and exfoliated urothelial cells than nonsmokers (oral mucosa cells, 684 ± 107 vs 370 ± 83, $P < 0.0005$; urothelial cells, 689 ± 72 vs 495 ± 57, $P < 0.0005$). There was a good correlation ($r = 0.69$; $P < 0.001$) between PAH-DNA adducts in oral and bladder cells from the same individuals. These results demonstrate that the immunohistochemical method has sufficient sensitivity to detect smoking exposure.

4-Aminobiphenyl–DNA. 4-Aminobiphenyl (4-ABP), an aromatic amine present in cigarette smoke, is an established animal carcinogen and in humans is associated with urinary bladder cancer. 4-ABP is metabolized *in vivo* to a reactive *N*-hydroxyarylamine, which binds covalently to DNA; the major adduct results from binding at the C8 position of guanine. Monoclonal antibodies recognizing 4-ABP–DNA were developed, characterized by ELISA, then applied in an immunohistochemical detection system (47).

There was a dose-related increase in specific nuclear staining in liver and bladder tissues from BALB/c mice treated with 4-ABP. DNA from liver tissue was also analyzed by alkaline hydrolysis to release 4-ABP, derivatization of the released material, and GC/MS analysis. A good correlation ($r = 0.98$; $P < 0.0001$) was found between relative fluorescence intensity and adduct levels determined by GC/MS. A similar immunoperoxidase method was used to investigate levels of 4-ABP–DNA in exfoliated oral and bladder cells from smokers and nonsmokers. Levels of 4-ABP–DNA in exfoliated urothelial cells were elevated in each of 20 smokers (mean relative staining intensity: 517 ± 137) compared with nonsmokers (313 ± 79; <005) (46). Significantly higher damage levels were also observed in oral mucosa cells of smokers compared with nonsmokers (552 ± 157 vs 326 ± 101; $P < 0.0005$) and there was a good correlation ($r = 0.59$; $P < 0.001$) between levels of 4-ABP–DNA in both cell types. This same method was also applied to stored paraffin tissue from bladder-cancer cases (48). Again adducts were increased in smokers compared with nonsmokers.

8-Hydroxydeoxyguanosine. We developed monoclonal antibodies and an ELISA for quantification of 8-OHdG in human samples and demonstrated that data determined by the ELISA correlated with that obtained on the same samples by HPLC with electrochemical detection (49). We also adapted the antibodies to immunoperoxidase detection of oxidative DNA damage (50). Oral mucosal cells from a total of 12 pairs of smokers and nonsmokers were analyzed. Higher levels of specific nuclear staining were observed in every smoker compared with his/her matched nonsmoker. Mean level of relative staining was elevated 1.6-fold in smokers (mean: 381 ± 70; $P < 0.01$) compared with nonsmokers (mean: 244 ± 43). But there was no association between staining intensity and the number of cigarettes smoked per day.

We are currently carrying out an antioxidant intervention using these assays as intermediate biomarkers. We are randomizing heavy smokers (≥20 cigarettes/day) to either placebo or 500 mg of vitamin C, 400 IU of vitamin E, and 12 mg of β-carotene/day for 6 months. Samples collected at 0, 1, 3, and 6 months include blood, exfoliated oral and bladder cells, and urine. PAH-DNA is measured in mononuclear cells by competitive ELISA, PAH-DNA, and oxidative DNA damage (8-OHdG) in buccal cells, and 4-ABP–DNA in exfoliated bladder cells by immunoperoxidase techniques. Oral and bladder cells are targets for smoking-induced cancers, so the demonstration that an intervention can modulate DNA damage in these tissues should provide support for using DNA adduct measurement as intermediate biomarkers.

Summary

Prospective, randomized clinical trials are clearly the most desirable means for testing the efficacy of an intervention in the prevention of cancer. However, as the studies carried out to date in humans indicate, they are extremely costly (the CARET study reportedly cost $42 million and the Harvard Physicians Study $22.5 million [51]). Studies that use biological indicators or markers in the causal pathway to disease are shorter and less costly. They cannot eliminate trials with cancer as the end point but can allow the more rapid initial testing of specific agents. Our studies and those of others suggest that DNA adducts are appropriate intermediate markers in intervention studies since they are in the causal pathway and can be readily measured in a high proportion of the population.

In summary, measurement of DNA damage can provide information on exposure to various chemical carcinogens, inter- and intraindividual variation in adduct levels, the relationship between genetic susceptibility factors and DNA damage, and the efficacy of intervention protocols designed to decrease cancer risk. As additional studies lead to early identification of individuals at increased risk, better targeting of interventions to high risk populations will also be possible.

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