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DNA base modifications in chromatin of human cancerous tissues

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Free radical-induced damage to DNA in vivo is implicated to play a role in carcinogenesis. Evidence exists that DNA damage by endogenous free radicals occurs in vivo, and there is a steady-state level of free radical-modified bases in cellular DNA. We have investigated endogenous levels of typical free radical-induced DNA base modifications in chromatin of various human cancerous tissues and their cancer-free surrounding tissues. Five different types of surgically removed tissues were used, namely colon, stomach, ovary, brain and lung tissues. In chromatin samples isolated from these tissues, five pyrimidine-derived and six purine-derived modified DNA bases were identified and quantitated by gas chromatography/mass spectrometry with selected-ion monitoring. These were 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, 5-(hydroxymethyl)uracil, 5-hydroxycytosine, 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine, 8-hydroxyadenine, xanthine, 2-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 8-hydroxyguanine. These compounds are known to be formed typically by hydroxyl radical attack on DNA bases. In all cases, elevated amounts over control levels of modified DNA bases were found in cancerous tissues. The amounts of modified bases depended on the tissue type. Lung tissues removed from smokers had the highest increases of modified bases above the control levels, and the highest overall amounts. Colon cancer tissue samples had the lowest increases of modified bases over the control levels. The results clearly indicate higher steady-state levels of modified DNA bases in cancerous tissues than in their cancer-free surrounding tissues. Some of these lesions are known to be promutagenic, although others have not been investigated for their mutagenicity. Identified DNA lesions may play a causative role in carcinogenesis.

Oxidative damage; Free radicals; DNA damage; Hydroxyl radical; Mutation

1. INTRODUCTION

Oxygen-derived species such as superoxide radical ($O_2^{\cdot-}$), H_2O_2, singlet oxygen and hydroxyl radical ($^{\cdot}OH$) are well-known to be cytotoxic, and they may be implicated in the etiology of a number of human diseases including cancer (reviewed in [1,2]). The superoxide radical is formed in almost all aerobic cells [3]. Any living system producing $O_2^{\cdot-}$ is expected to produce H_2O_2 by chemical or enzymatic dismutation of $O_2^{\cdot-}$. Endogenously generated oxygen-derived species may cause damage to biological molecules, including DNA, by a variety of mechanisms (reviewed in [4]). Under physiological conditions, however, neither $O_2^{\cdot-}$ nor H_2O_2 appears to produce modifications in DNA unless metal ions are present in the system [1,4]. Thus much of the toxicity of $O_2^{\cdot-}$ and H_2O_2 is thought to result from their metal ion-catalyzed conversion into highly reactive $^{\cdot}OH$ [1,4]. The hydroxyl radical produces a unique and extensive pattern of chemical modifications in DNA and nucleoprotein, including modified bases and DNA–protein cross-links (reviewed in [4–7]). Such DNA lesions may be promutagenic and may play a role in carcinogenesis [1,4,8,9]. Evidence exists that DNA damage by endogenous free radicals occurs and accumulates in vivo, and that there is a steady-state level of free radical-modified bases in cellular DNA [10–16]. Continuous endogenous damage to cellular DNA by free radicals and accumulation of such damage has been suggested to significantly contribute to carcinogenesis in humans [17–19]. Because of their ability to damage DNA, free radicals are thought to be involved in all stages of carcinogenesis [2,4,9]. Understanding the role of free radicals at the molecular level may lead to an understanding of cancer related to free radicals. Such an understanding in turn will depend on the characterization of the nature of DNA modifications caused by free radicals in living systems.

Chemical characterization of free radical-induced modifications in pyrimidine and purine bases of DNA can be achieved by a method incorporating the technique of gas chromatography/mass spectrometry with

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Abbreviations: $O_2^{\cdot-}$, superoxide radical; $^{\cdot}OH$, hydroxyl radical; 5-OH-5-Me-Hyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-Cyt, 5-hydroxycytosine; 5-OHMe-Ura, 5-(hydroxymethyl)uracil; 5,6-diOH-Ura, 5,6-dihydroxyuracil; FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring.

selected-ion monitoring (GC/MS-SIM), which can be applied to DNA itself or directly to chromatin [7,20,21]. By this method, a large number of pyrimidine- and purine-derived modified bases has been shown to be endogenously present in chromatin of cultured mammalian cells, including human cells [22–25]. The same method has recently been applied to show increases over control levels in the amounts of four purine-derived modified bases in DNA from neoplastic livers of feral fish exposed to carcinogens [26–28], and in the amounts of three purine-derived modified bases in DNA from cancerous female breast tissues [29].

In the present work, we have investigated endogenous levels of typical free radical-modified pyrimidines and purines of DNA in chromatin samples isolated from various human cancerous tissues and their cancer-free surrounding tissues.

2. MATERIALS AND METHODS

2.1. *Materials*

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Authentic compounds were purchased or synthesized as described previously [22,23]. Xanthine was purchased from Sigma Chemical Co. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were obtained from Pierce Chemical Co. Formic acid was from Mallinckrodt.

2.2. *Human tissues and isolation of chromatin*

The human tissues were obtained from Departments of Surgery, Medical School, Bydgoszcz, Poland, during surgery on cancer patients. Approximately 1–1.5 g of tissues were collected for each chromatin isolation. After surgical removal, cancerous tissues and their respective surgical margins were quickly frozen in liquid nitrogen and maintained in this condition until the isolation of chromatin. Histopathological evaluation of surgically removed tissues revealed that the surgical margin tissues were tumor free, while cancerous tissues exhibited the following characteristics: brain cancer (male patient), fibrillary astrocytoma; lung cancer I (female patient, smoker), squamous cell carcinoma; lung cancer II (female patient, smoker), adenocarcinoma; stomach cancer (male patient), mucinous carcinoma; ovary cancer (female patient), serous cystadenocarcinoma; colon cancer (male patient), lymphogranuloma (Hodgkin's lymphoma). Except for colon cancer, all tumors were primary tumors. Equal amounts (1 g) of cancerous tissues and their surrounding cancer-free tissues were used for chromatin isolation. Five separate tissue samples collected from the same tissue type were used. Frozen tissues were ground in porcelain grinders, and then suspended in sucrose buffer (0.25 M sucrose, 3 mM $CaCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM dithiothreitol, 50 mM Tris-HCl at pH 7.4) and homogenized in a Teflon-glass homogenizer. Subsequently, samples were filtered through a nylon sieve. Chromatin was then isolated as described previously [22].

2.3. *Hydrolysis, trimethylsilylation and gas chromatography/mass spectrometry*

To aliquots of chromatin samples (in 1 mM Tris buffer) containing 0.1 mg of DNA, 1 nmol of 6-azathymine and 2 nmol of 8-azaadenine were added as internal standards. Samples were then lyophilized. Samples of chromatin were hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min [25]. Samples were lyophilized and then trimethylsilylated with 0.1 ml of a BSTFA/acetonitrile (4/1; v/v) mixture in poly(tetrafluoro-ethylene)-capped hypovials under nitrogen at 130°C for 30 min. Analysis of derivatized samples was performed by GC/MS-SIM as described previously [22,25]. An aliquot (4 μl) of each derivatized sample was injected without any further treatment into the injection port of the gas chromatograph by means of an autosampler. A split ratio of 1:20 was used, resulting in ≈0.2 μg of hydrolyzed and derivatized DNA going through the GC column for each analysis.

3. RESULTS

The objective of this work was to examine whether typical free radical-modified pyrimidines and purines occur in chromatin of various human cancerous tissues and their cancer-free surrounding tissues, and to compare the quantities of these modified DNA bases in both types of tissues. Previously, elevated amounts over control levels of three purine-derived modified bases in DNA isolated from cancerous tissues of human female breast were observed [29]. This study was limited to determination of three purine lesions in DNA isolated from one type of human cancerous tissue. DNA as a whole was isolated and analyzed. In the present study, by contrast, we have examined chromatin isolated from five different human cancerous tissues and their cancer-free surrounding tissues. Moreover, five pyrimidine-derived and six purine-derived modified bases were identified and quantitated. The modified bases and their quantities are given in Tables I and II. We also searched for two other products, namely thymine glycol and 5-hydroxyuracil, however, we were unable to identify the presence of these compounds in the tissue samples examined. Fig. 1 illustrates the structures of the modified bases dealt with in the present work. 5-Hydroxycytosine (5-OH-Cyt) and 5-hydroxyuracil result from acid-induced modification of cytosine glycol; the former by dehydration and the latter by deamination and dehydration [30]. Similarly, 5,6-dihydroxyuracil (5,6-diOH-Ura) is formed by deamination of 5,6-dihydroxycytosine [30]. In addition to the modified bases found in chromatin in our previous publications [22–25], we have identified xanthine in the present work. This compound may arise from deamination of guanine, however, there is also a possibility that it may be formed by attack of $^{\bullet}OH$ at the carbon-2 of guanine.

Lung and colon cancerous tissues had the highest and the lowest increases, respectively, in the amounts of modified bases over the control amounts (Tables I and II). Cancer-free lung tissues from two patients had almost equal amounts of each modified base with the exception of two, namely 5-OH-5-Me-Hyd and 5,6-diOH-Cyt (Table I). Up to ≈7-fold increases in the amounts of both pyrimidine- and purine-derived lesions were observed in cancerous lung tissues with respect to their cancer-free surrounding tissues. In terms of increased amounts of lesions, there were differences between the two lung tissues examined. Essentially no

Table I

Amounts* of modified DNA bases (molecules per 10^5 DNA bases) in human tissues

Base	Brain		Lung I		Lung II	
	Control	Cancerous	Control	Cancerous	Control	Cancerous
5-OH-5-Me-Hyd	1.65±0.09	2.01±0.30	1.82±0.17	4.98±0.88*	6.10±0.55	10.5±1.27*
5-OH-Hyd	2.77±0.04	4.35±0.54*	2.87±0.60	9.32±1.78*	3.75±0.39	14.5±1.39*
5-OHMe-Ura	0.41±0.07	0.30±0.06	0.74±0.08	1.35±0.10*	0.71±0.07	1.03±0.11*
5-OH-Cyt	1.01±0.14	1.16±0.23	0.67±0.13	0.85±0.13	0.40±0.07	1.78±0.25*
5,6-diOH-Ura	0.49±0.08	0.99±0.17*	0.31±0.05	1.21±0.32*	1.16±0.18	3.87±0.70*
FapyAde	0.55±0.04	0.94±0.04*	0.54±0.06	1.16±0.16*	0.74±0.05	1.47±0.20*
8-OH-Ade	0.39±0.05	1.17±0.25*	3.06±0.15	3.60±0.88	3.12±0.10	8.04±0.85*
Xanthine	5.86±0.48	7.80±0.73	3.35±0.32	4.82±0.72	5.09±1.06	15.3±2.61*
2-OH-Ade	0.27±0.04	1.18±0.23*	0.75±0.22	5.52±0.70*	1.14±0.27	6.04±1.27*
FapyGua	1.03±0.07	3.30±0.62*	1.17±0.12	1.50±0.03*	0.96±0.10	3.07±0.68*
8-OH-Gua	1.36±0.03	2.49±0.47*	9.68±0.46	10.1±1.00	7.33±0.97	23.0±4.88*

*Each value represents the mean ± S.E.M. from measurements of chromatin samples isolated from five separate tissue samples.
*Significantly different from controls ($P \leq 0.05$ by Student's t-test).

increases were observed in the amounts of 8-hydroxypurines, i.e. 8-OH-Ade and 8-OH-Gua, in lung cancerous tissue I. In both lung cancerous tissues, the highest increases over the control levels were in the amounts of 2-OH-Ade (≈7- and ≈5-fold). The lung cancerous tissue II had the highest amounts of almost all modified bases when compared with other types of cancers examined. Stomach, ovary and brain cancerous tissues also had significantly higher amounts of lesions in their chromatin than their respective cancer-free surrounding tissues. In these cases, the highest increases over control levels were observed in the amounts of 8-OH-Gua (≈5-fold), 5-OH-Cyt (≈5-fold) and 2-OH-Ade (≈4-fold), respectively. Colon cancerous tissues had significant increases of modified bases only in a few instances. In most cases, the amount of each modified base was similar in cancer-free tissues. The most variable amounts of modified bases in cancer-free tissues were those of 8-OH-Ade and 8-OH-Gua, ranging from 0.39 to 3.12 8-OH-Ade/10^5 DNA bases, and from 0.94 to 9.68 8-OH-Gua/10^5 DNA bases. The amounts of 8-hydroxypurines were higher than those of formamidopyrimidines in both cancer-free and cancerous tissues of lung, stomach, and ovary. Colon and brain tissues had similar amounts of these compounds. These types of DNA products may arise from respective one-electron oxidation and reduction of carbon-8-OH adduct radicals of purines, which are formed by the addition of $^{\bullet}OH$ to the carbon-8 of purines [31].

4. DISCUSSION

The results obtained in the present work indicate that typical $^{\bullet}OH$-mediated products of DNA bases occur in

Table II

Amounts* of modified DNA bases (molecules per 10^5 DNA bases) in human tissues

Base	Colon		Stomach		Ovary	
	Control	Cancerous	Control	Cancerous	Control	Cancerous
5-OH-5-Me-Hyd	2.67±0.22	4.18±0.27*	2.01±0.29	3.19±0.79	1.95±0.20	6.42±0.78*
5-OH-Hyd	1.69±0.23	3.34±0.41*	1.87±0.14	3.27±0.04*	3.48±0.75	12.7±2.36*
5-OHMe-Ura	0.39±0.01	0.47±0.09	0.13±0.03	0.33±0.04*	0.63±0.19	3.17±0.32*
5-OH-Cyt	0.44±0.03	0.54±0.06	0.69±0.07	0.59±0.09	0.25±0.06	1.35±0.45*
5,6-diOH-Ura	0.41±0.09	0.92±0.06*	0.46±0.09	1.29±0.32*	0.43±0.08	1.55±0.44*
FapyAde	0.87±0.15	0.74±0.17	0.35±0.03	0.81±0.02*	0.81±0.19	1.08±0.18
8-OH-Ade	1.06±0.11	1.34±0.26	0.70±0.12	2.32±0.37*	1.49±0.31	3.32±0.48*
Xanthine	5.17±0.85	4.13±0.41	2.17±0.46	5.33±0.96*	6.84±0.51	9.68±1.13*
2-OH-Ade	0.61±0.11	0.64±0.11	0.23±0.06	0.42±0.02*	0.50±0.07	1.66±0.07*
FapyGua	2.27±0.07	2.66±0.72	0.55±0.09	1.65±0.39*	1.43±0.19	2.94±0.52*
8-OH-Gua	2.71±0.13	4.43±0.37*	0.94±0.19	5.08±1.02*	3.11±0.71	9.20±2.43*

*Each value represents the mean ± S.E.M. from measurements of chromatin samples isolated from five separate tissue samples.
*Significantly different from controls ($P \leq 0.05$ by Student's t-test).

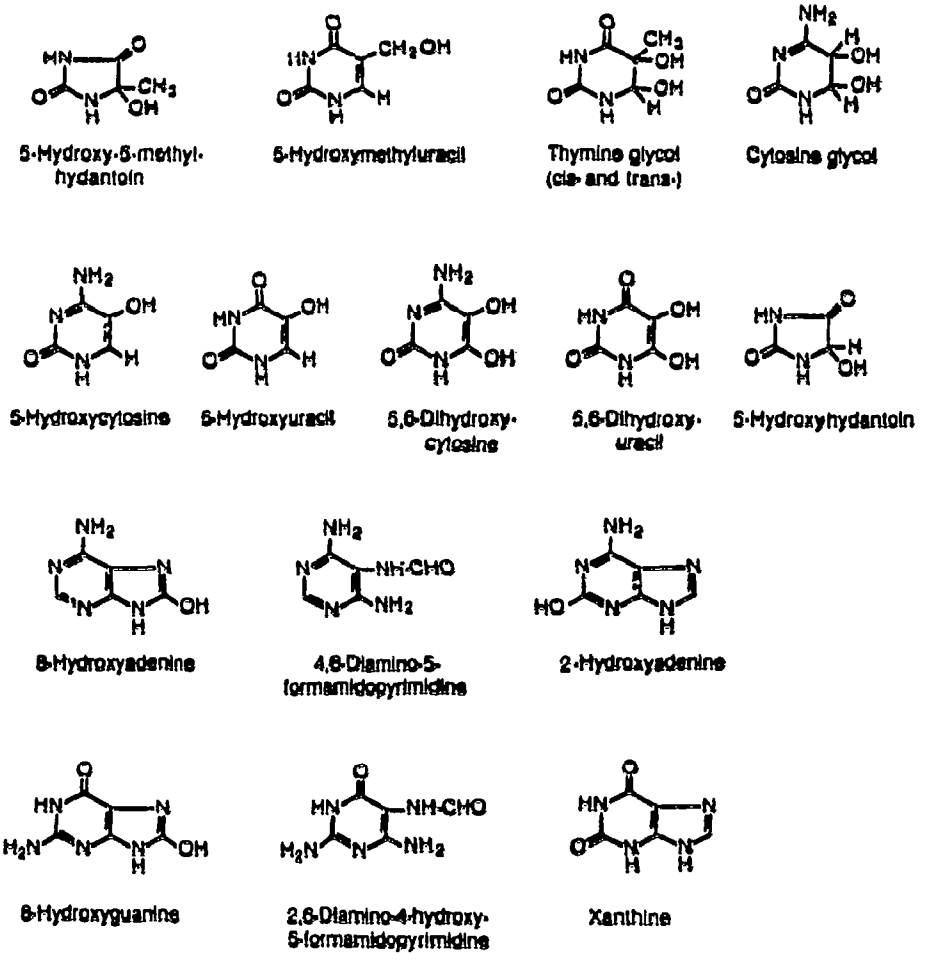

Fig. 1. Structures of the modified bases.

chromatin of both cancer-free and cancerous human tissues of various types. In all six tissue types examined, the endogenous amounts of most pyrimidine- and purine-derived DNA lesions in cancerous tissues were found to be consistently higher than in their respective cancer-free surrounding tissues. Our results are consistent with a recent observation that the amounts of three purine-derived modified bases in cancerous tissues isolated from breasts of several human female patients were higher than control levels [29], however, our study was not limited to one type of tissue or to a limited number of purine-derived lesions. We have examined six types of tissues, and have identified and quantitated eleven modified bases. In the present study, the increases in the amounts of modified bases over the control levels were no more than ≈7-fold in all tissue samples examined, as opposed to ≈17-fold observed previously in one type of female breast cancer tissue [29]. In contrast to the previous study mentioned above, we have also found significantly increased amounts of FapyAde over control levels in four types of tissues.

It is not known whether the DNA base lesions identified in the present work play a role in carcinogenesis, or are formed in greater amounts in cancerous tissues than in their cancer-free tissues as a result of the disease. There is little doubt that free radicals and DNA damage resulting from free radical reactions play an important role in carcinogenesis (reviewed in [1,32]). In the same context, a number of free radical-induced DNA base lesions have been examined for their biological consequences, and some of them have been found to possess mutagenic properties [8,33–40]. The majority of modified bases identified in the present work, however, have not been investigated for their biological consequences. It is conceivable that these lesions, too, may be promutagenic. DNA lesions measured here (except for xanthine) are known to be produced in DNA (as a whole) or in chromatin of mammalian cells upon treatment of cells with mutagenic agents such as hydrogen peroxide or ionizing radiation [11,24,25,41–48]. All these facts mentioned above indicate a possible causative role for free radical-modified bases in carcinogenesis.

Elevated levels of modified bases in cancerous tissues may be due to the production of large amounts of H_2O_2, which has been found to be characteristic of human tumor cells [49]. It is known that H_2O_2 treatment of mammalian cells causes formation of the herein identified DNA lesions in their chromatin, most likely via site-specific $^{\bullet}OH$ production [24]. Furthermore, evidence exists that tumor cells have abnormal levels and activities of antioxidant enzymes when compared with their respective normal cells (reviewed in [32]). Low levels of antioxidant enzymes, such as superoxide dismutase or catalase, in tumor cells may cause accumulation of $O_2^{\bullet-}$ and H_2O_2 with subsequent $^{\bullet}OH$-induced damage to DNA, resulting in greater amounts of modified DNA bases in tumor cells than in normal cells. Differences in the activities of antioxidant enzymes among individual tumors may account for the different amounts of modified DNA bases that were found in various cancerous tissues in the present work. In this respect, two lung cancerous tissues had the greatest amounts of modified DNA bases among the tissues examined here. This may be attributed in part to the fact that both patients were heavy smokers. Epidemiological studies have shown that smoking is the major cause of human lung cancer [50]. Cigarette smoke is known to cause DNA damage in human lung cells and cigarette smoke-induced generation of H_2O_2 with subsequent formation of $^{\bullet}OH$ has been implicated in causing DNA damage [51–53].

The types of role that individual free radical-induced DNA base modifications play in carcinogenesis are not known, and remain to be determined. High levels of modified DNA bases observed in various human tumor cells may be in part a result of the disease. Whatever the reason is for this phenomenon, high levels of modified DNA bases may contribute to the genetic instability of tumor cells, and thus to increased metastatic potential [54,55]. Epidemiological studies involving measurement of typical free radical-modified DNA bases in a large variety of individual tumor tissues and their respective normal tissues may provide insight into mechanisms of carcinogenesis related to oxygen-derived species. Measurement of pyrimidine- and purine-derived DNA lesions in tissues as described herein may prove to be useful in determining an association between free radical-producing agents and cancer risk.

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