DNA Methylation at the C-5 Position of Cytosine by Methyl Radicals: A Possible Role for Epigenetic Change during Carcinogenesis by Environmental Agents

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During carcinogenesis, methylation of the C-5 position of cytosines in the promoter region of tumor suppressor genes is often observed. Enzymatic DNA methylation is a widely accepted mechanism for this phenomenon. It is interesting to propose a free radical mechanism for 5-methyldeoxycytidine (m^5dC) production, because the C-5 position of cytosine is an active site for free radical reactions. When deoxycytidine (dC) and cumene hydroperoxide (CuOOH), a tumor promoter and a methyl radical producer, were reacted in the presence of ferrous ion at pH 7.4, the formation of m^5dC was observed. The same reaction also proceeded with *t*-butyl hydroperoxide (BuOOH). The formation of m^5dC was also observed in DNA by the CuOOH treatment. This is the first report of chemical DNA methylation at cytosine C-5 by environmental tumor promoters. We propose here that this reaction is one of the important mechanisms of de novo DNA methylation during carcinogenesis, because methyl radicals are produced by the biotransformation of various endogenous and exogenous compounds.

Introduction

DNA methylation is an important epigenetic mechanism of transcriptional control and plays an essential role in maintaining normal cellular function. During carcinogenesis, the methylation of CpG islands in the promoter regions of tumor suppressor genes occurs, and this can lead to a loss of the gene function or to gene silencing (*1, 2*). The hypermethylation of the promoter regions of these genes is frequently observed in human cancer (*3*). Therefore, during multistage carcinogenesis, both mutation and hypermethylation can lead to an inactive tumor suppressor gene. It is generally accepted that methylation occurs enzymatically by de novo DNA methyl transferases, such as DNMT3b (*4*). However, the exact mechanisms of hypermethylation, particularly in relation to environmental factors during carcinogenesis, are not clear.

Valinluck and Sowers reported that one possible mechanism of DNA hypermethylation is the formation of inflammation-induced 5-halogenated dC, such as 5-chloro-dC in DNA, which mimics m^5dC and induces inappropriate methylation by the maintenance DNMT1 enzyme within the CpG sequence (*5*). They also reported that a form of inflammation-induced oxidative DNA damage, 5-hydroxymethyldeoxycytidine, prevents DNMT1 methylation within CpG sequences and induces hypomethylation. They proposed that the chemical modification of DNA could cause heritable changes in cytosine methylation patterns, resulting in human tumor formation.

The formation of a mutagenic methyl radical-deoxyguanosine adduct, 8-methyl-2′-deoxyguanosine, has been detected in DNA after a treatment with BuOOH and ferrous ion in vitro or after the administration of 1,2-dimethylhydrazine to rats (*6*–*8*). We proposed a free radical mechanism to produce m^5dC in DNA or the nucleotide pool, because the C-5 position of cytosine is an active site for free radical reactions, in addition to the C-8 of purines and the C-6 position of pyrimidines, on the basis of quantum mechanical calculations (*9*). In this study, environmental tumor promoters, cumene hydroperoxide (CuOOH) and *t*-butyl hydroperoxide (BuOOH), which are known to generate methyl radicals (*10*), were tested for the formation of m^5dC from dC or in DNA.

Experimental Procedures

Materials. Deoxycytidine (dC), calf thymus DNA, poly(dG-dC)•poly(dG-dC), poly(dG)•poly(dC), and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were purchased from Sigma-Aldrich (St. Louis, MO). dC was purified by repeated rounds of HPLC (Capcell Pak C18, 5 μm, 10 mm × 250 mm, Shiseido Fine Chemicals, Japan; elution, 5% methanol in water) to remove the small amount of contaminating 5-methyl-dC. α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) was a product of Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). CuOOH (80% solution) was a product of Lancaster (Morecambe, England). BuOOH (70% solution) and ferrous sulfate ($FeSO_4•7H_2O$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The $FeSO_4$ solution (100 mM) was prepared just before use for reactions. The anti-5-methyl-dC monoclonal antibody was a gift from Dr. Kazuaki Watanabe, Toray Research Center (Kamakura, Japan) (*11*).

Reaction of dC with CuOOH/Fe^{2+} and Analysis of the Product by HPLC. Detailed reaction conditions are described in the legends to Figures 1−3. After the reaction, the solution was centrifuged, and an aliquot of the supernatant was injected into the HPLC column (YMC-Pak ODS-AM, 4.6 mm × 250 mm, particle size, 5 μm; elution, 5% methanol, 0.9 mL/min) connected with a photodiode array UV detector (Hewlett-Packard 1100 HPLC Detection System).

Reaction of DNA Polymer with CuOOH/Fe^{2+}. Detailed reaction conditions are described in the legend to Figure 4. After

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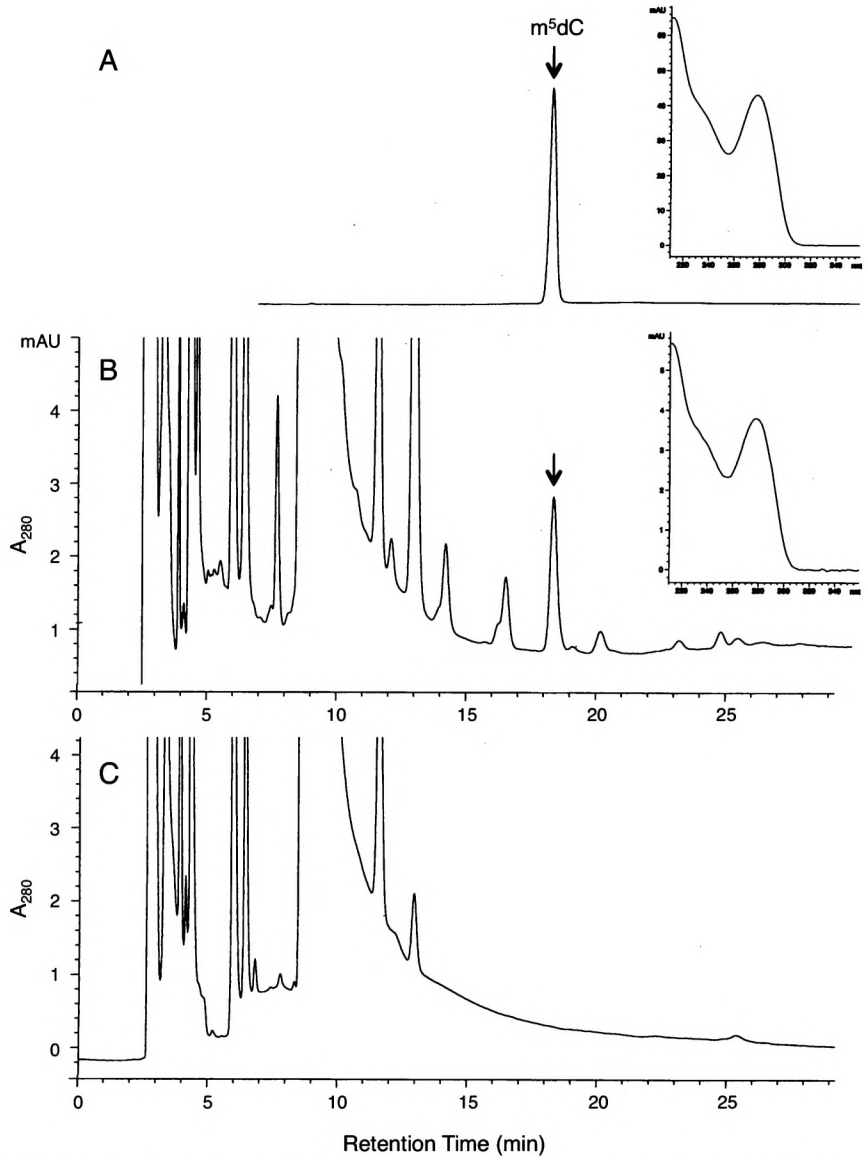

Figure 1. Detection of m^5dC in the reaction mixture of dC, Fe^{2+}, and CuOOH by HPLC. The reaction mixture (final volume, 0.32 mL), containing dC (final concentration, 5.46 mM), $FeSO_4$ (6 mM), and CuOOH (63 mM) in 20 mM phosphate buffer (pH 7.4), was reacted in a sealed plastic tube (tube volume, 2 mL) by vigorous shaking at 20 °C. After a 1 h of reaction, the solution was centrifuged, and an aliquot of the supernatant was injected into the HPLC apparatus. (A) Chromatogram of the m^5dC standard and its UV spectrum (inset), (B) chromatogram of the reaction mixture and UV spectrum of the peak at 18.5 min (inset), and (C) chromatogram of the control reaction mixture without CuOOH.

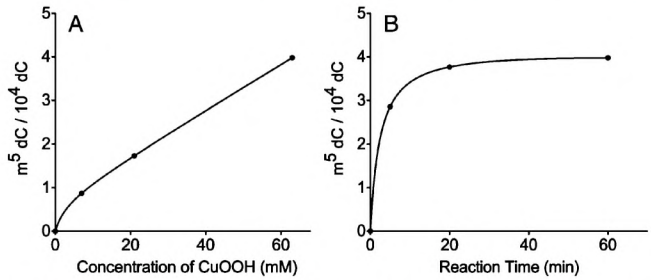

Figure 2. Dose and time dependency of m^5dC formation in the dC/CuOOH/Fe^{2+} reaction. (A) Dose dependency: The reaction conditions were the same as those in Figure 1, except that three different concentrations of CuOOH (7, 21, or 63 mM) were used. Mean values of duplicate experiments are plotted. (B) Time dependency: The reaction conditions were the same as those in Figure 1, except that the reaction was stopped at 5, 20, and 60 min. Mean values of duplicate experiments are plotted.

a 5 min reaction, the reaction mixture was centrifuged, and the supernatant (290 μL) was mixed with 87 μL of 5 M NaCl and 754 μL of cold ethanol and kept at 5 °C to precipitate the DNA. The DNA was recovered, washed with cold ethanol, dried under reduced pressure, and then dissolved in 260 μL of 1 mM EDTA (pH 8.0). For the LC/MS/MS analysis, a 170 μL aliquot of the sample was digested with 14 units of nuclease P1 and 4 units of alkaline phosphatase. For immunodot blot analysis, the DNA solution was centrifuged, and the supernatant was passed through a centrifugal filter device (Amicon Microcon YM-100) to recover the high molecular weight DNA (MW > 100000). The DNA trapped by the filter was dissolved in 150 μL of 1 mM EDTA (pH 8.0).

Detection of m^5dC in DNA by Immunodot Blot Analysis. The immunodot blot analysis was performed by basically the same method as previously reported (*12*). A calf thymus DNA sample (1 mg/mL PBS) was sonicated to obtain fragments of DNA. The DNA solution was then heat-denatured and diluted with 2 M ammonium acetate to an appropriate concentration (1−100 ng/mL). When the oligonucleotide was used, the sonication step was omitted. Single-stranded DNA and oligonucleotides (containing 0.1−10 ng DNA or 50 μg oligonucle-

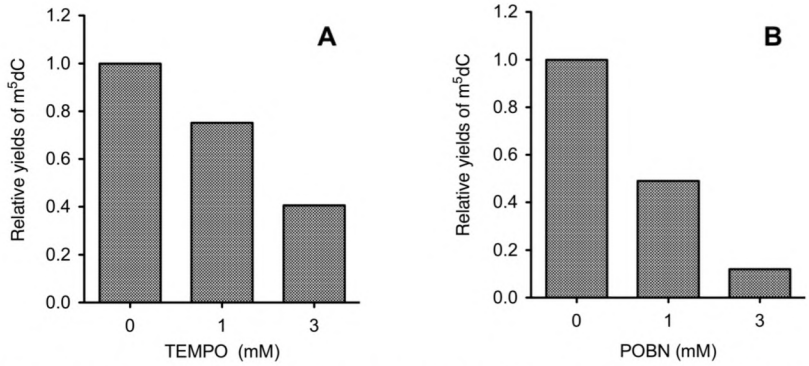

Figure 3. Inhibition of m^5dC formation from dC by TEMPO (A) or POBN (B). The reaction conditions were the same as those in Figure 1, except that the reaction time was 20 min. The reaction was conducted in the presence or absence of TEMPO and POBN. The ratio to the m^5dC yield without TEMPO or POBN (1.0) is shown.

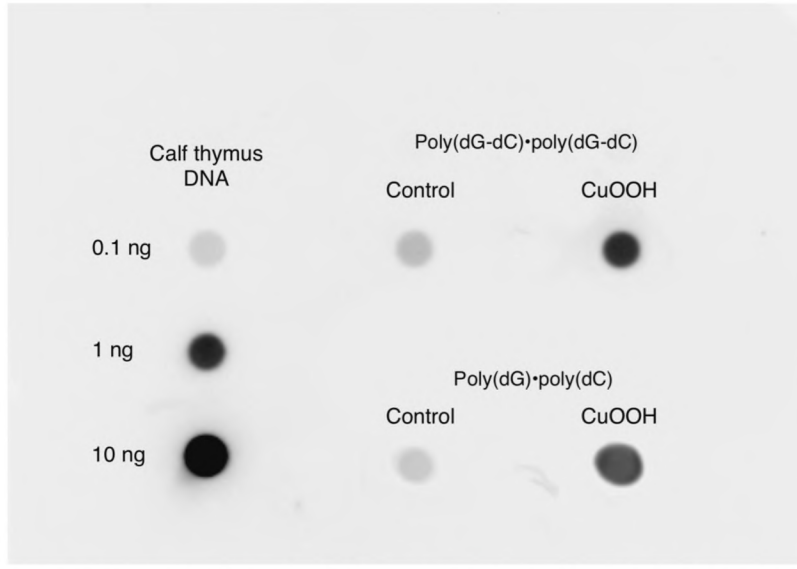

Figure 4. Detection of m^5dC in DNA polymers by an immunodot blot analysis. The reaction mixture (final volume, 0.32 mL) contained poly(dG-dC)•poly(dG-dC) or poly(dG)•poly(dC) (final concentration, 10 A_{260} OD units/mL), $FeSO_4$ (6 mM), and CuOOH (63 mM) in 20 mM phosphate buffer (pH 7.4) and was reacted in a sealed plastic tube (tube volume, 2 mL) by vigorous shaking at 20 °C. After 5 min, the polymers were recovered from the reaction mixture, as described in the Experimental Procedures, and were used for the analysis. As positive controls, m^5dC in various amounts of calf thymus DNA was visualized. As negative controls, DNA polymers without treatment were analyzed.

otide /100 μL sample) were immobilized on a nitrocellulose membrane (0.45 μm, Bio-Rad Laboratories, CA) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories). The wells were rinsed with 200 μL of 2 M ammonium acetate. The filter was subsequently removed from the support, and the DNA was cross-linked to the nitrocellulose using a Spectrolinker XL 1000 UV (Spectronics Co., NY). The membrane was washed twice for 5 min with PBS-Tween 20 (0.05%) (PBS-T) containing 2% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, United Kingdom) (blocking solution). The membrane was then incubated overnight at 4 °C with the blocking solution containing an anti-m^5dC monoclonal antibody (1.3 μg/mL) (*11*). The membrane was washed three times with PBS-T and was then incubated with the secondary antibody diluted 1:75000 in the blocking solution (ECL Anti-Mouse IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody, from sheep, GE Healthcare) for 2 h at room temperature. The membrane was washed four times with PBS-T. The enzymatic activity was visualized with an Amersham ECL advance Western blotting detection kit (GE Healthcare). The chemiluminescence output from the membrane was imaged using a CCD imager (Light Capture AE-6972, ATTO, Tokyo, Japan).

LC/MS/MS Analysis. The LC/MS/MS data were acquired on a Waters Micromass Quattro Ultima Pt triple quadrupole mass spectrometer with an ESI source (Waters Corp., Milford, MA). It was operated in the positive ion mode with a potential of 35 V. The desolvation temperature was 350 °C, and the ion source temperature was 120 °C. The collision energy was 11 eV. HPLC was performed using a Waters Alliance 2695 system (Waters Corp.), with a Capcell Pak C18 MG column, 5 μm, 2.0 mm × 250 mm (Shiseido Fine Chemicals, Japan); column temperature, 40 °C; elution, 8% aqueous methanol containing 10 mM ammonium formate; and elution speed, 0.2 mL/min.

Results

Reaction of dC with CuOOH/Fe^{2+}. When dC was reacted with CuOOH in the presence of Fe^{2+} at pH 7.4, the formation of m^5dC was clearly identified by HPLC equipped with a photodiodo array UV detector (Figure 1). The retention time and the UV spectrum of the reaction product were exactly the same as those of the authentic m^5dC. Its formation was dependent on the concentration of CuOOH (Figure 2A), and the reaction was rather rapid, due to its radical character. The reaction was approximately 70% complete within 5 min (Figure 2B). The methyl radical is produced by the reduction of CuOOH by Fe^{2+}. It is reasonable to speculate that the methyl radical formation rate and the m^5dC formation rate are dependent upon

Table 1. Yield of m^5dC from dC by BuOOH/Fe^{2+} Treatment[a]

reaction condition		yield of $m^5dC/10^4dC$
1 N H_2SO_4		25.4
0.35 N H_2SO_4		21.3
pH 4.5	air	6.01
	N_2	5.94
pH 7.4	air	1.76
	N_2	2.19

[a] dC (final concentration, 5.46 mM) and $FeSO_4$ (4 mM) were mixed in 0.32 mL of 20 mM sodium acetate buffer (pH 4.5), 20 mM phosphate buffer (pH 7.4), or in H_2SO_4 solutions (0.35 or 1 N), and the reaction was started by adding BuOOH (final concentration, 16 mM) in a sealed plastic tube (tube volume, 2 mL) by vigorous shaking at 20 °C. In some experiments, the oxygen in the solution was removed by flushing with nitrogen gas before starting the reactions. After a 30 min reaction, the solution was centrifuged, and an aliquot of the supernatant was injected into the HPLC apparatus. For the reactions in H_2SO_4 solutions, the supernatant was neutralized with 5 M NaOH, and then, an aliquot was injected into the HPLC apparatus.

the CuOOH concentration with the same concentration of Fe^{2+}. When a radical scavenger, TEMPO or POBN, was added to the reaction mixture at a concentration up to 3 mM, the m^5dC formation was inhibited (Figure 3). It should be mentioned that POBN is an efficient trapping agent for methyl radicals (*8*), while TEMPO, in addition to combining with methyl radicals, oxidizes Fe^{2+}, which is an important factor to produce methyl radicals (*13*). From these results, it can be concluded that this reaction proceeds via a free radical mechanism, probably via a methyl radical.

Reaction of dC with BuOOH/Fe^{2+}. The formation of m^5dC from dC was also observed after a reaction with BuOOH/Fe^{2+} at pH 7.4 (Table 1). Acidification of the reaction conditions increased the yield of m^5dC. When oxygen was removed from the reaction mixture at pH 7.4, the yield increased by 25%. The methyl radicals produced in the reaction may partly react with oxygen under aerobic conditions to form methyl peroxy radicals and then further decompose to formaldehyde.

Reaction of DNA Polymers with CuOOH/Fe^{2+}. After a double-stranded homopolymer, poly(dG)•poly(dC), or an alternating copolymer, poly(dG-dC)•poly(dG-dC), was reacted with CuOOH in the presence of Fe^{2+} at pH 7.4, the formation of m^5dC was examined by an immunodot blot analysis. The formation of m^5dC was clearly detected in both DNA polymers after the treatment (Figure 4). As a positive control, we analyzed 0.1, 1, and 10 ng of calf thymus DNA, because it contains 1.39 mol % m^5dC. The chemiluminescence intensity increased depending upon the calf thymus DNA concentration. The control DNA polymers without treatment also showed weak chemiluminescence. This means that commercial DNA polymers contain a small amount of m^5dC. We considered the immunodot blot analysis to be semiquantitative; therefore, the exact amount of m^5dC in the reaction mixture was analyzed by the LC/MS/MS method.

Confirmation of m^5dC Formation in the Reaction Mixtures by LC/MS/MS Analysis. In the LC/MS analysis, the standard m^5dC exhibited an MH^+ ion at *m/z* 242, and product ion analysis from *m/z* 242 with 11 eV revealed a fragment BH_2^+ ion at *m/z* 126 that is formed by the loss of 2′-deoxyribose (Figure 5). Therefore, the m^5dC in the reaction mixture was analyzed by LC/MS/MS, by monitoring the *m/z* 242 → 126 transition. In Figure 6, chromatograms of the LC/MS/MS analysis of standard m^5dC (A), dC-BuOOH/ Fe^{2+}(B), and DNA polymer- CuOOH/ Fe^{2+}(C) are shown. In both the dC- and the polymer DNA-product analysis, a 242 → 126 transition peak

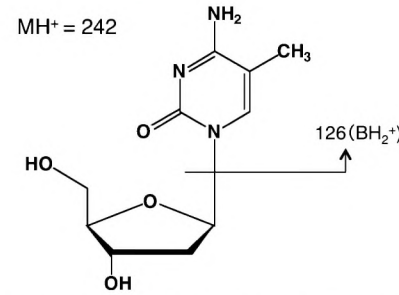

Figure 5. MH^+ ion of m^5dC and the product ion BH_2^+ in the LC/MS/MS analysis.

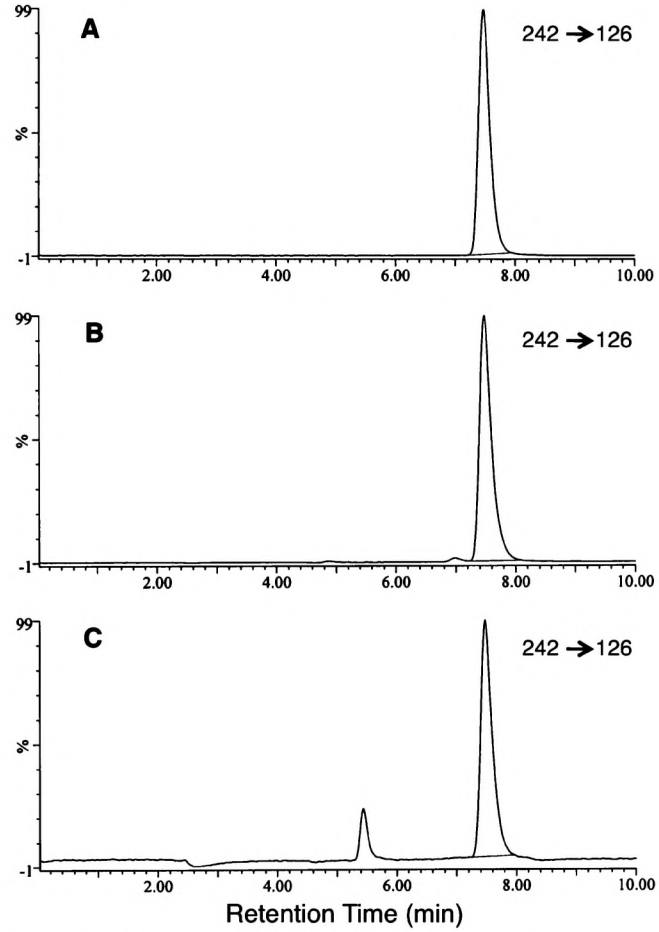

Figure 6. LC/MS/MS analysis of the reaction products. (A) Standard m^5dC (356 ng/mL), (B) dC-BuOOH/Fe^{2+} reaction mixture (reaction conditions were the same as in Table 1, at pH 7.4 and with air), and (C) hydrolysate of poly(dG-dC)•poly(dG-dC)-CuOOH/Fe^{2+} reaction product (reaction conditions were the same as in Figure 4). A 2 μL portion of each sample was injected. The transition *m/z* 242 → 126 was monitored.

appeared at 7.47 min, which is the same retention time as that of authentic m^5dC.

On the basis of the LC/MS/MS analysis, the yield of m^5dC in the dC-BuOOH/Fe^{2+} reaction was calculated to be $1.97/10^4$ dC, which is comparable to that estimated by HPLC-UV ($1.76/10^4$ dC, see Table 1). On the other hand, the yield of m^5dC in the DNA polymer/CuOOH/Fe^{2+} reaction was $2.97/10^5$ dC, while that from dC with the same reaction condition was $2.85/10^4$ dC (see Figure 2B). Therefore, the yield of m^5dC formation is 10-fold lower in DNA than in the dC monomer.

Discussion

A generally accepted concept of the mechanism of de novo DNA methylation during carcinogenesis is the enzymatic

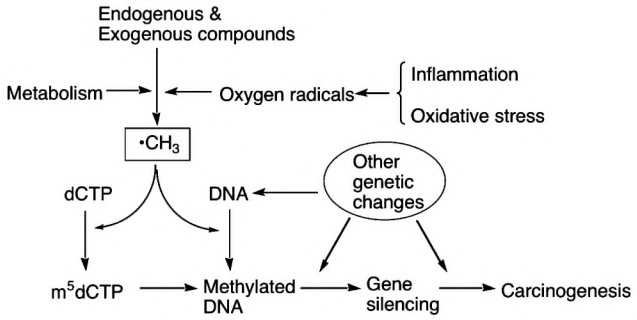

Figure 7. Hypothetical formation of m^5dC in nucleotides and DNA via methyl radicals.

reaction by DNMT3b, using *S*-adenosylmethionine as the methyl donor. Correlations between DNMT3b polymorphisms and neoplastic outcomes have been reported (*14, 15*). It was also reported that tumor suppressor gene inactivation during cadmium-induced transformation is correlated with the overexpression of the de novo DNA methyltransferase DNMT3b (*16*). In the present study, a free radical mechanism to produce m^5dC in DNA or the nucleotide pool was proposed, because the C-5 position of cytosine is an active site for free radical reactions. We observed the methylation of the C-5 position of cytosine in the nucleoside and DNA by the tumor promoters, BuOOH and CuOOH, in the presence of Fe^{2+} via a free radical mechanism. The generation of methyl radicals from organic hydroperoxide tumor promoters in vitro and in isolated mouse keratinocytes has been previously characterized by ESR (*10*). In addition to these chemicals, the generation of methyl radicals by the chemical and biological transformation of various carcinogens has been characterized in vitro and in vivo. For instance, methyl hydrazine derivatives, such as 1,2-dimethylhydrazine (*17*) and procarbazine (*18*), are metabolized to methyl radicals. Acetaldehyde, which is an important human carcinogen related to smoking, drinking, and inflammation (*19, 20*), generates methyl radicals upon treatment with xanthine oxidase (*21*), peroxynitrite (*22*), and iron (II)/hydrogen peroxide (*22*). The amino acid methionine produces a methyl radical upon γ-irradiation (*23*) and by the treatment of its sulfoxide derivative with peroxynitrite (*24*).

During tumor promotion in mouse skin by cigarette smoke condensate, hypermethylation in the promoter regions of the HoxA5, p16, and MGMT genes and their inactivation are important mechanisms of clonal expansion (*25*). Cigarette smoke is known to generate oxygen and carbon radicals (*26*). CuOOH treatment also reportedly induces malignant carcinomas in DMBA, TPA-carcinogenesis experiments with mice (*27, 28*). Therefore, DNA hypermethylation is an important mechanism of tumor promotion and progression.

Our results indicate that the formation of m^5dC in DNA does not seem to be specific to the CpG sequence, and the yield is rather low. Even if the methylation is a rare reaction, the m^5dC thus produced in DNA is not repaired, and its formation in CpG sequences would accumulate during continuous cell divisions by the maintenance DNA methyltransferase, DNMT1. When these modifications occur by chance in the promoter sequences of tumor suppressor genes, these cells will acquire a growth advantage over the surrounding cells, which will be further accelerated with other genetic changes.

It is interesting to speculate that m^5dCTP is formed from dCTP by methyl radicals in the nucleotide pool and then is incorporated into DNA (Figure 7), because we found that the yield of m^5dC as the monomer is much higher than that in DNA in the present study. It has been reported that m^5dCTP introduced into cultured CHO V-79 cells by electroporation is incorporated into DNA and induces gene silencing (*29*).

In conclusion, we found the methyl radical mediated formation of m^5dC from dC or in DNA, by a treatment with CuOOH or BuOOH in the presence of ferrous ion at pH 7.4. We have extended our finding to the following hypothesis. Methyl radicals are produced from the metabolism of carcinogens or from endogenous compounds attacked by oxidants generated by inflammation, ionizing radiation, and other oxidative stresses, and they modify dCTP and DNA to form m^5dC (Figure 7). The accumulation of this chemical modification may be one of the mechanisms of epigenetic change to induce gene silencing and carcinogenesis.

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References

(1) Feinberg, A. P., and Tycko, B. (2004) The history of cancer epigenetics. *Nat. Rev. Cancer 4*, 43–153.
(2) Herman, J. G., and Baylin, S. B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med. 349*, 2042–2054.
(3) Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. (2001) A gene hypermethylation profile of human cancer. *Cancer Res. 61*, 3225–3229.
(4) Okano, M., Xie, S., and Li, E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine5) methyltransferases. *Nat. Genet. 19*, 219–220.
(5) Valinluck, V., and Sowers, L. C. (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. *Cancer Res. 67*, 946–950.
(6) Gasparutto, D., Dherin, C., Boiteux, S, and Cadet, J. (2002) Excision of 8-methylguanine site-specifically incorporated into oligonucleotide substrates by the AlkA protein of *Escherichia coli*. *DNA Repair 1*, 437–447.
(7) Hix, S., Morais, M. D. S., and Augusto, O. (1995) DNA methylation by tert-butylhydroperoxide-iron(II). *Free Radical Biol. Med. 19*, 293–301.
(8) Netto, L. E. S., RamaKrishna, N. V. S., Kolar, C., Cavalier, E. L., Rogan, E. G., Lawson, T. A., and Augusto, O. (1992) Identification of C8-methylguanine in the hydrolysates of DNA from rats administered 1,2-dimethylhydrazine, evidence for in vivo DNA alkylation by methyl radicals. *J. Biol. Chem. 267*, 21524–21527.
(9) Kochetkov, N. K., Budovskii, E. I., Sverdlov, E. D., Simukova, N. A., Turchinskii, M. F., and Shibaev, V. N. (1971) *Organic Chemistry of Nucleic Acid, Part A*, pp 166−171, Plenum Press, London and New York.
(10) Taffe, B. G., Takahashi, N., Kensler, T. W., and Mason, R. P. (1987) Generation of free radicals from organic hydroperoxide tumor promoters in isolated mouse keratinocytes, Formation of alkyl and alkoxyl radicals from *tert*-butyl hydroperoxide and cumene hydroperoxide. *J. Biol. Chem. 262*, 12143–12149.
(11) Sato, K., Shimode, Y., Hirokawa, M., Ueda, Y., and Katsuda, S. (2008) Thyroid adenomatous nodule with bizarre nuclei: A case report and mutation analysis of the p53 gene. *Pathol. Res. Pract. 204*, 191–195.
(12) Leuratti, C., Singh, R., Lagneau, C., Farmer, P. B., Plastaras, J. P., Marnett, L. J., and Shuker, D. E. (1998) Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. *Carcinogenesis 19*, 1919–1924.
(13) Bar-On, P., Mohsen, M., Zhang, R., Feigin, E., Chevion, M., and Samuni, A. (1999) Kinetics of nitroxide reaction with iron(II). *J. Am. Chem. Soc. 121*, 8070–8073.
(14) Singal, R., Das, P. M., Manoharan, M., Reis, I. M., and Schlesselman, J. J. (2005) Polymorphisms in the DNA methyltransferase 3b gene and prostate cancer risk. *Oncol. Rep. 14*, 569–573.
(15) Lee, S. J., Jeon, H. S., Jang, J. S., Park, S. H., Lee, G. Y., Lee, B. H., Kim, C. H., Kang, Y. M., Lee, W. K., Kam, S., Park, R. W., Kim, I. S., Cho, Y. L., Jung, T. H., and Park, J. Y. (2005) DNMT3B polymorphysms and risk of primary lung cancer. *Carcinogenesis 26*, 403–409.
(16) Benbrahim-Talla, L., Waterland, R. A., Dill, A. L., Webber, M. M., and Waalkes, M. P. (2007) Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of *de novo* DNA methyltransferase. *Environ. Health Perspect. 115*, 1454–1459.

(17) Augusto, O., Du Plessis, L. R., and Weingrill, C. L. V. (1985) Spin-trapping of methyl radical in the oxidative metabolism of 1,2-dimethylhydrazine. *Biochem. Biophys. Res. Commun. 110*, 625–631.
(18) Goria-Gatti, L., Iannone, A., Tomasi, A., Poli, G., and Albano, E. (1992) In vitro and in vivo evidence for the formation of methyl radical from procarbazine: a spin-trapping study. *Carcinogenesis 13*, 799–805.
(19) Salaspuro, V., and Salaspuro, M. (2004) Synergistic effect of alcohol drinking and smoking on in vivo acetaldehyde concentration in saliva. *Int. J. Cancer 111*, 480–483.
(20) Matsuse, H, Fukushima, C, Shimoda, T, Sadahiro, A, and Kohno, S. (2007) Effects of acetaldehyde on human airway constriction and inflammation. *Novartis Found Symp. 285*, 97–106.
(21) Nakao, L. S., Kadiiska, M. B., Mason, R. P., Grijalba, M. T., and Augusto, O. (2000) Metabolism of acetaldehyde to methyl and acetyl radicals: in vitro and in vivo electron paramagnetic resonance spin-trapping studies. *Free Radical Biol. Med. 29*, 721–729.
(22) Nakao, L. S., Ouchi, D., and Augusto, O. (1999) Oxidation of acetaldehyde by peroxynitrite and hydrogen peroxide/iron(II). Production of acetate, formate, and methyl radicals. *Chem. Res. Toxicol. 12*, 1010–1018.
(23) Makino, K. (1979) Studies on spin-trapped radicals in γ- irradiated aqueous solutions of DL-methionine by high performance liquid chromatography and ESR spectroscopy. *J. Phys. Chem. 83*, 2520–2523.
(24) Nakao, L. S., Iwai, L. K., Kalil, J., and Augusto, O. (2003) Radical production from free and peptide-bound methionine sulfoxide oxidation by peroxynitrite and hydrogen peroxide/iron(II). *FEBS Lett. 547*, 87–91.
(25) Watson, R. E., Curtin, G. M., Hellmann, G. M., Doolittle, D. J., and GoodmanJ, I. (2004) Increased DNA methylation in the HoxA5 promoter region correlates with decreased expression of the gene during tumor promotion. *Mol. Carcinog. 41*, 54–66.
(26) Church, D. F., and Pryor, W. A. (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect. 64*, 111–126.
(27) Shvedova, A. A., Kisin, E. R., Murray, A. R., Kommineni, C., Vallyathan, V., and Castranova, V. (2004) Pro/antioxidant status in murine skin following topical exposure to cumene hydroperoxide throughout the ontogeny of skin cancer. *Biochemistry (Moscow) 69*, 23–31.
(28) Murray, A. R., Kisin, E. R., Kommineni, C., Vallyathan, V., Castranova, V., and Shvedova, A. A. (2007) Pro/antioxidant status and AP-1 transcription factor in mouse skin following topical exposure to cumene hydroperoxide. *Carcinogenesis 28*, 1582–1588.
(29) Holliday, R., and Ho, T. (1991) Gene silencing in mammalian cells by uptake of 5-methyl deoxycytidine-5′-triphosphate. *Somat. Cell Mol. Genet. 17*, 537–542.

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