7-Methylguanine adducts in DNA are normally present at high levels and increase on aging: Analysis by HPLC with electrochemical detection

(cancer/mitochondrial DNA/endogenous DNA adducts)

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ABSTRACT The 7-methylguanine adduct in the DNA of rat liver is determined as an indicator of exposure to exogenous and endogenous methylating agents. A method for the analysis of 7-methylguanine adducts has been developed by combining the selectivity of separation of reversed-phase HPLC with the specificity and high sensitivity of electrochemical detection. The sensitivity of the method is about 10,000-fold that of optical methods and is sufficient to determine the endogenous background of DNA methylation. DNA from the liver of normal young rats (6 months old) contains 7-methylguanine at a level of 1 residue per 31,000 bases in mitochondrial DNA and 1 residue per 105,000 bases in nuclear DNA. These levels increase about 2.5-fold in old rats (24 months old). We attribute this strikingly high level of adducts to endogenous methylation, which could contribute to aging and cancer.

Endogenous damage to DNA may be a major contributor to both aging and cancer (1-4). Oxidative damage has been postulated as one major endogenous contributor to aging (2, 3, 5). It is reasonable that endogenous alkylation of DNA may also be significant because a specific DNA repair glycosylase for methylated adducts has been described (6) and potential methylating agents are present in the cell (7, 51). The ³²P-postlabeling procedure of Randerath *et al.* (8) has been used to show that some adducts accumulate in rodents with age, though their identification has been difficult. An adduct that is elevated in DNA from senescent mouse heart tissue has been found by Gaubatz (9), by using Randerath's method, and has tentatively been identified as 7-methylguanosine deoxynucleotide.

Exposure of DNA to methylating carcinogens results in the formation of a number of methylated base adducts, including 7-methylguanine (m⁷Gua), 3-methyladenine, and O⁶-methylguanine (10). The major adduct is m⁷Gua, since the N-7 position of the base guanine lies exposed in the major groove of the DNA helix (11-13). This DNA adduct persists for a long time in cellular DNA since it is repaired enzymatically with low efficiency (14-16). The presence of m⁷Gua does not interfere with DNA replication (17) and appears to be of minor harm to the cell in comparison to some of the other methylated adducts. These properties indicate that determination of this adduct in tissue DNA samples may be informative in assessing the past exposure to methylating agents, either endogenous or exogenous.

Several assays for m⁷Gua have been described (18): labeling with [³H]acetic anhydride and analysis by using HPLC, immunoassay, and mass spectrometry with multiple ion detection. These methods, however, are not specific for purine and pyrimidine nucleotides and lack the sensitivity required for the analysis of biological samples. Despite the

excellent sensitivity of the ³²P-postlabeling procedure developed by Randerath *et al.* (19), this method has not yet found wide use in detecting DNA damage from low molecular weight alkylating agents due to the difficulty in separating adducts that possess similar chromatographic properties from each other and from the vast excess of the labeled normal deoxynucleotides (20).

Electrochemical detection in HPLC has been useful for the sensitive measurement of many important organic compounds (21) and for the 8-hydroxyguanine oxidation adduct in DNA (2, 22, 23). In this report, we describe HPLC with electrochemical detection (HPLC-EC) for the determination of m⁷Gua in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) of rat liver, and we also demonstrate the age-dependent increase of m⁷Gua in DNA from rat livers.

MATERIALS AND METHODS

Chemicals and Enzymes. m⁷Gua, calf thymus DNA, *Escherichia coli* tRNA, proteinase K, RNase A and RNase T1 were supplied by Sigma. BamHI was obtained from Boehringer Mannheim. Dimethyl sulfate and diethyl sulfate were from Aldrich. Synthetic ring-opened m⁷Gua (ro-m⁷Gua; 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine) was prepared by alkaline treatment of m⁷Gua (24). To prepare ro-m⁷Gua-containing DNA, calf thymus DNA (1 mg/ml) was treated with dimethyl sulfate (12.5 mM) in 0.2 M sodium cacodylate, pH 7.2/1 mM EDTA for 3 hr at 37°C. The imidazole ring opening of m⁷Gua in DNA was achieved by dialysis of methylated DNA against 0.2 M NaOH (24).

HPLC-EC. The HPLC system consisted of a Waters model 6000A solvent delivery pump, a model U6K injector, a Kratos (Westwood, NJ) Spectroflow 773 UV detector, and a reversed-phase Supelcosil C-18 column (particle size 5 μm, 25 cm × 4.6 mm). Electrochemical detection was accomplished by a Bioanalytical Systems (West Lafayette, IN) LC-4B amperometric detector with a glassy-carbon electrode. The mobile phase was 0.05 M potassium phosphate, pH 5.5/methanol, 90:10 (vol/vol), which was filtered through a 0.45-μm filter (Millipore). The flow rate was 1.0 ml/min in all experiments.

Isolation of DNA. Nuclear and mitochondrial fractions were isolated from the livers of female Sprague-Dawley rats (≈200 g of body weight) by conventional differential centrifugation (25). Two procedures were then used for nucleic acid isolation. DNA from the nuclear fraction of rat liver was isolated as described by Gupta (26). The DNA concentration was estimated spectrophotometrically by using an A₂₆₀ of 20

Abbreviations: m⁷Gua, 7-methylguanine; HPLC-EC, HPLC with electrochemical detection; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; ro-m⁷Gua, ring-opened m⁷Gua (2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine).

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equals 1 mg/ml. The yield of nDNA was usually about 2 mg per g of liver, and the A_{260}/A_{280} ratio was 1.82 ± 0.04 . mtDNA from rat liver was isolated as previously described (2). The yield of mtDNA was usually about 60 μ g per liver, with an A_{260}/A_{280} ratio of 1.76 ± 0.3 . The mtDNA preparation was free of nDNA as judged by digestion of 750 ng of DNA by the restriction enzyme *Bam*HI followed by agarose gel electrophoresis and staining with ethidium bromide. Extensive RNase treatment with RNase T1 (20 μ g/ml) and RNase A (100 μ g/ml) at 37°C for 30 min was performed to remove any contaminating RNA from DNA samples.

Quantitation of m⁷Gua. The removal of m⁷Gua by cleavage of the N-glycosidic bond was achieved by heating DNA at 100°C for 30 min in 5 mM phosphate (pH 7.4). After the solution was cooled, depurinated DNA was precipitated by the addition of 2 volumes of cold ethanol (−20°C) and was centrifuged for 30 min at $10,000 \times g$. The supernatant was decanted and reduced to near dryness under reduced pressure. After addition of HPLC buffer to the sample, aliquots were analyzed by HPLC-EC.

RESULTS

To determine the most efficient conditions for rapid and selective determination of m⁷Gua, we investigated the electrochemical behavior of m⁷Gua. The hydrodynamic voltammogram indicates that a high applied potential is required for the detection of m⁷Gua. The oxidation potential at half-maximum height was approximately +1.03 V. The potential of +1.1 V versus the Ag/AgCl electrode was chosen to obtain an optimal balance between high sensitivity and low background current. At this applied potential, a relative response (defined as the ratio of the peak areas of electrochemical detection at a potential of +1.1 V to the UV absorbance at 260 nm) for m⁷Gua is about 10^4 .

To determine the linearity of detection, a standard calibration curve for m⁷Gua was constructed. The response is linear between 20 fmol and 2 pmol (data not shown).

A key factor in the sensitivity of this approach is the extent to which trace amounts of modified residues can be resolved from the unmodified residues. Guanine is electrochemically active at +1.1 V, but it is about 7 times less active than m⁷Gua. To eliminate the signal from guanine residues in DNA, a neutral thermal hydrolysis procedure was employed to selectively release m⁷Gua from DNA. The glycosidic bond of 7-methylated deoxyguanosine is unstable (27, 28) and breaks easily on heating at neutral pH, in contrast to deoxyguanosine. Analysis of depurinated DNA indicated

FIG. 1. HPLC-EC chromatograms of nDNA (A) and mtDNA (B) from rat livers. Conditions: column, Supelcosil C-18; applied potential, +1.1 V versus Ag/AgCl, 20-nA range; mobile phase, 0.05 M potassium phosphate, pH 5.5/methanol, 90:10 (vol/vol); flow rate, 1.0 ml/min. RT, retention time.

Table 1. m⁷Gua content in DNA from rat liver

Source	Age, months	Analyses, no.	m ⁷ Gua, pmol per μ g of DNA*
nDNA	6	3	0.031 ± 0.004
	24	3	0.082 ± 0.007
mtDNA	6	4	0.105 ± 0.02
	24	3	0.217 ± 0.03

*The values are given as the means $\pm$ SD.

that m⁷Gua in DNA was consistently released quantitatively (>97%) and chromatographed as a single major peak on HPLC. Added guanine is well resolved from m⁷Gua in our HPLC analysis and elutes $\approx$ 2.5 min earlier.

We exclude the possibility that the m⁷Gua, known to be present in tRNA, contributes to our values. (i) The analysis of a control tRNA sample by our procedure did not produce a detectable amount of m⁷Gua, which is consistent with the fact that m⁷Gua in RNA is stable in neutral aqueous solution, whereas this residue in DNA is easily depurinated (28, 29). (ii) Extensive RNase hydrolysis of DNA samples has been performed during isolation. (iii) No m⁷Gua was seen when we incubated 20 units (A_{260}) of *E. coli* tRNA with RNases and then followed the same procedure described for the DNA samples.

Typical chromatograms obtained from nDNA and mtDNA of rat livers are shown in Fig. 1. Both DNA samples contained a peak that comigrated with authentic m⁷Gua when analyzed by reversed-phase HPLC-EC. The contents of m⁷Gua in DNA from rat livers determined by this method are shown in Table 1. Endogenous levels of m⁷Gua are estimated in nDNA and mtDNA from young (6 months old) and old (24 months old) rat livers. The endogenous level of m⁷Gua in mtDNA is almost 3 times greater than that in nDNA.

When isolated rat liver mtDNA and nDNA were incubated with dimethyl sulfate, approximately equal amounts of m⁷Gua (34.5 versus 36 pmol/ μ g of DNA) were formed, indicating that mtDNA is not inherently more sensitive to alkylation under these conditions. The level of m⁷Gua in nDNA and mtDNA increased about 2- to 2.5-fold in old rats compared to young rats. In young rats (6 months old), m⁷Gua in nDNA varied from 5 to 7 residues per 10^6 normal bases, which increased to 15 to 20 residues per 10^6 bases in nDNA from old rats (24 months old). The HPLC-EC analysis of liver DNA from 3-, 6-, 12-, and 24-month-old rats, summarized in Fig. 2, shows the increase of m⁷Gua during aging.

The adduct ro-m⁷Gua, induced by alkali or x-irradiation cleavage of the imidazole ring of m⁷Gua, has been reported to inhibit the elongation of DNA strand synthesis *in vitro* (30).

FIG. 2. Nuclear m⁷Gua content plotted versus liver DNA from chronological age of Sprague-Dawley rat (female). The data represent the means $\pm$ SD for three independent analyses.

FIG. 3. HPLC-EC chromatogram of dimethyl sulfate-treated calf thymus DNA dialyzed with 0.2 M NaOH followed by the neutral thermal hydrolysis procedure. Chromatographic conditions were as described in Fig. 1. RT, retention time.

ro-m⁷Gua is also electrochemically detectable from dimethyl sulfate-treated calf thymus DNA dialyzed with 0.2 M NaOH followed by the neutral thermal hydrolysis procedure; it has a relative response of about 10⁴ (Fig. 3). Because ro-m⁷Gua displays a very short retention time on reversed-phase HPLC, it may be necessary to improve chromatographic conditions to apply this method for the determination of this adduct in biological samples.

We have treated DNA with diethyl sulfate and found that the procedure described is equally effective for 7-ethyl-guanine, which elutes about 7 min after m⁷Gua. We found no detectable 7-ethylguanine (<1 per 10⁶ bases) in normal rat (24-month-old) liver DNA.

DISCUSSION

Extensive m⁷Gua is present at steady-state levels in nDNA and mtDNA *in vivo* and increases during aging. Among the methylated bases formed from DNA exposed to various methylating agents, m⁷Gua has been proposed to be the most abundant adduct and is repaired enzymatically with low efficiency. In contrast, the repair of O⁶-methylguanine is carried out efficiently by an alkyl transferase that removes the methyl group, and the guanine residue remains in the nucleic acid (31). The existence of an efficient glycosylase for 3-methyladenine (6), in contrast to m⁷Gua (14–16), would make it unlikely to be as abundant an adduct in DNA. Although m⁷Gua in DNA has not previously been considered to be very harmful to the cell, spontaneous hydrolysis (*t*_{1/2} ≈ 100 hr at 37°C) (15), caused by the labile nature of the N-glycosidic bond of m⁷Gua, can produce apurinic sites in DNA at a very high rate. We calculate from the data in Table 2 that every cell in an old rat has 70,000 depurinations per day from loss of m⁷Gua. This can be contrasted with an estimation of about 10,000 apurinic sites per day, which occurs from loss of purines (32). Such defects are repaired very efficiently, but in view of their frequent occurrence in DNA they may nevertheless contribute to inactivation or muta-

genesis of cells (33). Increasingly, knowledge is being acquired of the importance of DNA damage in mtDNA as well as in nDNA in carcinogenesis (34) and the relationship of endogenous DNA damage to cancer and aging (2, 3, 35). However, the very small amount of damaged bases has restricted determination of the steady-state level of adducts in DNA. The development of a sensitive and selective HPLC-EC method has allowed the determination of endogenous levels of a major alkylated product, m⁷Gua.

m⁷Gua is found at about a 3-fold higher level in mtDNA than in nDNA from the same cells. Assuming 1 × 10⁷ daltons for mtDNA and 1 × 10⁴ mtDNA copies (36), it can be calculated that about 10,000 m⁷Gua residues are present in the mitochondrial genomes of a young rat liver cell (Table 2). An analogous calculation yields 174,000 m⁷Gua residues for the nuclear genome of the same cell. This excess of methylation in mtDNA compared to nDNA is presumably due to several factors. Numerous studies (34, 37–39) indicate that some types of chemical carcinogens modify mtDNA more efficiently than nDNA. Since mtDNA lacks histones and is active in replication and transcription, it may be more accessible to mutagens than nDNA, which is highly compact and protected by proteins. Another explanation for the relatively high levels of m⁷Gua might be lack of, or not very efficient, repair enzymes in mitochondria (34); mitochondria lack nucleotide excision repair and recombinational DNA repair (40, 41).

Endogenous oxidative damage, as measured by 8-hydroxydeoxyguanosine adducts, is 15 times more frequent in mtDNA than in nDNA (2). This is presumably because the main source of oxidants, but not alkylating agents, is mitochondrial in origin.

Endogenous methylation and oxidation appear to be major contributors to the damage of both mtDNA and nDNA. Endogenous methylation of DNA could be an unavoidable by-product of the presence of the reactive methylating agent S-adenosylmethionine in the nucleus (7). Higher eukaryotic organisms normally methylate DNA enzymatically with S-adenosylmethionine to form 5-methylcytosine (42). Both prokaryotes and eukaryotes help to defend their DNA from aberrant methylation by DNA glycosylases specific for methylated bases (6). Specific DNA repair glycosylases also defend against oxidative (43) and deamination (44) damage, suggesting that both types of damages are important as well.

A plausible somatic damage theory of aging is that the amount of maintenance and repair of somatic tissues is always less than that required for indefinite survival. Thus, point mutations, chromosome abnormalities, and loss of 5-methylcytosine (45) could occur with time from DNA damage and contribute to both aging and cancer. An increase in the steady-state level of adducts with age is not required. In the case of oxidative damage, the preliminary, but not definitive, evidence suggests that there is no marked increase of adducts with age (2, 35), but an increase with age in oxidized protein accumulation has been observed (46). The increase in methylated adducts observed here (Table 1 and Fig. 2) might possibly be due to a decrease in DNA repair. DNA repair has been reported to decline with age in isolated

Table 2. Occurrence of m⁷Gua in rat liver

Source	Age, months	m ⁷ Gua, pmol per μg of DNA	Residue per bases	Residues per genome*	Residues per cell
mtDNA	6	0.105	1 per 31,000	1	10,000
	24	0.217	1 per 15,000	2.1	21,000
nDNA	6	0.031	1 per 105,000	87,000	174,000
	24	0.082	1 per 40,000	230,000	460,000

*For mtDNA, the values given are the number of residues per mitochondrion genome, and for nDNA, the values given are per haploid genome.

rodent myocardial cells (47). Whether this, in turn, is due to DNA or protein damage is unclear.

Though damage to nDNA has received the most attention, damage to mtDNA might also be of major importance in contributing to aging and cancer. Movement of fragments of mtDNA into the nucleus have been detected (48, 49) and could cause a variety of deleterious effects. Such transfers might be increased by endogenous or exogenous carcinogens, particularly if the mtDNA receives high levels of damage (2) and if minimally degraded mtDNA is released from within the mitochondrial membrane (50). In addition, cumulative damage to mtDNA might decrease energy supplies in old cells, which in turn might decrease cell defenses.

The use of HPLC-EC for the rapid, specific, and sensitive determination of m⁷Gua and other 7-alkylated guanines should be useful in monitoring the effect of alkylating agents *in vivo* and is suited for analyzing these adducts in the DNA of human tissues. It will be of interest to understand the human variation in the levels of m⁷Gua, modulating factors, and significant exogenous contributors to DNA alkylation.

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