2. State of validation of biomarkers of carcinogen exposure and effect

2.1. Generic biomarkers

2.1.1. Bulky DNA adducts

David H. Phillips and Stan Venitt
Institute of Cancer Research, London, UK

'Bulky adducts' are taken, in general, to include aromatic moieties with two or more aromatic rings, and some large extended non-aromatic or aliphatic structures, for example the N7-guanine adduct of aflatoxin B1, the N^2-guanine adduct of benzo[*a*]pyrene and the C8-guanine adduct of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).

Animal studies

Dose–response for adduct formation and carcinogenesis

In a review of the relationship between DNA adduct levels and tumour incidence in laboratory rodents, Poirier and Beland [1] summarised the data from experiments using 2-acetylaminofluorene, 2-aminobiphenyl, aflatoxin B1, *N,N*-diethylnitrosamine, or 4-(*N*-methyl-*N*-nitrosoamino)-1-(3-pyridyl)-1-butanone in a total of nine different combinations of carcinogen, species, sex and target organ. Of these combinations, there were five in which dose–response relationships for DNA adduct formation reflected those for tumorigenesis and, in these, linearity with dose for both tumours and DNA adducts appeared to be the norm at the lowest doses. In two situations, the levels of DNA adducts formed were low, presumably below the threshold for extensive tumorigenesis. In two other combinations, even though DNA adducts increased linearly with dose, tumours did not appear at the lower doses. These data suggest that when extrapolating from high doses to low doses within an animal model, the extent of DNA adduct formation will generally reflect the extent of tumorigenesis.

Surrogate vs target tissue

The tissue specificity of carcinogenesis in experimental animals following dosing with an indirectly acting carcinogen is usually dependent on the species, sex and route of administration. DNA-adduct formation has also been found to be dependent on these variables and the distribution and levels of adducts may not always parallel the pattern of carcinogenesis. Examples include tamoxifen [2,3], aristolochic acid [4,5]

and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) [6]. Peripheral blood lymphocytes (PBLs) are the cells most easily obtained for human monitoring and in some animal studies DNA adducts have been detected in PBLs from animals, for example in rats given benzo[*a*]pyrene by ip injection [7], and in rats given 3-nitrobenzanthrone by intratracheal instillation [8]. Taken together, these results suggest that the use of surrogate tissues, such as PBLs, for monitoring human exposure to carcinogens using DNA adducts, while useful, will not provide assurance for absence of exposure and absorption of carcinogens.

Human studies

Exposure–response

Few studies have examined whether there is a quantitative relationship between DNA adduct levels and the degree of exposure of humans to environmental pollutants. Two studies suggest that there is a non-linear dose–response.

Lewtas et al. [9] compared 76 coke-oven workers in Ostrava in the Czech Republic with another population exposed to environmental levels of polycyclic aromatic hydrocarbons (PAHs) from air pollution in Teplice. At low-to-moderate environmental exposures to carcinogenic PAHs, there was a significant positive correlation between DNA adduct levels in PBLs and exposure. However, at the higher occupational levels, the exposure–DNA adduct relationship became non-linear. Under these high exposure conditions, the relative DNA adduct level per unit of exposure (DNA-binding potency) was significantly lower than measured at environmental exposures.

Van Schooten et al. [10], using ^{32}P-postlabelling, examined DNA adduct formation in PBLs and bronchoalveolar lavage (BAL) cells in several populations of smokers. They observed a saturation of DNA adduct formation in both PBLs and BAL cells, suggesting less efficient adduct formation at higher doses. A similar non-linear dose–response was found in PBLs from smoking and non-smoking groups of aluminium workers exposed to high levels of PAHs [10].

Inter- and intra-individual variation

It is likely that several factors (for example, time of sampling and degree of exposure) will determine the levels of biomarkers, such as DNA adducts, measured in one individual. Such intra-individual variation has been documented by Besaratinia et al. [11] in a ^{32}P-postlabelling study of DNA adducts in PBLs and induced-sputum (IS) cells in 9 smokers and 9 non-smokers in which samples were taken once-weekly for three weeks. In most cases, the magnitude of intra-individual variation appeared to be smaller than that recorded between individuals, which ranged from 4-fold to 8-fold. Clearly, the effects of intra-individual and inter-individual variation will have to be taken into account in designing and interpreting biomonitoring studies of human populations.

Background levels

In a meta-analysis of the relationship between the levels of bulky DNA adducts and the risk of cancer, Veglia et al. [12] noted a wide variation in adduct levels in controls — from 0.4 to 7.9 adducts per 10^8 nucleotides. This nearly 20-fold variation in adduct levels in control populations, if typical of populations in general, clearly poses major problems in interpreting the results of biomonitoring studies using DNA adducts.

Case–control studies, prospective studies

The first study to demonstrate that DNA adducts could be biomarkers of cancer risk was a nested case–control study, in which urinary aflatoxin adducts were found to be significantly associated with subsequent development of liver cancer in Chinese men [13]. By far the most extensive studies of DNA adducts as markers for human biomonitoring have been those linking cancer with tobacco smoking and/or air pollution [12,14–19]. The following statement summarised the findings following a comprehensive review [17]:

Smoking-related DNA adducts have been detected by a variety of analytical methods in the respiratory tract, urinary bladder, cervix and other tissues. In many studies the levels of carcinogen-DNA adducts have been shown to be higher in tissues of smokers than in tissues of nonsmokers. Some but not all studies have demonstrated elevated levels of these adducts in the peripheral blood and in full-term placenta. Smoking related adducts have also been detected in cardiovascular tissues. Collectively, the available biomarker data provide convincing evidence that carcinogen uptake, activation and binding to cellular macromolecules, including DNA, are higher in smokers than in nonsmokers.

Covered by this review is a nested case–control study of male smokers that found that those who subsequently developed lung cancer had approximately twice the level of smoking-related leukocyte DNA adducts than those that did not develop the disease [14]. The results of a recent prospective study [18] accord with the statement above, indicating a slightly higher risk of lung cancer with higher levels of adducts in PBLs among smokers and suggesting that bulky DNA adducts may have a weak association with lung cancer risk. In a nested case–control study of never-smokers and ex-smokers who had not smoked for at least ten years [19] there was a significant excess (OR, 4.04; 95% CI, 1.06–15.42) in PBL adduct levels in never-smokers with lung cancer compared with controls. A positive association was found between DNA adducts and ozone concentration.

The relative advantages and disadvantages of case–control and prospective studies are well known [20] and apply to biomonitoring using DNA adducts. Prospective cohort designs (including nested case–control studies) are generally accepted as being the more reliable but the most complicated, time-consuming and expensive of studies. Nevertheless, it seems sensible to encourage investigators to employ this approach, rather than persevere with cheaper, quicker but inevitably less rewarding case–control studies.

Conclusions

While there are many studies demonstrating DNA adducts as biomarkers of carcinogen exposure, and a few demonstrating that they can be biomarkers of cancer risk, studies also indicate large inter-individual variations in adduct levels among subjects with apparently similar degrees of exposure. At the same time, there is relatively little information on intra-individual variation, i.e. how adduct levels may vary in a given individual over time. Furthermore, there are a few studies that have indicated non-linearity in dose–response at high levels of exposure, and further work is needed to verify such findings.

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2.1.2. Protein adducts

Bo A.G. Jönsson[1] and Peter Farmer[2]
[1] *University of Lund, Sweden*
[2] *University of Leicester, UK*

DNA adducts are enzymatically repaired, which presents problems because of their low concentrations *in vivo* and in calculation of the target dose [1]. In contrast, protein adducts are generally stable *in vivo*: haemoglobin (Hb) has a reasonably long lifetime of about 4 months and human serum albumin a half-life of about 3 weeks. Thus, adducts of these proteins are suitable as biomarkers of exposure. Correlations between the levels of DNA and protein adducts have been found [2,3].

Since the 1970s, when the use of protein adducts as biomarkers of exposure and risk was pioneered by the group of Professor Lars Ehrenberg [4,5], many methods have been described for the analysis of these adducts. Gas chromatography (GC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has been widely used, and more recently, liquid chromatography (LC) coupled to MS/MS.

Biomarkers of exposure

The use of protein adducts for exposure determination has been extensively reviewed [6–8]. Dose–response relationships in mice exposed to radioactive ethylene oxide were first determined by Ehrenberg and his colleagues more than 30 years ago [4], and tissue doses were determined from the degree of protein alkylation. In 1976 the suggestion was made to use specifically Hb adducts for this purpose [5], and experiments in animals were carried out to demonstrate the suitability of this approach for alkylating agents such as ethylene oxide and *N*-nitrosodimethylamine. The Hb adducts were shown to have the same life span as that of mouse Hb, demonstrating their stability in this haemoprotein and the lack of any repair mechanisms. Comparison of DNA and Hb alkylation using ^{14}C-labelled ethylene oxide has also been studied in mice by Segerbäck [9] and in rats by Potter et al. [10], demonstrating the quantitative relationships between these adducts.

The development of the N-terminal valine adduct approach to monitoring Hb alkylation [11] allowed very detailed studies of the dose–response of ethylene oxide adduct formation in rats and mice and the relationship of these products with biological markers of effect. For example, Hemminki et al. [12] investigated the absorption, distribution, elimination, Hb adducts (at N-terminal valine) and DNA adducts (by ^{32}P-postlabelling) of a series of olefins administered by inhalation to the rat. The olefins included ethylene, which is metabolised to ethylene oxide. Walker et al. carried out very extensive studies on rats and mice exposed repeatedly to ethylene oxide and determined the formation and persistence of the N-terminal valine adducts [*N*-(2-hydroxyethyl)valine] in Hb [13]. DNA adducts were compared with Hb adducts and it was shown that the relationships between the N-terminal valine adduct in Hb and the DNA adduct [*N*-7-(2-hydroxyethyl)guanine] varied with level of exposure, interval since exposure, species and tissue [14]. Subsequently comparisons of N-(2-hydroxyethyl)valine and DNA alkylation [*N*-7-(2-hydroxyethyl)guanine] and *hprt* mutation were made in mice exposed to ethylene or ethylene oxide [15]. The dose–response curves for the Hb and DNA adducts after ethylene treatment were found to be supralinear, indicating that metabolism of ethylene to ethylene oxide was saturated at the higher doses.

Other examples of compounds where dose–response relationships have been determined in animals are methyl methanesulphonate [16], ethyl methanesulphonate [17], the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [18], and the heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) [19].

Studies of human exposure showed that the formation of the N-terminal valine adduct following occupational exposure to ethylene oxide correlated strongly with the airborne concentration of the epoxide [7]. Strong correlations were also seen for propylene oxide and butadiene. Correlations of Hb adducts of ethylene oxide with *hprt* mutants, chromosomal aberrations, micronuclei, and sister chromatid exchanges (SCEs) were investigated in humans [20]. Haemoglobin adducts were the most sensitive of the endpoints for detection of ethylene oxide exposure. In a separate study, N-terminal valine adducts were compared with SCEs, micronuclei, chromosomal aberrations, DNA single strand breaks and a DNA repair index [21]. The adducts were significantly correlated with SCEs.

Many other genotoxic compounds have been studied in an analogous fashion. Haemoglobin binding indices have been determined for several aromatic amines, including aniline, toluidines, 2,4-dimethylaniline, *p*-chloroaniline, 4-aminobiphenyl (4-ABP), benzidine etc., or for metabolic precursors of aromatic amines such as nitrobenzene and 2-acetylaminofluorene [for reviews see 22,23]. Nitrotoluene is discussed in detail below.

Examples of the use of protein adducts to assess exposure to food-related carcinogens are given in Sections 2.2.1 (heterocyclic aromatic amines), 2.2.3 (N-nitroso compounds), 2.2.4 (acrylamide) and 2.2.6 (aflatoxin) of this Report.

Biomarkers of health effects

Acrylamide and neurological effects

The analytical method for acrylamide-protein adducts has been well validated by a number of research groups [24–28]. Background levels in the normal population have been described [28,29]. Clear relationships were found between the oral intake of acrylamide and Hb adducts, and inter-individual variation in adduct levels was low [30]. There is also a relationship between air-borne acrylamide and Hb adducts in exposed workers [31]. The adducts seem to be stable *in vivo* [32].

Two studies have looked at the neurological effects of exposure to acrylamide using the N-terminal Hb adduct approach, initially developed by the group of Lars Ehrenberg [11]. In the first, Hb adduct levels and neurological health effects were studied in Chinese workers exposed to acrylamide and acrylonitrile. Significant correlations were found between the acrylamide adduct levels and a neurotoxicity index [33]. The second study was of workers exposed to acrylamide and methyloacrylamide in Sweden during the construction of a railway tunnel. As in the Chinese study, an exposure–response relationship between Hb adducts and neurological health effects was found. In addition, a no observed adverse effect level of the Hb adducts was determined [32].

Organic acid anhydrides, isocyanates and airways disease

The analytical method for protein adducts of allergenic hexahydrophthalic and methylhexahydrophthalic anhydrides has been extensively validated [34] and it has been determined that the anhydrides bind mainly to serum albumin [35]. Furthermore, very high correlations ($r = 0.92–0.97$) were found between daily exposure determined

10–12 times over a month in 10 exposed workers and adduct levels at the end of the month, indicating low inter-individual variation in exposure. In addition, the adducts were stable *in vivo* [36].

Few animal studies have been presented but these adducts can be considered as biomarkers of effective dose since conjugates between serum albumin and anhydrides induce allergy in animals [37]. Dose–response relationships have also been successfully determined by analysis of protein adducts in airways disease including type-1 allergy. Rosqvist et al. [38] studied organic acid anhydrides using a cross-sectional approach. Dose–response relationships between plasma protein adducts of hexahydrophthalic anhydride and symptoms from eyes and nose were reported as well as with anhydride-specific IgE and IgG antibodies.

Many analytical methods have been reported for determination of protein adducts of isocyanates and related amines (see e.g. [39–41]). Most of these methods use a hydrolysis step that releases free amine; however, the hydrolysis conditions used vary and the widely differing recoveries make comparisons difficult. Thus, the hydrolysis conditions should be standardised. It has been shown that the adducts in plasma are exclusively bound to serum albumin and that very few low molecular weight isocyanate metabolites are hydrolysed to free amine in plasma compared with the protein adducts [41–43], making it unnecessary to dialyse the plasma prior to hydrolysis. In exposure chamber studies of healthy volunteers, air levels of isocyanates and protein adduct concentrations were found to be related [44]. However, there seem to be large inter-individual variations [45]. Such variations have also been found in studies of exposed workers [46,47]. Background adduct levels have been described [48]. In a cross-sectional study of diisocyanate exposure, significant associations were reported between plasma protein adducts of isocyanates and specific antibodies and work-related airways disease [49].

Nitrotoluene-related health effects

In workers exposed to nitrotoluenes an association has been found between Hb adducts and adverse health effects such as cataract, hepatomegaly, splenomegaly, inertia, somnolence, nausea and dizziness [50–53]. The analytical method has been validated [3,54–56], at least for most of the compounds analysed. Relationships have been found between external exposure and adduct levels [50] and urinary levels of nitrotoluenes and adduct levels [53]. There is no information on levels found in the reference population but levels of non-exposed Chinese workers have been reported [52,53]. Furthermore, there are no reports on inter-individual variation but the high associations with health effects indicate that the Hb adducts may be precise biomarkers of exposure.

Arylamines, acrylamide and cancer

For 4-ABP-protein adducts, several groups have reported validation of the method; for biomonitoring of arylamines, see [3]. The same method was used for analysis of the other arylamines and has been validated for these compounds. Most work on characterisation of the adducts has been performed on 4-ABP. Little inter-individual variation

was found in cigarette smokers in different countries but a clear difference was found between adduct levels in smokers and non-smokers and in persons exposed to environmental tobacco smoke compared with non-exposed non-smokers. However, there are indications that 4-ABP adducts are not fully stable *in vivo*. The persistence of 4-ABP adducts in human Hb has been investigated in a population withdrawing from smoking. Although the adduct declined at a rate faster that was expected on the basis of the life span of human Hb, it persisted much longer than cotinine [57]. Background levels of arylamine-Hb adducts have been described by several authors [3].

In a recent case–control study, Gan et al. [58] found significant associations between bladder cancer and Hb adduct levels of three different arylamines, 2,6-dimethylaniline, 5,3-dimethylaniline and 3-ethylaniline. The associations were still significant when only non-smokers were studied. In addition, 4-ABP-Hb adducts in women have been shown to be associated with smoking-related diseases (cancer and airways) in a case–control study [59].

The N-terminal Hb adduct approach has also been applied in assessments of human cancer risk. The effective dose of the chemical is calculated from the protein adduct level and the cancer risk is then obtained using an approach originally developed for radiation [60]. For example, in the study by Hagmar et al. [32], of exposure to acrylamide during construction of a railway tunnel, it was calculated that the risk for the workers and the people living in the area of developing cancer due to the exposure was very low. Thus, the use of protein adducts in this case was extremely important for the communication of risk to the public. The low cancer risk was due to the short exposure duration. A life-time dose, ten times lower compared with the no observed adverse effect level for neurological symptoms, would generate an excess risk of about 1 cancer case per 1000 individuals [61]. In addition, the study by Hagmar et al. [32] elucidates another strength of protein adducts. At the time of the investigation, the workers had already stopped using acrylamide; however, because of the life-time of 120 days of the adducts it was still possible to estimate the exposure levels during the work.

Conclusions

One problem with the use of Hb adducts or adducts with other blood proteins in studies of cancer is the relatively short half-lives of these proteins. While Hb adducts reveal exposure over a period of months it is often the life-time dose that best predicts the risk of cancer. There have, however, been some studies of adducts of proteins with longer half lives, e.g. histones [62] and collagen [63,64]. Their usefulness in determining dose–response relationships remains to be established.

Nonetheless, protein adducts will be important in future studies of dose–response relationships. However, because of the expense and labour intensiveness of GC- and LS-MS methods, which makes them unsuitable for large-scale human population studies of environmental exposures, new strategies must be developed, e.g. the use of immunological methods such as the enzyme-linked immunosorbent assay (ELISA). Such methods

are widely used for a number of proteins and also for low molecular weight compounds; they are sensitive, easy to perform and the analytical equipment is relatively cheap. On the other hand, their selectivity is often rather low because of cross-reactivity with other compounds. It is therefore, necessary during the work-out of the methods to compare them with a more selective method such as MS.

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2.1.3. Chromosomal damage

Raluca Mateuca and Micheline Kirsch-Volders
Free University of Brussels, Belgium

Chromosomal damage reflects cellular phenotypic changes resulting from gene–environment interactions that are expressed as structural or numerical chromatid/chromosome modifications. Combinations of these phenotypic changes at the level of individual cells will define the phenotype of the tissue or organism. Biomarkers of chromosomal damage are thus expected to show: inter-cellular variation related to differences in cell cycle stage at the time of exposure; inter-individual differences related to genotype; inter-specific differences related to chromosome number and gene maps.

However, they will have relatively low specificity (except for particular cases) since different mutagens can induce the same type of chromosomal damage. Biomarkers of chromosomal damage will allow: good assessment of systemic/global early effects induced by single or multiple, acute or chronic exposures; possible assessment of cumulative effect over a relatively long period of time if a suitable cell type is considered (e.g., T-lymphocytes); possible assessment of chromosomal damage in both somatic and germ cells.

Validation of chromosomal changes as biomarkers of exposure or effect has focused on the following aspects: sound scientific/mechanistic basis of the methodology [e.g. accurate identification of first mitotic divisions by incorporation of 5-bromo-2'--deoxyuridine (BrdU) or cytokinesis block]; dose dependency; reproducibility in experimental systems *in vitro* and *in vivo* (e.g., bone marrow, spermatids, spermatozoids); background levels in non-exposed populations; predictivity for the assessed disease; applicability.

Chromosomal aberrations and micronuclei as biomarkers of genotoxicity and cancer risk

The two most commonly used biomarkers of chromosomal damage, chromosomal aberrations (CAs) and micronuclei (MN), are used in biomonitoring or molecular epidemiological studies of environmental cancer. At the time the CA test was adopted by the OECD guidelines for genotoxicity testing, extensive coordinated validation studies were not required [1]. However, the CA test has been widely accepted and considered as validated *in vitro* as well as *in vivo* through its intensive application in many laboratories. The *ex vivo/in vitro* cytokinesis-block MN assay is more recent and has undergone the current validation procedure for the acceptance of a new test in the international guidelines. Major steps in the validation were performed either by the HUMN[1] working group for human biomonitoring, which examined the major confounding factors (culture conditions, scoring criteria, age, smoking, genotype, exposure) [2–6], or by an inter-laboratory collaborative exercise coordinated by the SFTG (Societé Française de Toxicologie Génétique) for *in vitro* genotoxicity studies. The details of this important validation study can be found in a special issue of *Mutation Research* (2006) [7]. In parallel, ECVAM[2] finalised a document stating that the *in vitro* MN assay is a scientifically valid alternative to the *in vitro* CA assay for genotoxicity testing.

Chromosomal aberrations are used routinely for the assessment of genotoxicity both *in vitro*, in human primary lymphocytes and cell lines, and *in vivo*, in rodent bone marrow and spermatids. The CA assay has a key position in the test battery for genotoxic compounds and its protocol is defined by OECD guidelines[3]. Additionally, the use of fluorescence *in situ* hybridisation (FISH) chromosome painting methods to detect structural and numerical CAs may provide increased efficiency and specificity for identifying certain kinds of CAs induced *in vivo* [e.g. translocations, stable symmetrical rearrangements derived from chromatid-type aberrations (CTAs), hyperploidy] (for review see [8]).

The MN assay is also used both *in vitro* and *in vivo* for genotoxicity testing. The *in vivo* MN assay in rodent bone marrow plays a crucial role in the test battery aimed at hazard identification for mutagens. The *in vitro* MN assay has, since its modification with the cytochalasin-B block, been promoted as an alternative test for the *in vitro* CA assay. Additionally, the combination of the MN assay and FISH with probes labelling the pan (peri-)centromeric region of the chromosomes enables a distinction to be made between MN containing a whole chromosome (centromere-positive MN) and an acentric chromosome fragment (centromere-negative MN) (for review see [8]). Protocols

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1 http://www.humn.org

2 ECVAM Validation Management Team (Albertini S, van Benthem J, Corvi R, Hoffmann S, Maurici D, Pfuhler S, Vanparys P). Report on the Micronucleus test *in vitro*. ECVAM, 2006, in preparation. Available from: http://ecvam.jrc.cec.eu.int/index.htm

3 http://ecb.jrc.it/testing-methods

for human primary lymphocytes and cell lines were validated and harmonised [9,10], and are now in the final phase of acceptance in the OECD guidelines. Besides its capacity to detect MN (a biomarker of chromosome breakage and/or whole chromosome loss), the cytokinesis-block MN assay can provide additional measures of genotoxicity and cytotoxicity: nucleoplasmic bridges (NPB, a biomarker of DNA misrepair and/or telomere end-fusions), nuclear buds (NBUD, a biomarker of gene amplification), cell division inhibition (by estimation of the nuclear division index), necrosis and apoptosis (for review see [11]). For this reason, the cytokinesis-block MN test can be considered as a 'cytome' assay covering chromosome instability, mitotic dysfunction, cell proliferation and cell death [12].

For biomonitoring purposes, assessment of CAs and MN is usually done in peripheral blood lymphocytes (PBLs) as a surrogate tissue. Scoring of MN in erythrocytes is also possible; however, it is known that in humans, micronucleated erythrocytes are quickly eliminated by the spleen [13]. Therefore, scoring of MN in erythrocytes can be recommended only when assessed shortly after acute exposure. Recent advances in flow cytometry have the potential to provide a rapid analysis of micronucleated reticulocytes by separating the very youngest erythrocytes (the transferrin–positive reticulocytes) [13]. The methodology was successfully applied by Grawé et al. [14] and Abramsson et al. [13] for human biomonitoring. MN can also be analysed in skin, buccal, nasal and urothelial cells. Data on MN levels in different tissues are not usually available since most occupational exposure studies focus on only the tissue that is relevant for a specific mutagen/carcinogen exposure. An example of the relative sensitivity of the MN assay in different tissues is provided on the CRIOS website[4] and concerns workers occupationally exposed to formaldehyde (FA). Thus, a prospective study of 29 mortician students (22 males and 7 females) who were about to take a 85 day course in embalming, found a 12-fold increase in MN frequency in epithelial cells from the buccal area during the study period, from 0.046±0.17/1000 cells preexposure to 0.60±1.27/1000 cells at the end of the course ($p < 0.05$) [15]. In blood cells, the frequency of micronucleated lymphocytes increased by 28%, from 4.95±1.72/1000 cells to 6.36±2.03/1000 cells ($p < 0.05$). No significant increase in MN was observed in nasal cells of FA-exposed students (from 0.41±0.52/1000 cells to 0.50±0.67/1000 cells, $p = 0.26$). A dose–response relationship was observed between cumulative exposure to FA and increases in buccal cell MN in the 22 male subjects but not in the 7 female subjects. The study concluded that low-level exposure to FA is associated with cytogenetic changes in epithelial cells of the mouth and in blood lymphocytes. Another study, conducted by Titenko-Holland et al. [16], employed the FISH technique on specimens of exfoliated buccal and nasal cells from mortuary science students following a 90 day embalming course. A significant increase in total MN frequency was observed in buccal cells of the students after the course (from 0.6/1000 to 2/1000, $p = 0.007$), whereas no significant increase was observed in nasal cells (from 2

[4] http://cdfc00.rug.ac.be/healthrisk/

to 2.5/1000, $p = 0.2$). The frequency of MN in cells of the nasal mucosa, oral mucosa and in lymphocytes was also evaluated for 25 anatomy students exposed to FA over an 8 week period [17]. A higher frequency of MN was observed in nasal and oral exfoliative cells after FA exposure (3.85 ± 1.48 vs 1.20 ± 0.676 and 0.857 ± 0.558 vs 0.568 ± 0.317, paired t-test: $p < 0.001$ and $p < 0.01$, respectively), whereas no significant increase in the frequency of lymphocyte MN was found ($p > 0.05$). These results indicate that differences in MN levels between specific tissues could be related to exposure time/dose and individual sensitivity to FA.

The sensitivity of DNA damage assays in PBLs as a measure of exposure by inhalation or oral ingestion depends on the solubility, reactivity, uptake and metabolism of the considered mutagen. A positive response in PBLs is therefore a major signal for genotoxic risk. A negative response does not exclude a tissue-specific genotoxic effect at other sites. CAs are used to evaluate exposure to chemical carcinogens. Examples of occupational exposure where it was demonstrated that CA should be recommended to perform surveillance in occupational settings (e.g., styrene and ethylene oxide) [18–22] can be found on the CRIOS website. The MN assay can also be recommended for surveillance purposes, in particular for those exposures which trigger spindle inhibition/cell cycle dysfunction (e.g., cytostatics, pesticides) [23–30] (for review see CRIOS[5]).

Knowledge about the predictivity of CAs and MN for cancer risk as assessed in PBLs is crucial. An association between high frequency of CAs and cancer risk was first reported by several Nordic [31–33] and Italian [34] cohort studies. A case–control study nested within the joint Nordic and Italian cohorts indicated that the association between CA frequency and cancer risk was not explained by tobacco smoking or known occupational exposure to carcinogens, suggesting that a high frequency of structural CAs as such is predictive of an increased cancer risk, irrespective of the cause of the initial CA increase [35]. Several studies have also addressed the cancer risk predictivity of CA subclasses [36–39]. An increased risk of cancer incidence was limited to chromosome-type aberrations (CSAs) in a nested case–control study carried out in Taiwan [36]. In contrast, in a large Nordic and Italian cohort study, a significantly elevated cancer risk was observed in the Nordic cohorts for subjects with both high CSAs and high chromatid-type aberrations (CTAs), while the results of the Italian cohort did not indicate any clear-cut difference in cancer predictivity between the CSA and CTA biomarkers [37]. A significant association between the overall cancer incidence and the presence of CSAs was recently found in a large Czech cohort study of healthy individuals [38]. Supporting the previously published data, a recent study performed on 6430 healthy individuals from a Central European cohort showed that a high frequency of CAs in PBLs, and in particular CSAs, is associated with increased risk of cancer [39].

The possibility of a link between MN induction and cancer development was first addressed by the Nordic and Italian cohort studies [31–33], which found that high MN frequencies in PBLs were not predictive of an increased cancer risk. However, these studies

5 http://cdfc00.rug.ac.be/healthrisk/

did not have sufficient power and/or follow up time to allow conclusions to be drawn concerning the cancer predictivity of MN. Moreover, most of the data had not been obtained by using the more sensitive *ex vivo/in vitro* cytokinesis block methodology. A recent analysis of new results from the HUMN project indicates that an increased frequency of MN in PBLs predicts the cancer risk in humans [40]. The analysis was performed on a total of 6718 subjects from 10 countries (20 laboratories) who were screened for MN frequency between 1980 and 2002. To standardise for inter-laboratory variability, subjects were classified according to the percentiles of MN distribution within each laboratory as low, medium, or high frequency. A significant overall increase in cancer incidence in subjects with medium (RR = 1.84; 95% CI = 1.28–2.66) and high MN frequency (RR = 1.53; 95% CI = 1.04–2.25) was observed. Moreover, the same groups showed decreased cancer-free survival ($p = 0.001$ and $p = 0.025$, respectively), which was present in all national cohorts and for all major cancer sites, especially urogenital (RR = 2.80; 95% CI = 1.17–6.73) and gastro-intestinal cancers (RR = 1.74; 95% CI = 1.01–4.71). Moreover, results from a case–control study of lung cancer in smokers showed that both spontaneous and nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced MN (OR = 2.06; 95% CI = 1.60–2.65 and OR = 2.32; 95% CI = 2.32–4.80, respectively), NPB (OR = 29.05; 95% CI = 7.48–112.80 and OR = 45.52; 95% CI = 4.48–422.17) and NBUD (OR = 6.53; 95% CI = 2.37–18.01 and OR = 10.10; 95% CI = 4.67–21.87, respectively) in PBL cultures are associated with increased lung cancer risk [41]. These results indicate that the integration of various cytogenetic biomarkers within one assay may improve cancer risk prediction.

Therefore, we can conclude that despite some shortcomings of the published studies (low statistical power and follow-up time for MN in the Nordic-Italian cohorts [31–33]) and observations that merit additional research (e.g. dose–effect relationships, adaptive response), CAs and MN in human lymphocytes can be considered as predictive for cancer risk assessment (for review see [8]). A final validation of CAs and MN as good predictors for cancer risk would require two additional steps: (1) to show that reducing the level of the biomarker by intervention strategy also reduces cancer risk; and (2) to show the extent to which levels in surrogate tissue (lymphocytes) are predictive of cancer risk in target tissue.

Conclusions

In conclusion, the CA and MN assays can be recommended as biomarkers of chromosomal damage to assess cancer risk. Their major disadvantage is that manual scoring is extremely time consuming and requires skilled personnel. The applicability of the CA and MN tests as biomarkers in molecular epidemiology needs automation. For CAs, semi-automated procedures are available although they entail a heavy workload; mis-classification by the software (except for gaps) is not a real concern, since the final decision is left to the scorer. For MN also, it is expected that the development of suitable

software will allow fully automated scoring; particular attention should therefore be paid to selection of adequate positive controls and detection of false negatives/positives. Further technical development and validation of the automated procedure are still needed. The scoring of MN is by definition a cell by cell analysis, which assesses the formation of MN during the metaphase-anaphase transition. Therefore it is essential to take into account cell division. Both flow cytometry and image analysis can fulfil these requirements, as long as adequate preparation methods, staining methods and selection criteria are applied. The recommended methodology will depend on the question being asked (cumulative low level exposure, recent acute exposure or effectiveness of chemo-prevention/intervention trials), cell type (erythrocytes or lymphocytes), application (biomonitoring or hazard assessment) and the sensitivity, specificity and reproducibility of the results obtained with a given methodology. An assessment of inter-laboratory variation is therefore recommended. Full validation of a methodology should take into account these three parameters, and, in particular, the capacity to discriminate accurately between small MN induced by clastogens and large MN induced by aneugens. In practice, this consists in evaluating the proportions of false negatives and false positives for a selected number of clastogens and aneugens with a given method. Without this validation step no data can be accepted. These reference chemicals would therefore serve as controls in later studies.

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2.1.4. DNA base oxidation and repair

Steffen Loft and Peter Møller
University of Copenhagen, Denmark

Biomarkers of oxidatively damaged DNA

Oxidative damage to DNA can involve any base or sugar moiety, and strand breaks and a very large number of possible lesions have been described [1]. Among the many specific products of oxidative damage to DNA, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the most studied because of the relative ease with which it can be measured and its mutagenic properties: replication of DNA containing 8-oxodG results in G-T transversions [2,3]. The major problem found in measuring oxidised bases in DNA is the occurrence of spurious oxidation during sample preparation. DNA extraction is a critical issue and the derivatisation required for gas chromatography-mass spectrometry (GC-MS) analysis is particularly problematic as described by the European Standards Committee on Oxidative DNA Damage (ESCODD), which organised a large inter-laboratory validation exercise with 25 member laboratories in which coded samples with defined numbers of 8-oxodG and other oxidised guanine lesions were analysed [4,5]. The comet assay detects DNA strand breaks and abasic sites; base oxidations can be detected if repair enzymes, such as formamidopyrimidine glycosylase (FPG) or endonuclease III, which nick DNA at oxidised purines and pyrimidines, respectively, are also used. This method is recommended for detection of these lesions with minimum risk of spurious oxidation [4-6]. However, for exact determination of specific base oxidations, chromatographic assays, in particular those based on MS, are required.

The urinary excretion of products of damaged nucleotides from cellular pools or DNA may also be important as a biomarker of exposure to relevant carcinogens and may be used to predict cancer risk. Of the many oxidative damage products, 8-oxodG is also the most studied in urine with assays based on high performance liquid chromatography-electrochemical detection (HPLC-EC), capillary electrophoresis (CE)-EC, GC-MS, HPLC-MS/MS and enzyme-linked immunosorbent assay (ELISA) [7]. Two small laboratory validation studies have shown concurrence between the chromatographic methods ($r = 0.95$ and $r = 0.99$ were obtained in two exercises where laboratories analysed the same samples) [8], whereas ELISA yielded several-fold higher concentrations although with reasonable correlations ($r = 0.42$, 0.83, and 0.88 were obtained in three different trials; the highest correlations being achieved when HPLC-purified fractions of 8-oxodG were used before the antibody-based detection step) [9–11].

Biomarkers of capacity to repair oxidatively damaged DNA

The complex repair pathways of oxidised bases need to be taken into account in the interpretation of the levels in DNA. Oxidised guanine in DNA is mainly repaired by oxoguanine glycosylase (OGG1), which removes 8-oxoguanine (8-oxoGua) opposite cytosine in the DNA strand [12–14]. In addition, repair of 8-oxodG may to some extent occur by nucleotide excision repair and nucleotide incision [2]. A specialised enzyme (MTH1/NUDT1) cleaves phosphates from 8-oxodGTP, thus removing it from the nucleotide pool. If incorporated during DNA synthesis, 8-oxodGTP is highly mutagenic and mice deficient in this enzyme develop tumours [15]. MYH, a base excision repair protein, which removes adenine misinserted opposite oxidised guanine, works in concert with MTH1 [16,17].

A number of biomarkers and assays have been developed for assessment of the capacity for repair of oxidatively damaged DNA. The capacity for nicking of labelled oligonucleotides containing, e.g., oxidised bases or ethenoadducts, by extracts of cells or tissues is measured relatively easily [18,19]. Similarly, cell extracts can be applied to nucleoids with a defined level of oxidative damage, e.g. 8-oxodG induced by a photosensitiser, and nicking assessed by the comet assay [22]. The *OGG1* gene has a common genetic polymorphism with a variant Ser326Cys, which appears to increase susceptibility to reactive oxygen species *in vitro*; however, 8-oxodG levels and incision activity in leukocytes and some target tissues generally show no difference between the genotypes [21,22].

Validation of biomarkers of oxidatively damaged DNA

Animal studies

Almost a decade ago, the ability of more than 50 compounds to cause oxidative damage to DNA was investigated in tissues of animals [23]. However, thorough assessments of the dose–response relationship are sparse and formal validation studies in animals have not been attempted like those done, e.g., for the determination of strand breaks by the comet assay, which encompassed an analysis of 208 different chemicals tested

dose-dependently at various exposure times [24]. The interpretation of many of the animal experimental studies is hampered because of suboptimal procedures that have yielded too high baseline levels of 8-oxodG due to spurious oxidation. Exposure to ionizing radiation and ingestion of potassium bromate can be considered as model agents that oxidize DNA. Linear dose–response relationships were observed in lungs of mice after topical irradiation and detection of FPG sites, whereas the level of of 8-oxodG was unaltered at the same doses [25]. An improved method of DNA extraction for the analysis of 8-oxodG by HPLC increased the sensitivity and suggested a threshold of effect [26]. Also administration of potassium bromate in the drinking water generated 8-oxodG in the kidney (target organ) in a dose-dependent manner, albeit with a threshold [27]. It is not possible to distinguish between technical (limit of detection) and biological effects of these thresholds. Consequently, the role of oxidative DNA damage in experimental carcinogenesis is hypothesised, but not well established [2,3].

The limited influence of base excision repair capacity on urinary excretion in the steady state was demonstrated by the observation of only 26% lower 8-oxoGua excretion and unchanged 8-oxodG excretion in mice with complete OGG1 knockout [28].

Human studies

Biomarkers of oxidative DNA damage have been used in human studies for more than 20 years [29]. A large number of cancer risk factors, e.g. smoking and exposure to air pollution, have been shown to affect the level of oxidatively damaged DNA, the repair capacity in leukocytes or the urinary excretion of repair products [30]. Similarly, a recent review of 139 studies addressing possible effects of dietary or supplement-based antioxidants concluded that the levels of these biomarkers may be reduced [31]. Many of these studies looked at aspects of inter- and intra-individual variation.

The intra-individual variation of urinary 8-oxodG excretion in two subjects investigated over a 10 day period showed high variation (37% and 57%, respectively) [32]. A study with six experimental series over a period of eight months yielded inter-individual, intra-individual, and assay variation as follows: 57% (53–68% in various series), 48% (18–106%), and 14%, respectively (mean CV and range) [33]. However, in a large number of studies with up to 6 repeated samplings for up to 3 years in hundreds of individuals and various exposures (including air pollution, antioxidant supplements, fruit and vegetable diets, low calorie diets etc.) the residual variation has usually been around 20% and the assay variation 13% or less [34–36]. A study in human volunteers fed different doses of ^{15}N-labelled heavily oxidised DNA showed no urinary excretion of ^{15}N-labelled 8-oxoGua and 8-oxodG, suggesting that these urinary biomarkers do not originate from the diet [37]. In addition, the OGG1 Ser326Cys polymorphism has been shown not to affect the excretion of 8-oxodG [38].

In a steady state, the urinary excretion of 8-oxodG and similar products should, in principle, reflect the average rate of oxidative damage in the whole body, whereas the level of lesions in DNA from target cells or surrogate cells, such as white blood cells,

should reflect the balance between damage and repair only in these cell types [39]. In this context, a study with more than 100 subjects showed no correlation on an individual level between the levels of 8-oxodG in leukocytes and the excretion of 8-oxodG and 8-oxoGua measured by chromatographic methods [40].

The cellular DNA damage biomarkers appear to vary over time in the same subject; this variation is dependent on external exposures that could be diet [41,42], occupational stress [43] and ambient air pollution [35,44]. Probably the most reliable overall assessment of the inter-individual variation comes from the ESCODD study, which used standardised assay protocols and each laboratory analysed the level of DNA damage in mononuclear blood cell (MNBC) DNA from young healthy male subjects recruited in the respective countries. The median (range) inter-individual variations (CV) in 8-oxodG or FPG sites of MNBC DNA were 43% (33–188%) and 49% (30–100%), respectively [5]. Another study of 99 subjects, in which FPG sites and 8-oxodG were measured in lymphocyte samples from the same subject, indicated a somewhat lower CV for the FPG sites compared with the 8-oxodG measurements, i.e. the means (SDs) were 0.24 (0.08) and 0.92 (0.42) lesions per 10^6 dG, respectively [45]. An analysis of the reported data indicated that the variation in FPG sites in different studies is about threefold, i.e. 0.24–0.75 lesions/10^6 dG [46].

Case–control studies of biomarkers of oxidatively damaged DNA and repair in leukocytes consistently indicate an increased level of damage and decreased repair capacity among cases. Similarly, urinary biomarkers of oxidatively damaged DNA may be elevated in patients with cancer. However, it should be emphasised that oxidative stress and other alterations can easily be induced by cancer and thus influence the levels of both DNA damage and repair, hence biomarker-based case–control studies are likely to be compro-mised by reverse causality [47]. Case–control studies of genetic polymorphisms in DNA repair enzymes and cancer risk are not likely to suffer from the same bias. The common variant Ser326Cys in *OGG1*, of as yet unknown effect on the function of the protein, appears to be a risk factor for lung cancer [48], whereas a rare variant in *OGG1* and germ line mutations in the corresponding mismatch repair gene *MYH* appear to be important risk factors for hereditary colon cancer. These data clearly indicate the importance of oxidatively damaged DNA in some cancers. Hence, epidemiological studies of polymorphisms, mechanisms of action of gene products and levels of oxidative damage in surrogate and target tissues would be highly interesting.

Prospective cohort studies with biomarkers of oxidatively damaged DNA are necessary to determine whether a high level of such DNA damage implies a high risk of cancer. However, such studies will be challenging given the large number of subjects and long follow-up time required and the need to avoid spurious oxidation of DNA during collection and/or storage of samples. It is crucial that these issues are addressed when new cohorts are being established. Until validated biomarkers of oxidatively damaged DNA are available, biomarkers of DNA repair functions, e.g. in leukocytes, and urinary excretion of repair products could be used in cohort studies. In this context, high urinary

excretion of 8-oxodG was recently shown to be associated with increased risk of lung cancer in never-smokers, although not in former or current smokers in a prospectively based nested case–cohort study [38]. Fortunately, many new assays of repair capacity and oxidised DNA bases and nucleosides in urine are becoming available. Hopefully, such studies will elucidate the role of oxidatively damaged DNA in human cancer. Ultimately, the efficacy of cancer prevention treatments could be assessed by monitoring of biomarkers of oxidatively damaged DNA.

Conclusions

A few biomarkers of DNA base, especially guanine, oxidation have been subject to inter-laboratory assay validation, problems have been characterised and there is some consensus as to the true levels in cellular DNA. Experimental systems have documented the role of DNA base damage and repair in carcinogenesis and many biomarker studies have demonstrated consistent relations with exposures, interventions, host factors and risk factors relevant for cancer. Case–control studies support the role of DNA base oxidation and repair in cancer risk, although they may be compromised if the disease itself affects the biomarkers of exposure. Prospective studies are difficult because very large numbers of subjects are necessary and cellular DNA is susceptible to spurious oxidation during storage. In contrast, urinary biomarkers and measures of repair capacity may be unaffected by storage of samples, allowing prospective studies with nested-in-cohort case–control designs to be undertaken.

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2.1.5. Lipid peroxidation-induced DNA damage

Urmila Nair and Jagadeesan Nair
German Cancer Research Center, Heidelberg, Germany

Biomarkers of lipid peroxidation-induced DNA damage

Reactive oxygen (ROS) and reactive nitrogen species (RNS) generate DNA-reactive aldehydes from lipid peroxidation (LPO) such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), acrolein (Acr), and crotonaldehyde (Cro), which can cause DNA damage either through direct reaction with the DNA bases or through the generation of more reactive bifunctional epoxides, resulting in exocyclic DNA adducts. These can result from endogenous processes as well as from environmental exposures.

The major endogenous LPO-derived exocyclic DNA adducts are:

— Etheno-derived DNA adducts, which are generated by reaction of DNA with HNE, such as 1,N^6-ethenodeoxyadenosine (εdA), 3,N^4-ethenodeoxycytidine (εdC), 1,N^2-ethenodeoxyguanosine (1,N^2εdG) and N^2,3-ethenodeoxyguanosine (N^2,3εdG). Etheno-DNA adducts can also be formed from the human carcinogen vinyl chloride and the rodent carcinogen urethane.

— The MDA-derived major DNA adduct pyrimido[1,2-*a*]purin-10(3H)-one (M_1G). Oxopropenyl transfer from base propenal to dG residues can also produce M_1G.

— Cyclic 1,N^2-propanodeoxyguanosine adducts, which are products of the reaction of α,β-unsaturated aldehydes or enals, such as acrolein, crotonaldehyde and HNE, with guanine, yielding adducts such as Acr-dG, Cro-dG and HNE-dG.

Ultrasensitive and specific methods have been developed that allow the *in vivo* detection of background levels of these adducts arising from endogenous lipid peroxidation, and the study of their formation and role in experimental and human carcinogenesis. The literature has been comprehensively reviewed [1–6].

Validation of biomarkers of lipid peroxidation-induced DNA damage

Etheno-DNA adducts

Background or endogenous levels of εdA and εdC have been detected using an ultra-sensitive and specific immunoaffinity (IA)-^{32}P-postlabelling method [7] in all rodent and human tissues investigated so far. These include human liver, pancreas, colon, lung, kidney, cerebrum, cerebellum and white blood cells (WBCs); and several rat and mouse tissues; the levels ranged from 0–27 adducts per 10^9 parent bases. The levels were highly variable and affected by several factors including lifestyle and diet [8–11]. Similar background levels of εdA [12] and εdC [13] have also been measured in rodent and human tissue using high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS).

Background levels of N^2,3-εdG in human liver DNA, determined by an IA-gas chromatography (GC)-MS technique, ranged from 6×10^{-8} to 7×10^{-7} (molar ratio N^2,3-εG/G); similar levels were found in unexposed rats [14]. 1,N^2-εdG was also recently demonstrated in liver DNA of untreated female rats using on-line HPLC separation with MS/MS detection by multiple reaction monitoring (MRM) for direct quantification [15]. Low levels of ethenoguanines (1,N^2-εG and its isomer N^2,3-εG) have been detected in urine from unexposed healthy human subjects [16].

The IA-^{32}P-postlabelling method for εdA and εdC [7] has been used in several animal and human studies [8–11,17–29]. The levels found by the IA-^{32}P-postlabelling method were comparable with those found by radio-immunoassay [17]. Rodent studies reveal that the levels of εdA and εdC are significantly increased by cancer risk factors contributing to lipid peroxidation and oxidative stress. Increased εdA and εdC levels were observed due to copper accumulation; LPO and dietary curcumin treatment in LEC rats [18,19]; ageing in ROS-overproducing OXYS rats [20]; iron overloading [11,21]; overproduction of nitric oxide in mouse [22]; progression from benign to malignant in mouse skin and accumulation of arachidonic acid metabolites [23]; and glucose 6-phosphate dehydrogenase (G6PD) deficiency in mouse brain [24]. 1,N^6-ethenoadenine was first detected in rat urine after chloroethylene oxide exposure [30].

Several known cancer risk factors associated with oxidative stress increase the level of εdA and εdC in target organs in clinical situations. Patients suffering from genetic metal storage disorders like Wilson's disease and primary haemochromatosis [18,25], chronic pancreatitis [26], Crohn's disease, ulcerative colitis, and familial adenomatous polyposis [26,27] show an increased level of etheno-DNA adducts. A high ω-6-polyunsaturated fatty acid diet increased etheno-DNA adducts in women but not in men [28] and a significant inverse correlation was observed for εdA in WBCs and vegetable or vitamin E consumption in healthy women [29].

An immunohistochemical assay for εdA adducts in specific cells and tissue sections has been validated using 50 samples of patients affected by diseases predisposing to hepato-carcinogenesis. Excess hepatic DNA damage was seen in livers of all patients with alcohol-related hepatitis, fatty liver, fibrosis and cirrhosis as compared with asymptomatic livers. However, no change was observed in livers of patients with hepatitis [31].

Several methods have been developed for human urine analysis. Urinary levels of 1,N^6-ethenoadenine measured by liquid chromatography (LC)-ESI-MS/MS and isotope dilution GC-negative ion chemical ionization (NICI)-MS in healthy individuals were found to be in agreement [32]. A ^{32}P-postlabelling method has recently been reported for the measurement of εdC as deoxynucleoside in human urine [33]. MS-based methods appear to detect higher absolute adduct levels in normal subjects. In Japanese women urinary levels of εdA were associated with salt-induced inflammation and lipid peroxidation [34]; however, short-term fasting did not affect the urinary adduct levels in women [35]. Studies have revealed higher levels of urinary 3,N^4-ethenocytosine and εdC in smokers than in non-smokers [36,37].

Malondialdehyde-DNA adducts

Several analytical methods including ^{32}P-postlabelling, GC-MS, immuno-slot-blot and immunoassay have been developed to detect and quantify the M_1G adduct [5,38]. A study using a recently developed method with aldehyde-reactive probe labelling and LC-MS/MS, designed to mimimise the occurrence of artifactual oxidation, confirmed that the high levels of M_1G reported in animal tissues had been overestimated by 1–3 orders of magnitude. Background levels of M_1G in brain, liver, kidney, pancreas and lung of control rats were found to range from 0.8–1.8 $M_1G/10^8$ nucleotides [39]. The levels of M_1G detected in human liver and pancreas by GC-MS and in breast, lung and white blood cells by ^{32}P-postlabelling ranged from 2.2 adducts/10^9 to 1.1 adducts/10^6 nucleotides [10,40–44]. An ultrasensitive immuno-enriched ^{32}P-postlabelling method with a detection limit of 200 amol M_1dG from 10 μg of DNA, reproducibly detected background levels (6/10^9 nucleotides) of the adduct in human breast and liver tissue samples [45].

An early study using HPLC with fluorescence detection reported very high levels of M_1G-dR in rat and human urine [46], but a more recent study, using IA purification in combination with atmospheric pressure chemical ionization (APCI)-MS/MS, demonstrated much lower levels in healthy human volunteers [47].

Women and men on a high sunflower oil diet, rich in polyunsaturated fatty acids, showed increased M_1dG adduct levels compared with those on a rapeseed oil diet rich in monosaturated fatty acids [48]. An immuno-slot-blot technique [41] has been used in several human studies of larger sample size, to measure adduct levels in WBC, gastric antral mucosa and colorectal biopsies [41,49–51]. This immunoassay, which gave slightly lower values than an HPLC-^{32}P-postlabelling method, was used to study the relationship between diet and adenomas in human colorectal mucosa. Adduct levels were affected by diet and there was a trend for higher levels in individuals with adenomas [51].

Patients with hepatic metastases from colorectal cancer were treated with curcumin before surgery and no differences in M_1G adduct levels were found in the pre- and post-surgical samples of normal and malignant liver [52]. An immunohistochemical assay revealed higher MDA-DNA adducts (mean relative staining intensity) in smokers than in non-smokers with large inter-individual variations [53].

Propano-derived DNA adducts

Endogenously generated cyclic propano-dG adducts have been detected in tissues of rodents and humans with a ^{32}P-postlabelling method for Acr- and Cro-dG adducts. Acro- and Cro-dG adducts have been reported in untreated mice and rat liver and skin, lung, colon, brain, prostrate, kidney and mammary glands and in human breast and leukocytes [54,55]. Using a modified ^{32}P-HPLC method, specific adducts such as Acr-dG3 and Cro-dG1 have been detected in human lung, Acr-dG3 and Cro-DNA in colon, and total adducts in breast tissue [56]. An improved ^{32}P-postlabelling method with solid-phase extraction for adduct enrichment has recently been reported for the detection of all five enal-derived PdG adducts, i.e. Acr, Cro, Pen, Hep, and HNE adducts, in a single DNA sample and applied on rat liver DNA samples [57].

Cro-dG adducts were detected in the skin DNA of mice treated topically with crotonaldehyde [58]. A ^{32}P-postlabelling-HPLC method was used for detecting HNE-dG adducts and in CCl_4-treated F344 rat liver a significant increase in adducts was observed [59].

The level of adducts in human gingival tissue was found to be increased in smokers [60]. Using the modified ^{32}P-HPLC method referred to above for specific adducts such as Acr-dG3 and Cro-dG1, it was found that tumour tissues had higher levels of adducts than controls [56].

Conclusions

Over the years, evidence has accumulated that exocyclic lesions play a role in several human cancers [4]. The data suggest that promutagenic exocyclic DNA adducts could be useful biomarkers to investigate the potential role of oxidative stress and LPO in human cancers associated with certain lifestyles, chronic infections and inflammatory processes and to verify whether the levels of these adducts and associated malignancies can be reduced by chemopreventive strategies. The main gaps in validation are lack of inter-laboratory comparison using the same method and DNA samples, and confirmation of adduct levels using two different methods on the same DNA sample. The major problem could be the detection limits of different methods and the amount of DNA required for the analysis. For example, ^{32}P-postlabelling methods for etheno and propano adducts or the immuno-slot-blot technique for M_1dG require just a few micrograms of DNA, while the MS based methods require > 100 μg DNA for analysis. Eventually, the feasibility of applying these methods in larger studies in terms of time, effort and cost has to be evaluated. Several techniques that have been reported in the literature that have high sensitivity and specificity have not yet been applied in human studies, and the promising among them need to be validated in human pilot biomonitoring studies. As more assay methods suitable for use in human studies are identified, the relationships between oxidative stress factors and the levels of additional specific adducts or groups of adducts can be used more efficiently as surrogates to predict cancer endpoints.

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