Detection of Endogenous Malondialdehyde-Deoxyguanosine Adducts in Human Liver

Ajai K. Chaudhary, Munetaka Nokubo,* G. Ramachandra Reddy, Suresh N. Yeola, Jason D. Morrow, Ian A. Blair, Lawrence J. Marnett†

Endogenous DNA adducts may contribute to the etiology of human genetic disease and cancer. One potential source of endogenous DNA adducts is lipid peroxidation, which generates mutagenic carbonyl compounds such as malondialdehyde. A sensitive mass spectrometric method permitted detection and quantitation of the major malondialdehyde-DNA adduct, a pyrimidopurinone derived from deoxyguanosine. DNA from disease-free human liver was found to contain 5400 adducts per cell, a frequency comparable to that of adducts formed by exogenous carcinogens.

DNA damage is implicated in a variety of human diseases, including cancer (*1–6*). Until recently, it has been assumed that the major sources of DNA damage in humans are activated derivatives of exogenous chemicals (*7*). However, there is a growing realization that normal cells contain DNA adducts derived from electrophiles generated endogenously—for example, by oxidative metabolism and inflammation (*8–10*). Identification and quantitation of endogenous DNA adducts should provide an estimate of the baseline of DNA damage to which damage by exogenous chemicals can be compared. Once the biological potential of various endogenous and exogenous adducts has been evaluated, it may be possible to more realistically estimate the risk associated with exposure to a particular chemical.

Considerable attention has been focused on the extent and consequences of DNA modification by oxygen radicals and related reactive oxygen species (*11*, *12*). However, DNA is a quantitatively minor target for oxygen radical attack in intact cells (*13*). The polyunsaturated fatty acids of phospholipid membranes are likely to be the most abundant target for free radical attack (*14–16*). In fact, enhanced lipid peroxidation occurs in a variety of human diseases, and products of lipid peroxidation are detectable in tissues and fluids of healthy individuals (*17*). Thus, genotoxic products of lipid peroxidation may be important mediators of DNA damage associated with oxidative stress.

Malondialdehyde (MDA) is the most abundant carbonyl compound and the major mutagenic and carcinogenic product generated by lipid peroxidation (*18–23*). MDA reacts with deoxynucleosides in vitro to produce a variety of adducts, but at neutral pH the major adduct is a pyrimidopurinone produced from deoxyguanosine (M_1G-deoxyribose) (Fig. 1) (*24*, *25*). M_1G-deoxyribose has been detected after modification of calf thymus and plasmid DNA and has been implicated in the induction of G to T transversions by MDA (*26*). Thus, M_1G-deoxyribose is a biologically important adduct that is a potentially useful dosimeter of MDA-induced DNA modification.

To investigate whether M_1G-deoxyribose is present in human DNA, we studied liver tissue, which is sensitive to lipid peroxidation induced by a range of agents. Livers from six individuals (four male and two female) were obtained from Tennessee Donor Services and stored at −80°C (*27*). Purified DNA was prepared from isolated nuclei, digested to deoxynucleosides, and the purines released by mild acid hydrolysis. M_1G was converted to a pentafluorobenzyl (PFB) derivative that was analyzed by gas chromatography–electron capture negative chemical ionization mass spectrometry (GC-ECNCI MS). Quantitation was by selected ion monitoring relative to an internal standard of [$^2H_2$]M_1G-PFB (Fig. 2). The internal standard was characterized by absorption, nuclear magnetic resonance, and mass spectrometry (*25*).

Two peaks from endogenous M_1G were observed in the channel corresponding to m/z = 186 at 10.51 and 12.75 min. These signals arose from N7-PFB and N9-PFB derivatives, respectively. An interfering peak coeluted with the peak at 12.75 min, so analyses were carried out with only the N7-PFB derivative. In the selected ion current chromatogram of N7-M_1G-PFB from human liver DNA (Fig. 3), the retention times of the endogenous regioisomeric derivatives were 0.1 s longer than those from the dideuterated internal standard in the channel corresponding to m/z = 188; this difference was due to the expected isotope effect. M_1G was present in the DNA from all six individuals at levels ranging from 5 to 11 adducts per 10^7 bases. The average value was 9 adducts per 10^7 bases, which corresponds to 5400 adducts per cell. With each experiment, a sample of the dideuterated internal standard was subjected to the workup conditions in the absence of DNA to control for possible deuterium-hydrogen exchange. The isotopic content remained constant during the workup of all samples (99.9% [$^2H_2$]M_1G-deoxyribose).

To verify that the material coeluting with the authentic standard was a PFB derivative of M_1G, we prepared 9.3 mg of DNA from the liver of a 17-year-old male who died of AVM rupture and subjected it to the isolation and identification procedure without addition of the dideuterated internal standard. After derivatization of the sample, we recorded the mass spectra of the N7-PFB derivative (Fig. 4) and the N9-PFB derivative (*28*) and found that they were identical to those obtained from authentic standards. Thus, the material isolated from human liver reacts to form PFB derivatives that coelute with authentic standards and display identical mass spectra to that of M_1G-PFB. The absence of peaks at $m/z > 186$ (Fig. 4) suggests that the peak at 186 is not a fragment of a contaminant that coelutes with M_1G-PFB.

We then examined whether the M_1G detected in human liver DNA was generated artifactually during workup by performing two control experiments. In the first, we isolated DNA from rat liver in the presence of butylated hydroxytoluene and vitamin C (10 mM each), antioxidants that prevent lipid peroxidation during preparation of mi-

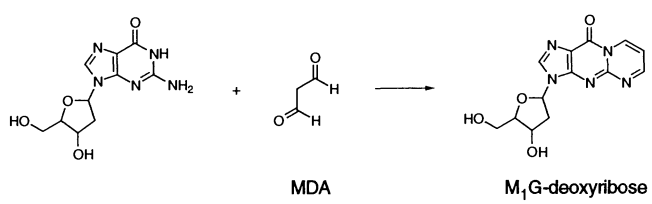

Fig. 1. Formation of M_1G-deoxyribose by reaction of MDA with 2′-deoxyguanosine.

A. K. Chaudhary, A. B. Hancock Jr. Memorial Laboratory for Cancer Research, and Departments of Pharmacology and Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232–0146, USA.
M. Nokubo, G. R. Reddy, L. J. Marnett, A. B. Hancock Jr. Memorial Laboratory for Cancer Research, and Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232–0146, USA.
S. N. Yeola, J. D. Morrow, I. A. Blair, A. B. Hancock Jr. Memorial Laboratory for Cancer Research, and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232–6602, USA.
*Present address: Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho Itabashi-ku, Tokyo 173, Japan.
†To whom correspondence should be addressed.

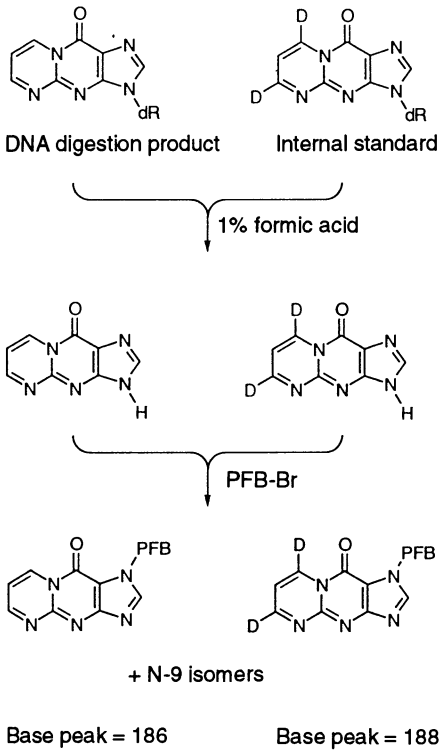

Fig. 2. Analytical method for determination of M_1G in DNA.

crosomal fractions and incubation of microsomal fractions with cumene hydroperoxide (*29*). We observed no decrease in the level of M_1G in the presence of the antioxidants. In the second control experiment, we added deoxyguanosine that had been labeled in the guanine ring with four ^{13}C atoms and one ^{15}N atom to the DNA hydrolysis mixture at an amount equivalent to that of the deoxyguanosine in the DNA. The mixture of deoxynucleosides was then carried through the workup and derivatization procedures and analyzed by GC-ECNCI MS. Any M_1G-deoxyribose formed by reaction of MDA with [^{13}C,^{15}N]deoxyguanosine during the time required for DNA hydrolysis would be detectable after workup as a

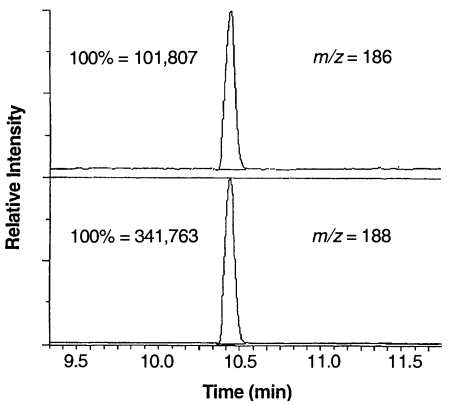

Fig. 3. Selected ion current chromatogram of human liver DNA analyzed for M_1G (*33*).

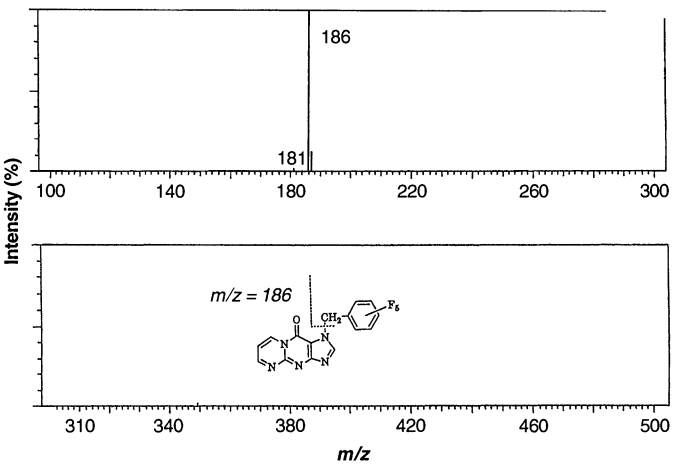

Fig. 4. GC-ECNCI mass spectrum of N7-M_1G-PFB isolated from human liver. DNA (9.3 mg) was isolated and subjected to enzymatic hydrolysis and C_{18} SPE purification as in Fig. 2 without the addition of dideuterated internal standard. The SPE eluate was purified by high-performance liquid chromatography (HPLC) with an octadecyl silyl column (4.6 mm by 250 mm, 5 μm; Beckman). Solvent A was 50 mM aqueous ammonium acetate and solvent B was acetonitrile. A linear gradient was run as follows: 6 to 10% B (0 to 5 min), 10 to 15% B (5 to 10 min), 15 to 20% B (10 to 13 min), and 20% B (13 to 15 min) at a flow rate of 1 ml/min. The fraction eluting between 12.5 and 14.5 min was collected and further purified with a C_{18} column (4.6 mm by 250 mm, 5 μm; Supelco). The mobile phase was acetonitrile:water (50:50) at a flow rate of 1 ml/min. The fraction eluting between 3 and 5 min was collected and evaporated to dryness. The derivatization, postderivatization cleanup, and GC-ECNCI MS conditions were as described in Fig. 2. A blank reaction was carried through all steps to monitor any carryover during HPLC purification.

PFB derivative with a base peak at m/z = 191. The selected ion current chromatogram at m/z = 191 revealed no material coeluting with the N7- or N9-PFB derivatives of M_1G. Thus, both control experiments indicate that M_1G is not generated artifactually during workup (*30*).

To test whether the M_1G detected in liver can be produced from endogenously generated MDA, we treated five Sprague-Dawley rats with 0.1 mg of CCl_4 per kilogram of body weight in corn oil (0.9 ml/kg) by oral intubation. Control animals received corn oil alone. Carbon tetrachloride administration induces intense lipid peroxidation in vivo and stimulates production of MDA and isoprostanes. Four days after CCl_4 administration, isoprostane levels in the liver of the treated rats increased 16-fold, whereas MDA levels increased 3.5-fold. Liver DNA from treated rats contained 3.8 ± 0.8 M_1G residues per 10^7 bases (n = 5), compared to 2.1 ± 0.5 per 10^7 bases in control animals (n = 5) ($P < 0.05$, Wilcoxon rank sum test).

These findings provide strong evidence that lipid peroxidation occurs continuously in healthy individuals and results in a substantial amount of endogenous DNA modification. The levels of M_1G (~one adduct per 10^6 bases) are comparable to those of 8-oxodeoxyguanosine, a major product of oxygen radical damage to DNA (*11*). This level is comparable to the highest levels of adducts reported in tissues from individuals exposed to exogenous carcinogens (for example, cigarette smoke–derived adducts in human lung) (*31*). Our ability to obtain direct mass spectroscopic confirmation of the presence of M_1G in human DNA suggests that it may be possible to identify the structures of other endogenous adducts that have been detected in human and rodent DNA (*32*). These structures may provide insight into the pathways of endogenous DNA damage that lead to cancer and other genetic diseases.

REFERENCES AND NOTES

1. E. C. Miller and J. A. Miller, *Pharmacol. Rev.* **18**, 805 (1966).
2. B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens* (Plenum, New York, 1983).
3. P. C. Hanawalt and A. Sarasin, *Trends Genet.* **2**, 124 (1986).
4. R. Fishet *et al.*, *Cell* **75**, 1027 (1993).
5. F. S. Leach *et al.*, *ibid.*, p. 1215.
6. N. Papadopolous *et al.*, *Science* **263**, 1625 (1994).
7. *Chemical Carcinogens. Second Edition, Revised and Expanded* (American Chemical Society, Washington, DC, 1984).
8. B. N. Ames and L. S. Gold, *Mutat. Res.* **250**, 3 (1991).
9. T. Lindahl, *Nature* **362**, 709 (1993).
10. L. J. Marnett and P. C. Burcham, *Chem. Res. Toxicol.* **6**, 771 (1993).
11. E.-M. Park *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3375 (1992).
12. J. R. Wagner, C.-C. Hu, B. N. Ames, *ibid.*, p. 3380.
13. R. A. Floyd, in *Proceedings of the First Oxygen Society Meeting*, K. A. J. Davies, Ed. (Pergamon, New York, in press).
14. P. A. Cerutti, *Science* **227**, 375 (1985).
15. V. E. Kagan, *Lipid Peroxidation in Biomembranes* (CRC Press, Boca Raton, FL, 1988).
16. T. A. Dix and J. Aikens, *Chem. Res. Toxicol.* **6**, 2 (1993).
17. J. D. Morrow *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9383 (1990).
18. F. Bernheim, M. L. C. Bernheim, K. M. Wilbur, *J. Biol. Chem.* **174**, 257 (1948).
19. H. Esterbauer, in *Free Radicals, Lipid Peroxidation and Cancer*, D. C. H. McBrien and T. F. Slater, Eds. (Academic Press, New York, 1982), pp. 101–128.
20. D. R. Janero, *Free Radical Biol. Med.* **9**, 515 (1990).
21. F. H. Mukai and B. D. Goldstein, *Science* **191**, 868 (1976).
22. A. K. Basu and L. J. Marnett, *Carcinogenesis* **4**, 331 (1983).
23. J. W. Spalding, *Nat. Toxicol. Prog. Tech. Rep.* **331**, 5 (1988).

24. H. Seto, T. Okuda, T. Takesue, T. Ikemura, *Bull. Chem. Soc. Jpn.* **56**, 1799 (1983).
25. L. J. Marnett *et al.*, *J. Am. Chem. Soc.* **108**, 1348 (1986).
26. M. Benamira *et al.*, *Carcinogenesis*, in press.
27. The four males were 17, 29, 35, and 36 years old and weighed 125, 130, 232, and 174 pounds, respectively, whereas the two females were 23 and 26 years old and weighed 185 and 111 pounds, respectively. The causes of death were motor vehicle accidents (3), cardiac arrest, cardiorespiratory arrest, and arteriovenous malformation (AVM) rupture.
28. A. K. Chaudhary *et al.*, unpublished observations.
29. T. A. Dix and L. J. Marnett, *Science* **221**, 77 (1983).
30. Further evidence against artifactual generation of M_1G-deoxyribose during workup is its absence in rat testis DNA (M. Nokubo and A. Chaudhary, unpublished observations). The limit of detection in this experiment was three adducts per 10^9 bases.
31. D. H. Phillips *et al.*, *Int. J. Cancer* **46**, 569 (1990).
32. K. Randerath *et al.*, *Mutat. Res.* **295**, 11 (1993).
33. Tissue samples were homogenized with a Dounce homogenizer, and nuclei were prepared by centrifugation at 500*g*. DNA was isolated by phenol-chloroform extraction, purified by treatment with proteinase K and ribonucleases A and T_1, and then digested with micrococcal nuclease, spleen phosphodiesterase, alkaline phosphatase, and adenosine deaminase. A sample of M_1G-deoxyribose (2 ng) that had been labeled in the pyrimido ring with deuterium was added to the DNA digest. The hydrolysate was applied to a solid phase extraction (SPE) column (Bondelute, Varian, C_{18}, 1 ml) and the column was washed with 1% aqueous CH_3OH (10 ml). M_1G-deoxyribose was eluted with 25% aqueous CH_3OH (0.5 ml) and evaporated to dryness under N_2. The residue was dissolved in formic acid (2.5%, v/v, 200 μl) and the solution was heated at 60°C for 45 min. M_1G was recovered by evaporation under vacuum. The residue obtained after evaporation of formic acid was dissolved in anhydrous CH_3OH (150 μl) containing K_2CO_3 (2 mM) and PFB-Br (7.5 μl) and the solution was stirred at room temperature. After 90 min, the contents were evaporated under N_2 and the residue was extracted with CH_2Cl_2 (0.5 ml). K_2CO_3 was removed by filtration through a 0.2-μm nylon filter and the CH_2Cl_2 solution was applied to an SPE column (Bondelute, Varian, silica, 3 ml, preconditioned with 10 ml of CH_2Cl_2). The column was washed with CH_3OH:CH_2Cl_2 (1:99, v/v, 3 ml) and the PFB derivatives were eluted with CH_3OH:CH_2Cl_2 (10:90, v/v, 3 ml). The solvent was removed by evaporation under N_2 and the residue was dissolved in ethyl acetate (15 μl) for GC-ECNCI MS analysis. GC-ECNCI MS was carried out on a Nermag R1010C instrument interfaced with a Varian Vista 6000 gas chromatograph with the use of a 7.5-m SPB 1701 fused silica capillary column (0.25 μm inside diameter, 0.25 μm film thickness; Supelco). Helium was used as the carrier gas at a pressure of 7×10^{-2} torr. Injections were made in splitless mode with the injector temperature held at 250°C. The instrument was operated with an accelerating potential of −70 eV; the source temperature was held at 260°C and the emission current at 250 μA. The column temperature was programmed from 100°C (held for 0.2 min) to 260°C at 25°C per minute and then to 290°C at 5°C per minute. Selected ion monitoring was performed for ions m/z = 186 and m/z = 188, representing the $[M\text{-}PFB]^-$ ions for M_1G-PFB and $[^2H_2]M_1$G-PFB, respectively.
34. We thank D. Cylc for technical assistance and J. Swenberg for helpful discussions. Supported by NIH grants GM42056 (to J.D.M.), CA47479 (to L.J.M.), and ES00267. J.D.M. is a Howard Hughes Medical Institute Physician Research Fellow and recipient of a Career Development Award from the International Life Sciences Institute.

8 April 1994; accepted 14 July 1994

Control of Angiogenesis in Fibroblasts by p53 Regulation of Thrombospondin-1

Kristina M. Dameron, Olga V. Volpert, Michael A. Tainsky, Noël Bouck*

As normal cells progress toward malignancy, they must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth. In cultured fibroblasts from Li-Fraumeni patients, this switch was found to coincide with loss of the wild-type allele of the *p53* tumor suppressor gene and to be the result of reduced expression of thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis. Transfection assays revealed that p53 can stimulate the endogenous TSP-1 gene and positively regulate TSP-1 promoter sequences. These data indicate that, in fibroblasts, wild-type p53 inhibits angiogenesis through regulation of TSP-1 synthesis.

Angiogenesis is controlled by the local balance between factors that stimulate new vessel growth and factors that inhibit it. In most normal tissues, inhibitory influences predominate, and cells derived from these tissues usually do not stimulate angiogenesis. In contrast, tumor cells, which must attract new vessels in order to grow and metastasize efficiently (*1*), are potently angiogenic as a result of decreased production of inhibitors and increased secretion of inducers (*2*). As normal cells undergo genetic changes that lead to malignancy they must switch from an inhibitory to an angiogenic phenotype. In this report we examine the role that the loss of the tumor suppressor gene *p53* plays in enabling human fibroblasts to become angiogenic.

The p53 protein is mutated in human tumors more frequently than any other known oncogene or suppressor gene [(*3*) and references therein]. In normal cells it is hypothesized to act as a transcriptional regulator (*4*), enhancing the expression of genes that contain specific p53-binding sites and interacting with a variety of transcription factors to inhibit the expression of other genes (*4*, *5*). Wild-type p53 protein can mediate a number of cellular activities, including apoptosis, the maintenance of genetic stability, G_1 growth arrest, cell differentiation, and the suppression of tumorigenicity (*3*, *5*). The up-regulation by p53 of the gene encoding p21, an inhibitor of G_1 cyclin-dependent kinases, appears to be responsible for p53-mediated growth arrest (*6*). The p53-regulated effector molecules responsible for the other cellular phenotypes dependent on wild-type p53 remain to be identified.

To examine the effect of p53 on angiogenesis, we used fibroblasts cultured from Li-Fraumeni patients (*7*). These individuals have inherited one wild-type (wt) and one mutant allele of the *p53* gene and as a result have an elevated risk of developing sarcomas and other tumors in which the remaining wt allele is inactivated (*8*). At early passage, the Li-Fraumeni fibroblasts are diploid and have one mutant and one wt *p53* allele. On continued passage in culture, however, they spontaneously lose their wt allele, become aneuploid and immortal (although not tumorigenic), and retain only the mutant allele of *p53* (*7*).

We previously showed (*9*) that hamster fibroblasts could switch to an angiogenic phenotype upon loss of an unidentified tumor suppressor gene by decreasing their se-

Table 1. Effect of Li-Fraumeni fibroblast conditioned media on corneal neovascularization. Serum-free conditioned media described in Fig. 1 were tested for the ability to induce neovascularization when incorporated into a noninflammatory pellet and implanted into the normally avascular rat cornea (*17*). Simultaneous controls showed that antibodies to TSP-1 were not angiogenic when tested alone (0/3 corneas positive) and not inhibitory when tested with bFGF (3/3 corneas positive).

Media source	Anti–TSP-1	Positive corneas/number implanted for patient		
		041	172	087
Early-passage cells	−	0/8	0/3	0/9
	+	3/3	3/3	5/7
Late-passage cells	−	4/4	3/3	11/11
Mix of early- and late-passage cells	−	0/7	0/3	0/13
	+	3/4	3/3	4/4
Early-passage cells (+ bFGF)	−	0/3	0/3	0/4
	+	3/3	3/3	4/4

K. M. Dameron, O. V. Volpert, N. Bouck, Department of Microbiology-Immunology and R. H. Lurie Cancer Center, Northwestern University Medical School, Chicago, IL 60611, USA.
M. A. Tainsky, Department of Tumor Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX 70030, USA.
*To whom correspondence should be addressed.