[CANCER RESEARCH 51, 5430–5432, October 1, 1991]

Advances in Brief

Major Alterations in the Nucleotide Structure of DNA in Cancer of the Female Breast[1]

Donald C. Malins and Russom Haimanot

Environmental Biochemistry Program, Pacific Northwest Research Foundation, Seattle, Washington 98122

Abstract

DNA of invasive ductal carcinomas from five women was analyzed for structural alterations in the purine nucleotides using gas chromatography-mass spectrometry with selected ion monitoring. The results were compared to those for a normal DNA control. The carcinoma DNA showed dramatically higher concentrations of the base modifications 8-hydroxyguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 8-hydroxyadenine. For example, the concentration of total identified base modifications represented a more than 9-fold increase over the control value. Base modifications of this type, which arise from radical-induced hydroxylation and cleavage reactions of the purine ring, likely play a major role in initiation and probably contribute to the further transformation of neoplastic cells in cancer of the female breast.

Introduction

There is abundant evidence suggesting that the hydroxyl radical (•OH) produces alterations in the structural integrity of the DNA bases (1–3). *In vivo*, unless the modified DNA is promptly repaired, miscoding may occur in replication (4) which may result in the formation of neoplastic cells (1, 2, 4). In this regard, a study with *Escherichia coli* (4) indicated that the 8-hydroxydeoxyguanosine residue, which arises from the attack of the •OH on the purine ring (5), has an overwhelming effect on template-directed DNA synthesis in that it causes misreplication at its own position as well as at neighboring base positions. Thus, there is persuasive evidence for the concept that the introduction of oxygen into nucleotide structure is an initiating step in mutagenesis and carcinogenesis (2, 4).

We have shown, using the English sole (*Parophrys vetulus*) carcinogenesis model (6), that the •OH-induced base modifications 8-OH-Gua,[2] Fapy-G, 8-OH-Ade, and Fapy-A (Fig. 1) can be structurally and quantitatively elucidated in tissues using GC-MS/SIM (7–9). Studies with this vertebrate model also demonstrated that substantial elevations in 8-OH-Gua, Fapy-G, 8-OH-Ade, and Fapy-A occurred in the DNA from hepatic carcinoma tissue compared to baseline concentrations in normal tissue (7–9). Thus, it was suggested that the DNA modifications (which had not previously been identified in any animal system) likely played a causative role in the formation of the carcinomas (7–9).

A recent study is noteworthy with respect to the role played by the •OH in modifying DNA. It was shown *in vitro* (10) that the •OH reacts with calf thymus DNA to yield a variety of nucleotide base derivatives such as 8-OH-Gua and Fapy-G. This reaction was mediated by the iron ion-dependent superoxide radical-generating system hypoxanthine/xanthine oxidase. The reaction produced, for example, 8.80 nmol Fapy-G/mg DNA. Inhibition of product formation occurred with mannitol, dimethyl sulfoxide, superoxide dismutase, and catalase, thus indicating that •OH was formed from H_2O_2 by an O_2^--assisted Fenton reaction. Low concentrations of base modifications in normal DNA, such as from calf thymus (10) and English sole (7–9), are readily identified at or near threshold detection limits using the GC-MS/SIM technique. In normal cells, a primary defense against •OH-induced elevations in DNA base modifications is provided by the glycosylases and other enzymes that participate in the excision repair process, as well as by antioxidants such as glutathione (11–13). However, excessive concentrations of the •OH overcome normal defense mechanisms and leave the DNA at risk from oxidative modifications (13) that are believed to be causally related to carcinogenesis (2). The process of •OH-induced DNA modification is reflected in the present findings on invasive ductal carcinoma of the female breast.

Materials and Methods

Excised carcinoma tissue from five female patients was shown microscopically to contain invasive ductal carcinomas; however, examination of the excised surgical margin tissue revealed no evidence for neoplasia, although there was some evidence for other microscopic changes (*e.g.*, fibrocytic). Residual carcinoma-containing and excised surgical margin tissue was placed in liquid nitrogen immediately after removal and maintained at −70°C prior to extraction of the DNA, which was undertaken as described previously (14). DNA was hydrolyzed and TMS derivatives were prepared under an atmosphere of pure nitrogen (15, 16). The TMS derivatives were analyzed by GC-MS/SIM (7–9, 15, 16) using a Hewlett-Packard Model 5890 microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5970B mass selective detector. The injector port and interface were both maintained at 250°C. The column was a fused silica capillary column (15.0 m; 0.2 mm inner diameter) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness, 0.33 μm). The column temperature was increased from 120 to 176°C at 3°C/min and from 176 to 250°C at 6°/min, after initially being held for 1.5 min at 120°C. Helium was used as the carrier gas with a linear velocity of 23.5 cm/s through the column (15, 16). The amount of TMS hydrolysate injected onto the column was about 0.7 μg. Quantitation of the modified nucleotide derivatives was undertaken on the basis of the principal ions, such as *m/z* 442 for the TMS derivative of Fapy-G (15, 16).

Fapy-A, 2,4,5-triamino-6-hydroxypyrimidine sulfate, and 8-bromoadenine were purchased from Sigma Chemical Co. and 8-OH-Gua was obtained from the Chemical Dynamics Corp. The 8-OH-Ade and Fapy-G were synthesized in our laboratories from 8-bromoadenine and 2,4,5-triamino-6-hydroxypyrimidine sulfate, respectively, and purified by recrystallization (17).

Received 7/17/91; accepted 8/16/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by United States Army Medical Research and Development Command Grant DAMD17-88-Z-8043.

[2] The abbreviations used are: 8-OH-Gua, 8-hydroxyguanine; Fapy-G, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; Fapy-A, 4,6-diamino-5-formamidopyrimidine; GC-MS/SIM, gas chromatography-mass spectrometry with selected ion monitoring; TMS, trimethylsilyl.

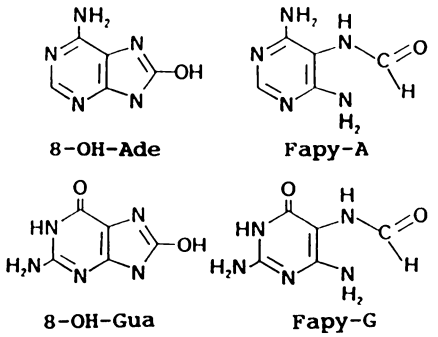

Fig. 1. Modifications arising from the attack of the •OH on C8 of the purine ring of adenine and guanine. As an example, the amine at position 6 of adenine promotes an electron deficiency at C-8 which results in the attack of the •OH, subsequent cleavage of the purine ring, and formation of the formamidopyrimidine structure (5, 8).

Results and Discussion

The cause of breast cancer is essentially unknown; however, cancer formation is likely to involve structural modifications in the nucleotide bases that affect template-directed DNA synthesis (4). Thus, it is significant that the present findings revealed dramatic differences in the concentrations of 8-OH-Gua, Fapy-G, and 8-OH-Ade with respect to the control and the carcinoma tissues (Fig. 2). The values for 8-OH-Gua, Fapy-G, and 8-OH-Ade in the control were 0.13 ± 0.03 (SD), 0.08 ± 0.08, and 0.22 ± 0.05 nmol/mg, respectively (Fig. 2). The respective values for the carcinoma tissues were 8- to 17-fold higher (1.26 ± 0.78, 1.33 ± 0.97, and 1.67 ± 1.86 nmol/mg DNA). In both the control and the carcinoma tissues, Fapy-A was present only at low levels near the limits of detection of the GC-MS/SIM technique (0.04 nmol/mg DNA) (Fig. 2). Accordingly, Fapy-A is not a prominent indicator of altered DNA in breast cancer in contrast to the other base modifications. There was not a significant difference between the calf thymus and surgical margin DNA with respect to any of the base modifications; however, a significant difference did exist between the DNA from the surgical margin and the carcinoma tissue with respect to 8-OH-Gua ($P \leq 0.01$), Fapy-G ($P \leq 0.03$), and 8-OH-Ade ($P \leq 0.05$). On a matched pair basis (surgical margin *versus* carcinoma), the concentrations of each of the above base modifications were substantially higher in the carcinoma, with the exception of FBT-5 which had relatively low concentrations of the base lesions (see Fig. 2 legend).

In studies with the English sole carcinogenesis model (7–9), we found that the relatively low concentrations of base modifications in normal tissues were within a relatively narrow range, close to the threshold of detection of the GC/MS-SIM method. In this regard, the present values with calf thymus DNA were not appreciably different from those obtained by ourselves (7–9) and other workers (10, 15, 16). Moreover, in an initial attempt to understand base level concentrations of the modified nucleotide derivatives in human tissues, we studied leukocytes from the blood of two apparently normal individuals. The values obtained were consistently low: 0.20 and 0.23; 0.12 and 0.14; 0.01 and 0.07; and 0.04 and 0.04 nmol/mg DNA for 8-OH-Gua, 8-OH-Ade, Fapy-G, and Fapy-A, respectively.[3] There is evidence to suggest that surgical margin tissue may not be microscopically normal (18). Nevertheless, as indicated, in terms of the DNA bases examined, the surgical margin DNA was not significantly different from the calf thymus DNA. Accordingly, the calf thymus data which have previously served as a standard for "normal DNA" (10, 15, 16) are compared to the carcinoma data in Fig. 2.

Overall, the present findings provide persuasive evidence for substantial •OH-induced alterations having taken place in the purine nucleotides of DNA from the breast carcinoma. Moreover, it seems unlikely that the radical attack on the DNA was essentially confined to 8-OH-Gua, Fapy-G, and 8-OH-Ade. It is probable, for example, that the pyrimidine nucleotides were also modified, although thus far we have not examined these modifications in sufficient detail to elucidate their relevance to carcinogenesis. Accordingly, the substantial base modifications reported are likely to reflect only a partial assessment of the oxidative changes inflicted on the breast DNA.

To our knowledge, the present study is the first to examine DNA base modifications in any mammalian tissue on a structural and quantitative basis and, as such, the findings provide a unique opportunity to evaluate their significance in relation to the pathobiology of breast cancer. In this respect, the presence of the relatively high concentration of 8-OH-Gua in the DNA of the carcinoma tissues seems especially relevant in view of the evidence demonstrating that 8-hydroxydeoxyguanosine has an overwhelming effect in causing misreplication in template-directed DNA synthesis (4). The significance of the other DNA modifications in this regard can be ascertained only from further studies. Considering the special need for maintaining the

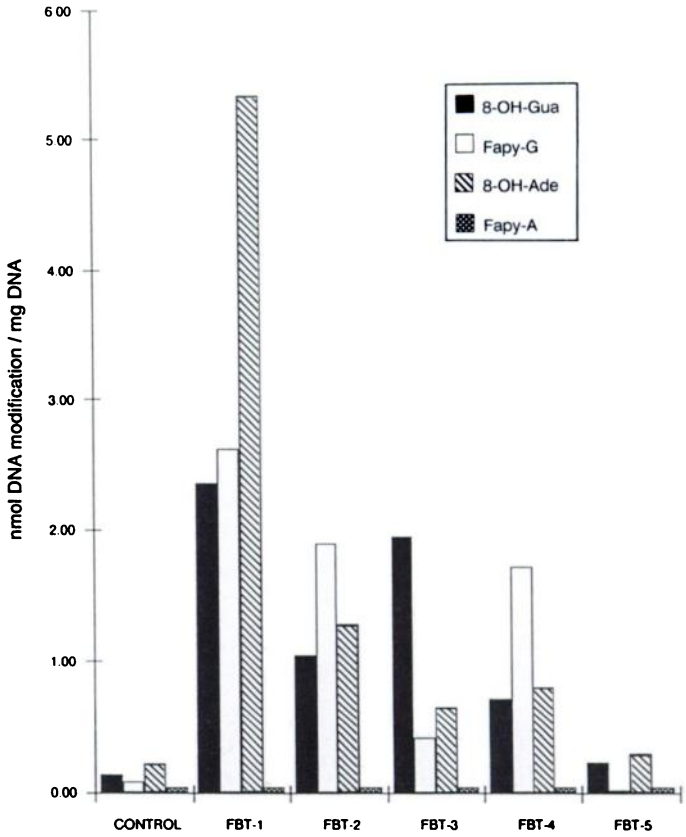

Fig. 2. DNA base modifications (nmol/mg DNA) in five female breast tumors (FBT) of the invasive ductal carcinoma type are compared to those of a (calf thymus) control. The DNA base modification concentrations represent an average of duplicate analyses by GC-MS/SIM. There were significant differences among the concentrations of 8-OH-Gua ($P \leq 0.01$), Fapy-G ($P \leq 0.02$), and 8-OH-Ade ($P \leq 0.05$) with respect to the carcinoma tissue and the control (five replicate analyses); however, the nucleotide base profile of FBT-5 was not significantly different, although the value for 8-OH-Gua was twice that of the control. The reason for this anomaly is not readily apparent, although it may be related to a paucity of neoplastic cells.

[3] D. C. Malins, R. Haimanot, and M. Bean, unpublished results.

structural integrity of DNA through enzymatic and other processes (11, 12), the substantial •OH-induced modifications in this molecule are likely to be causally related to the neoplastic transformations in the breast. However, the origin of the •OH that potentially initiates the base modifications is unclear, although one possibility is that this radical arises from H_2O_2 generated through the cytochrome P-450-mediated oxidation of estrogen (19).

A salient feature of tumor cells is their constitutive propensity to generate high concentrations of H_2O_2 (20). This suggests that the •OH attack on DNA in the carcinoma itself may be associated with escalations in H_2O_2 concentrations and subsequent formation of the •OH. That is to say, at some stage in tumor development, transformed cells become in effect "autotmutagenic," bringing about progressive alterations in the DNA as a consequence of constitutive H_2O_2 generation. Moreover, on the basis of the present results, the "antioxidant" capability attributed to tumor cells (21) may be accounted for, at least in part, by trapping of the •OH radical through its reactions with DNA. For example, in sample FBT-1 (Fig. 2) the combined concentrations of 8-OH-Gua and 8-OH-Ade alone are equivalent to 7.7 nmol of H_2O_2 or •OH, assuming a 1:1 mol ratio (5). In addition, the resistance to lysis associated with neoplastic cells (22) may also reflect to some degree the presently demonstrated tendency for DNA to act as a trapping agent for the •OH, which has well known cytolytic properties (23).

Studies of H_2O_2 generation in tumor cells (20) suggest that the relatively high concentrations of this oxidant lead to genetic instability (20, 24, 25). In this regard, the present findings suggest that the DNA base structure in the breast carcinoma is progressively modified by the •OH and is thus likely to produce a variety of genetically altered cell types, to possibly include those capable of metastasis. Overall, it seems likely that the •OH-induced base modifications of DNA found in female breast tumors will also be manifested by other types of cancer. In addition, the sensitive GC/MS-SIM methodology should prove to be valuable as a diagnostic tool for predicting, through DNA base modifications, the occurrence of cancer in a variety of tissues (9).

Acknowledgments

Appreciation is expressed to The Laboratory of Pathology of Seattle, Inc., for breast tissue and to Drs. William B. Hutchinson, Michael Bean, Eric Holmes, and John Houck for consultation.

References

1. Floyd, R. A., Watson, J. J., Harris, J., West, M., and Wong, P. K. Formation of 8-hydroxydeoxyguanosine, hydroxyl free radical adduct of DNA in granulocytes exposed to the tumor promoter, tetradecanoylphorbolacetate. Biochem. Biophys. Res. Commun., *137:* 841–846, 1986.
2. Troll, W., and Weisner, R. The role of oxygen radicals as a possible mechanism of tumor promotion. Annu. Rev. Pharmacol. Toxicol., *25:* 509–528, 1985.
3. Kasai, H., Nishimura, S., Kurokawa, Y., and Hayashi, Y. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. Carcinogenesis (Lond.), *8:* 1959–1961, 1987.
4. Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., and Nishimura, S. Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. Nature (Lond.), *327:* 77–79, 1987.
5. Steenken, S. Purine bases, nucleosides and nucleotides: aqueous solutions redox chemistry and transformation of their radical cations and e^- and OH adducts. Chem. Rev., *89:* 503–520, 1989.
6. Malins, D. C., McCain, B. B., Myers, M. S., Brown, E. W., Krahn, M. M., Roubal, W. T., Schiewe, M. L., Landahl, J. T., and Chan, S-L. Field and laboratory studies of the etiology of liver neoplasms in marine fish from Puget Sound. Environ. Health Perspect., *71:* 5–16, 1987.
7. Malins, D. C., Ostrander, G. K., Haimanot, R., and Williams, P. A novel DNA lesion in neoplastic livers of feral fish: 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Carcinogenesis (Lond.), *11:* 1045–1047, 1990.
8. Malins, D. C., and Haimanot, R. 4,6-Diamino-5-formamidopyrimidine, 8-hydroxyguanine and 8-hydroxyadenine in DNA from neoplastic liver of English sole exposed to carcinogens. Biochem. Biophys. Res. Commun., *173:* 614–619, 1990.
9. Malins, D. C., and Haimanot, R. The etiology of cancer: hydroxyl radical-induced DNA lesions in histologically normal livers of fish from a population with liver tumors. Aquat. Toxicol., *20*:123–130, 1991.
10. Auroma, O. I., Halliwell, B., and Dizdaroglu, M. Iron ion-dependent modification of bases of DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. J. Biol. Chem., *264:* 13024–13028, 1989.
11. Lindahl, T. DNA repair enzymes. Annu. Rev. Biochem., *51:* 61–87, 1982.
12. Wallace, S. S. AP endonucleases and DNA glycosylases that recognize oxidative DNA damage. Environ. Mol. Mutagen., *12:* 431–477, 1988.
13. Cotgreave, I. A., Moldeus, P., and Orrenius, S. Host biochemical defense mechanisms against prooxidants. Annu. Rev. Pharmacol. Toxicol., *28:* 189–212, 1988.
14. Dunn, B. P., Black, J. J., and Maccubbin, A. ^{32}P-Postlabeling analysis of aromatic DNA adducts in fish from polluted areas. Cancer Res., *47:* 6543–6548, 1987.
15. Dizdaroglu, M., and Bergtold, D. Characterization of free radical-induced base damage in DNA at biologically relevant levels. Anal. Biochem., *156:* 182–188, 1986.
16. Dizdaroglu, M., and Gajewski, E. Selected ion mass spectrometry: assays of oxidative DNA damage. Methods Enzymol., *186:* 530–544, 1990.
17. Cavalieri, L. F., and Bendich, A. The ultraviolet absorption spectra of pyrimidines and purines. J. Am. Chem. Soc., *7:* 2587–2594, 1950.
18. Ricketts, D., Turnbull, L., Ryall, G., Bakhshi, R., Rawson, N. S. B., Gazet, J-C., Nolan C., and Coombs, R. C. Estrogen and progesterone receptors in the normal female breast. Cancer Res., *51:* 1817–1822, 1991.
19. Deodutta, R., Floyd, R.A., and Liehr, J.G. Elevated 8-hydroxydeoxyguanosine levels in DNA of diethylstilbesterol-treated Syrian hamsters: covalent DNA damage by free radicals generated by redox cycling of diethylstilbestrol. Cancer Res., *51:* 3882–3885, 1991.
20. Szatrowski, T. P., and Nathan, C. F. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res., *51:* 794–798, 1991.
21. Nathan, C. F., Arrick, B. A., Murray, H. W., DeSantis, N. M., and Cohn, Z. A. Tumor cell anti-oxidant defenses: inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. J. Exp. Med., *153:* 766–782, 1981.
22. O'Donnel-Tormey, J., DeBoer, C. J., and Nathan, C. F. Resistance of human tumor cells *in vitro* to oxidative cytolysis. J. Clin. Invest., *76:* 80–86, 1985.
23. Ward, P. A., Till, G. O., Kunkel, R., and Beauchamp, C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J. Clin. Invest., *72:* 789–801, 1983.
24. Cifone, M. A., and Fidler, I. J. Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc. Natl. Acad. Sci. USA, *78:* 6949–6952, 1981.
25. Megidish, T., and Mazurek, N. A mutant protein kinase C that can transform fibroblasts. Nature (Lond.), *342:* 807–811, 1989.