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Endogenous formation and repair of oxidatively induced G[8-5 m]T intrastrand cross-link lesion

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ABSTRACT

Exposure to reactive oxygen species (ROS) can give rise to the formation of various DNA damage products. Among them, d(G[8-5 m]T) can be induced in isolated DNA treated with Fenton reagents and in cultured human cells exposed to γ-rays, d(G[8-5m]T) can be recognized and incised by purified *Escherichia coli* UvrABC nuclease. However, it remains unexplored whether d(G[8-5 m]T) accumulates in mammalian tissues and whether it is a substrate for nucleotide excision repair (NER) *in vivo*. Here, we found that d(G[8-5 m]T) could be detected in DNA isolated from tissues of healthy humans and animals, and elevated endogenous ROS generation enhanced the accumulation of this lesion in tissues of a rat model of Wilson's disease. Additionally, XPA-deficient human brain and mouse liver as well as various types of tissues of ERCC1-deficient mice contained higher levels of d(G[8-5 m]T) but not ROS-induced single-nucleobase lesions than the corresponding normal controls. Together, our studies established that d(G[8-5 m]T) can be induced endogenously in mammalian tissues and constitutes a substrate for NER *in vivo*.

INTRODUCTION

Endogenous and exogenous genotoxins induce DNA damage in living cells (1), which may give rise to mutations and altered gene function, cell senescence or apoptosis (2). Excess generation of reactive oxygen species (ROS) *in vivo* may produce a broad spectrum of single-nucleobase lesions that have been extensively characterized at the structural and biological levels (3). However, ROS may also induce a number of intrastrand cross-link lesions (4–16), which have been relatively poorly studied.

Previous studies showed that d(G[8-5 m]T), where the C8 of guanine is covalently bonded with the 5-methyl carbon of its neighboring 3′ thymine (Figure 1), can form in an oxygen-free aqueous solution of duplex DNA exposed to X- or γ-rays (4,6), in calf thymus DNA treated with Fenton reagents under aerobic conditions (9) and in DNA of cultured human cells exposed to γ-rays (17). Through independent generation of nucleobase radicals, it was found that d(G[8-5 m]T) and its structurally related intrastrand cross-link lesions are initiated from a single pyrimidine radical (4,14–16). In this mechanism, hydroxyl radical can abstract a hydrogen atom from the 5-methyl group of thymine to yield the methyl radical of the nucleobase (18), which may attack its adjacent 5′ guanine to yield d(G[8-5 m]T) (Figure 1) (4). We reasoned that hydroxyl radicals produced through endogenous Fenton-type reactions may also lead to the formation of this lesion. However, the endogenous formation of d(G[8-5 m]T) in mammalian tissues remains unexplored.

In vitro replication studies demonstrate that d(G[8-5 m]T) almost completely blocks high-fidelity DNA polymerases (17,19). Steady-state kinetic measurements show that yeast pol η-mediated nucleotide insertion opposite the thymine portion of d(G[8-5 m]T) is error-free; the polymerase, however, exhibits appreciable

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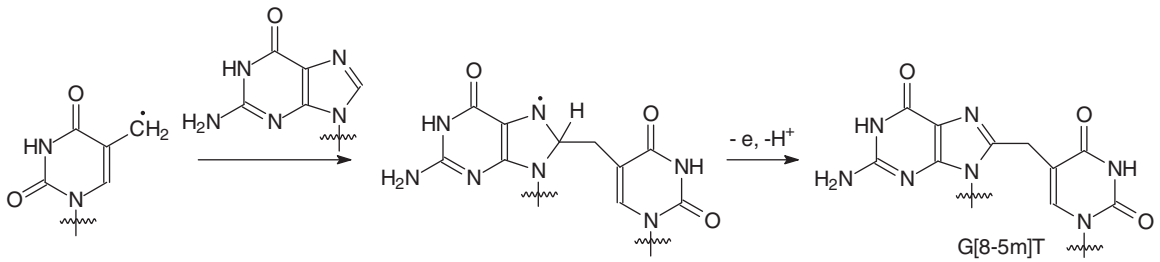

Figure 1. Mechanism for the formation of G[8-5 m]T intrastrand cross-link.

misincorporation of dAMP and dGMP opposite the guanine portion of the lesion (17). In contrast, bypass of this lesion by human pol η is nearly error-free, and the polymerase largely incorporates correct nucleotides opposite each cross-linked nucleobase (20). Along this line, replication studies using shuttle vector technology show that the structurely related d(G[8-5]C) lesion is highly blocking to DNA replication in *Escherichia coli* cells, and the guanine portion of the crosslink could lead to G→T and G→C mutations at frequencies of 8.7 and 1.2%, respectively (21). Previous studies also revealed that several intrastrand crosslink lesions, including the d(G[8-5 m]T), could be recognized and incised by *E. coli* UvrABC nuclease *in vitro* (22,23). This observation is in keeping with the finding that these lesions destabilized the DNA double helix (22). However, it is unclear whether these lesions are substrates for mammalian nucleotide excision repair (NER) *in vivo*.

In the present study, we assessed quantitatively the levels of d(G[8-5 m]T) in tissues of repair-competent and NER-deficient humans and laboratory animals using a highly sensitive and accurate LC–MS/MS/MS (MS^3) coupled with a stable isotope-dilution method.

MATERIALS AND METHODS

Detailed Materials and Methods can be found in online Supplementary Materials.

Materials and animals

The stable isotope-labeled d(G[8-5 m]T), 5-formyl-2′-deoxyuridine (5-FodU) and 5-hydroxymethyl-2′-deoxyuridine (5-HmdU), which contained a deuterium atom at the C2′ position and two ^{15}N atoms in the nucleobase component of the modified thymidine, were synthesized previously (9). LEA, $LEC^{+/-}$ and $LEC^{-/-}$ rats, $Xpa^{-/-}$ mice and wild-type littermates, and the ERCC1-deficient ($Ercc1^{-/\Delta}$) mice and repair-competent littermates (WT, $Ercc1^{+/-}$ and $Ercc1^{+/\Delta}$) were bred and genotyped as previously described (24–26).

Human brain samples

Human brain tissues were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore (contract HHSN275200900011C, Ref no. NO1-HD-9-0011). One sample from a Japanese XPA patient with neurologic disease was provided by Dr Nishigori. Details of the XP cases and controls are provided in Supplementary Table S1.

DNA extraction and enzymatic digestion

DNA was isolated from mammalian tissues using a high-salt method (27). Nuclease P1 (0.1 U/μg DNA), phosphodiesterase 2 (0.000125 U/μg DNA), EHNA (20 nmol) and a 20-μl solution containing 300 mM sodium acetate (pH 5.6), and 10 mM zinc chloride were added to 80–120 μg of DNA. The above digestion was continued at 37°C for 48 h. To the digestion mixture were then added alkaline phosphatase (0.05 U/μg DNA), phosphodiesterase 1 (0.00025 U/μg DNA) and 40 μl of 0.5 M Tris–HCl (pH 8.9). The digestion was continued at 37°C for 2 h and subsequently neutralized by addition of formic acid. To the mixture were then added appropriate amounts of isotopically labeled standard lesions, which included 5-HmdU, 5-FodU and d(G[8-5 m]T). The enzymes in the digestion mixture were subsequently removed by chloroform extraction twice. The resulting aqueous layer was subjected to offline HPLC enrichment of these three lesions.

LC–MS/MS/MS analysis

A 0.5 × 250 mm Zorbax SB-C18 column (particle size, 5 μm, Agilent Technologies) was used for the separation of the fractions containing d(G[8-5 m]T). An aqueous solution of 20 mM ammonium acetate (solution A) and methanol (solution B) was used as mobile phases for the analyses of d(G[8-5 m]T) after HPLC enrichment. The flow rate was 4 or 10 μl/min, and a gradient of 5 min 0–80% B and 30 min 80% B was employed for the separation.

The effluent from the LC column was directed to an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of the labeled and unlabeled d(G[8-5 m]T) in the positive-ion mode. The sensitivity for detecting this lesion was optimized by varying normalized collision energy (set to 31%) and activation Q (set to 0.25) of the LTQ mass spectrometer. The limit of quantification (LOQ) was estimated to be approximately 0.1 and 0.3 fmol when the flow rates were 4 and 10 μl/min, respectively.

LC–MS/MS analysis

A 3.0 × 100 mm Hypersil GOLD column (particle size, 5 μm, Thermo Scientific) was used for the separation of the fractions containing 5-HmdU and 5-FodU, and the flow rate was 50 μl/min. A solution of 0.1% (v/v) formic

acid in water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were employed as mobile phases, and a gradient of 35 min 0–70% B, 1 min 70–0% B and 14 min 0% B was used for the separation. The effluent from the LC column was directed to a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of the $[M-H]^-$ ions of the unlabeled and labeled 5-HmdU and 5-FodU in the multiple-reaction monitoring (MRM) mode. The MRM transitions were m/z 255→140 and m/z 258→142 for unlabeled and labeled 5-FodU, respectively, and m/z 257→124 and m/z 260→126 for unlabeled and labeled 5-HmdU, respectively. The S-lens RF amplitude and the collision energy were maintained at 97 V and 17 V, respectively. The LOQ was estimated to be ~40 fmol for both lesions.

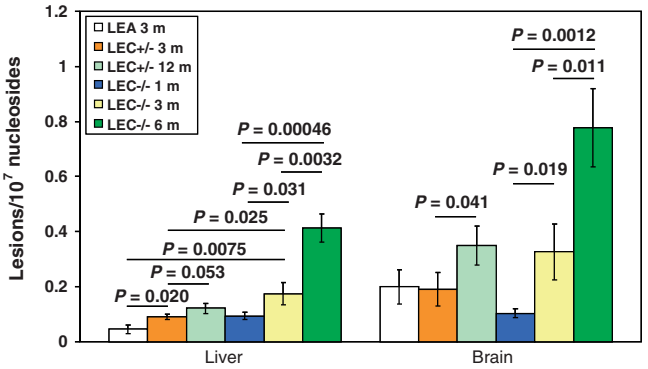

Figure 2. Levels of d(G[8-5 m]T) in nuclear DNA from the liver and brain of LEA (3 month old), $LEC^{+/-}$ (3 or 12 month old) and $LEC^{-/-}$ (1, 3 or 6 month old) rats. The values represent mean ± SD of results obtained from three rats, and the *P*-values were calculated by using unpaired two-tailed *t*-test.

RESULTS

Elevated accumulation of d(G[8-5 m]T) in liver tissues of an animal model of Wilson's disease

Transition metal ion-mediated Fenton reaction constitutes an important endogenous source of ROS (28). Wilson's disease (WD) is a single-gene disorder characterized by defective excretion of copper into bile, along with hepatic, neurological and renal abnormalities following copper toxicosis (29,30). The WD gene encodes a copper-dependent P-type ATPase (ATP7B) which is highly expressed in the liver, kidney and placenta (29,30). The Long–Evans Cinnamon (LEC) rat, which was naturally isolated from inbred Long–Evans Agouti (LEA) rats, has a deletion in the *Atp7b* gene (31) with copper-induced liver damage that worsens with age (32–34). Thus, LEC rat is a model for pathophysiology of liver injury in WD (34,35), while LEA rat is the healthy control. Our recent study revealed that oxidatively induced 8,5′-cyclopurine-2′-deoxynucleosides (cPu) are present at significant levels in liver and brain tissues of LEC rats (26). Since both cPu and d(G[8-5 m]T) can form from a single hydroxyl radical attack, we reasoned that d(G[8-5 m]T) should also be present in tissues of LEC rats.

By employing an offline enrichment along with $LC–MS^3$ using the stable isotope-dilution method (9), we measured the levels of d(G[8-5 m]T) in DNA isolated from the liver and brain tissues of LEA, $LEC^{+/-}$ and $LEC^{-/-}$ rats (Figure 2 and Supplementary Figure S1). Our results showed that d(G[8-5 m]T) was present at levels of ~0.5 lesions per 10^8 nucleosides in liver of 3-month old LEA rats, and the level of this lesion in liver of 3-month old rats followed the order of $LEA < LEC^{+/-} < LEC^{-/-}$. In addition, the level of d(G[8-5 m]T) in $LEC^{-/-}$ rats increased with age, though the liver of 12-month old $LEC^{+/-}$ rats contained only slightly higher level of this lesion than that of the corresponding 3-month old animals (Figure 2).

Similar to what was found with the cPu lesions (26), we observed an age-dependent accumulation of d(G[8-5 m]T) in brain of $LEC^{-/-}$ and $LEC^{+/-}$ rats (Figure 2). In contrast to the observations made for the liver, there was no significant difference in the level of this lesion in brain of 3-month old LEA, $LEC^{+/-}$ and $LEC^{-/-}$ rats, which is consistent with the absence of neurological abnormalities or brain damage in these animals (36).

d(G[8-5 m]T) is an endogenous substrate for mammalian NER

To examine whether d(G[8-5 m]T) constitutes a substrate for NER *in vivo*, we quantified this lesion in brain (cerebellum) of XPA patients and the age/gender-matched, repair-proficient individuals (Figure 3a and b and Supplementary Figure S2). It turned out that the levels of d(G[8-5 m]T) ranged from 0.04 to 0.4 lesions per 10^7 nucleosides in normal human brain and from 0.15 to 0.72 lesions per 10^7 nucleosides in brain of XPA patients. While we made an effort to match samples of control and XPA patients on the basis of age and gender, the relatively large variation in the lesion level among the different brain samples could emanate from multiple other factors, including heterogeneity in race, genetic background, nutrition, or agonal state, which could not be controlled. In spite of this variation, DNA from the brain of XPA patients contained higher levels of d(G[8-5 m]T) than that of the matched repair-proficient individuals, supporting the involvement of XPA in the repair of d(G[8-5 m]T) in human brain.

To seek further evidence about the role of NER in repairing d(G[8-5 m]T), we assessed the levels of this lesion in liver of $Xpa^{-/-}$ mice and control littermates. The results showed that the level of d(G[8-5 m]T) is higher in liver of 20-week old $Xpa^{-/-}$ mice than 19-week old normal controls, again supporting the involvement of mammalian NER in repairing d(G[8-5 m]T) *in vivo* (Figure 3a).

To validate and extend the XPA tissue data, we measured the levels of d(G[8-5 m]T) in brain, liver and kidney of NER-deficient $Ercc1^{-/\Delta}$ mice (37) and control littermates. We found that the liver of 10- and 21-week old ERCC1-deficient mice contained significantly higher levels of d(G[8-5 m]T) than those of the age-matched controls (Figure 3c). However, there was no apparent difference in the levels of d(G[8-5 m]T) in the liver between the two age groups of ERCC1-deficient mice or among the three age groups of control mice.

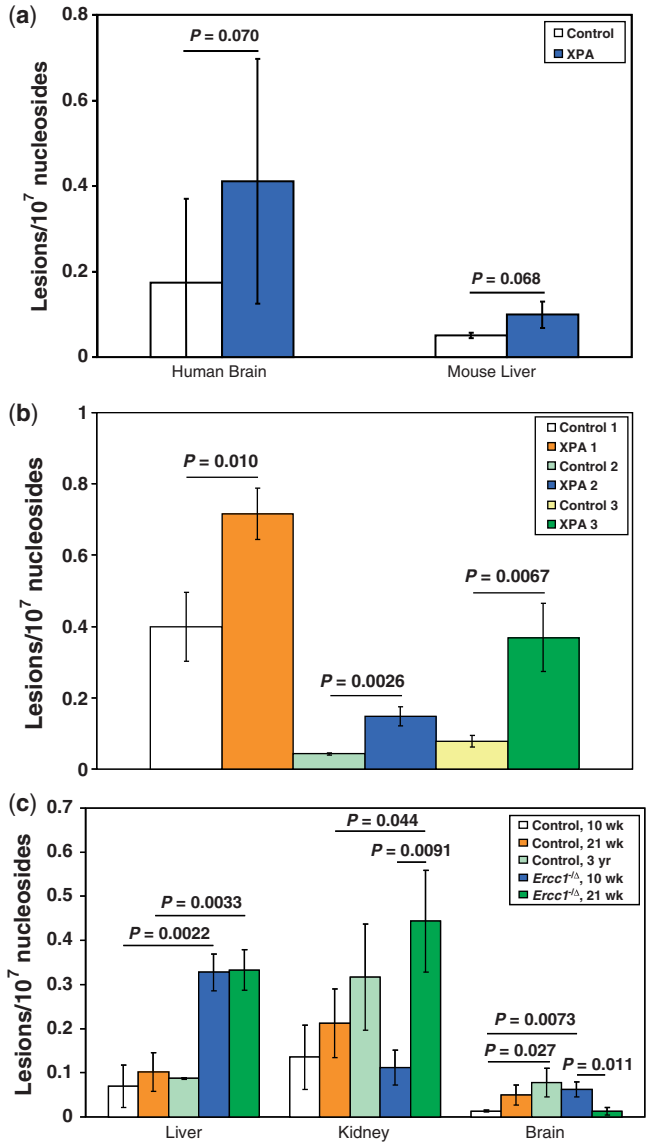

Figure 3. (**a**) Levels of d(G[8-5 m]T) in DNA from the brains of XPA patients and paired XPA-proficient individuals, and from the livers of $Xpa^{-/-}$ mice and age-matched wild-type (WT) littermates. For human brain samples, the data represent the mean ± SD of results from three pairs of age- and gender-matched human brain tissues; for mouse samples, the values represent mean ± SD of results obtained from two $Xpa^{-/-}$ mice and three wild-type littermates. (**b**) Levels of d(G[8-5 m]T) in DNA from individual XPA and the corresponding age- and gender-matched XPA-proficient human brain samples. The data for each brain tissue sample represent the mean ± SD of results from three independent enzymatic digestion, offline HPLC enrichment and LC–MS/MS/MS measurements. (**c**) Levels of d(G[8-5 m]T) in DNA isolated from the liver, kidney and brain tissues of $Ercc1^{-/\Delta}$ mice ($n = 3$) and the age-matched control littermates ($n = 3$). The values represent mean ± SD of results obtained from three mice per group. All the *P*-values were calculated by using unpaired two-tailed *t*-test except the *P*-values for the human brain data in (a) which were calculated using paired two-tailed *t*-test.

We also observed a significantly higher level of d(G[8-5 m]T) in kidney of 21-week old ERCC1-deficient mice than the corresponding control animals (Figure 3c), supporting the involvement of NER in the repair of d(G[8-5 m]T) in mouse kidney. No significant difference, however, was found for the level of this lesion in kidney of 10-week old ERCC1-deficient and control mice. Moreover, we found that the level of d(G[8-5 m]T) again increased with age in control mouse kidney, and this age-dependent accumulation of d(G[8-5 m]T) was more pronounced in kidney of the ERCC1-deficient mice (Figure 3c).

The brain of 10-week old $Ercc1^{-/\Delta}$ mice contained significantly more d(G[8-5 m]T) than that of the age-matched, repair-proficient littermates, and the level of this lesion increased with age in brain of the repair-proficient mice (Figure 3c). In stark contrast, the level of d(G[8-5 m]T) was significantly lower in brain of 21-week $Ercc1^{-/\Delta}$ mice than that of control littermates of the same age or the brain of 10-week $Ercc1^{-/\Delta}$ mice. This suggests that once a certain level of DNA damage is reached, it induces neuron loss, which could explain late-life cerebral atrophy, a hallmark of aging (38).

In the presence of molecular oxygen, the 5-methyl radical of thymine can also lead to the formation of two single-nucleobase lesions (39), namely, 5-FodU and 5-HmdU. Both 5-FodU and 5-HmdU are good substrates for base excision repair (BER) but not NER (40). Thus, comparing the levels of these two lesions and d(G[8-5 m]T) in tissues of NER-deficient and age-matched control animals may provide information about whether NER is specifically required for repairing d(G[8-5 m]T) but not 5-FodU and 5-HmdU. Our results showed that the levels of 5-FodU and 5-HmdU were approximately three orders of magnitude higher than that of d(G[8-5 m]T) (Figure 4, and representative LC–MS/MS results are depicted in Supplementary Figures S3 and S4). There was no significant difference ($P > 0.5$) in the levels of 5-FodU and 5-HmdU between XPA patients and control individuals (Figure 4a). Similar observations were made for the kidney and brain of $Ercc1^{-/\Delta}$ mice and age-matched normal littermates at both 10 and 21 weeks of age (Figure 4c). The absence of difference of 5-FodU and 5-HmdU levels in NER-proficient and deficient backgrounds showed that the elevated level of d(G[8-5 m]T) observed in NER-deficient animals and humans is not attributable to the enhanced rate of formation of this lesion in the repair-deficient background. However, there was significantly more 5-FodU and 5-HmdU in liver of $Ercc1^{-/\Delta}$ mice than that of normal littermates at these two ages, suggesting that there might be an elevated rate for the formation of these two lesions in liver of $Ercc1^{-/\Delta}$ mice than control animals (Figure 4c). Furthermore, it should be noted that the level of 5-FodU was several fold higher than that of 5-HmdU in the liver and kidney, whereas the levels of the two lesions were very similar in the brain (Figure 4c), suggesting that there might be a tissue-specific difference in the repair of these two lesions (41).

DISCUSSION

Previous studies revealed that d(G[8-5 m]T) could be induced in isolated DNA *in vitro* (4,6,9) and in cultured human cells exposed to γ-rays (17). However, it remains unknown whether this lesion can accumulate endogenously in mammalian tissues. Our results showed that

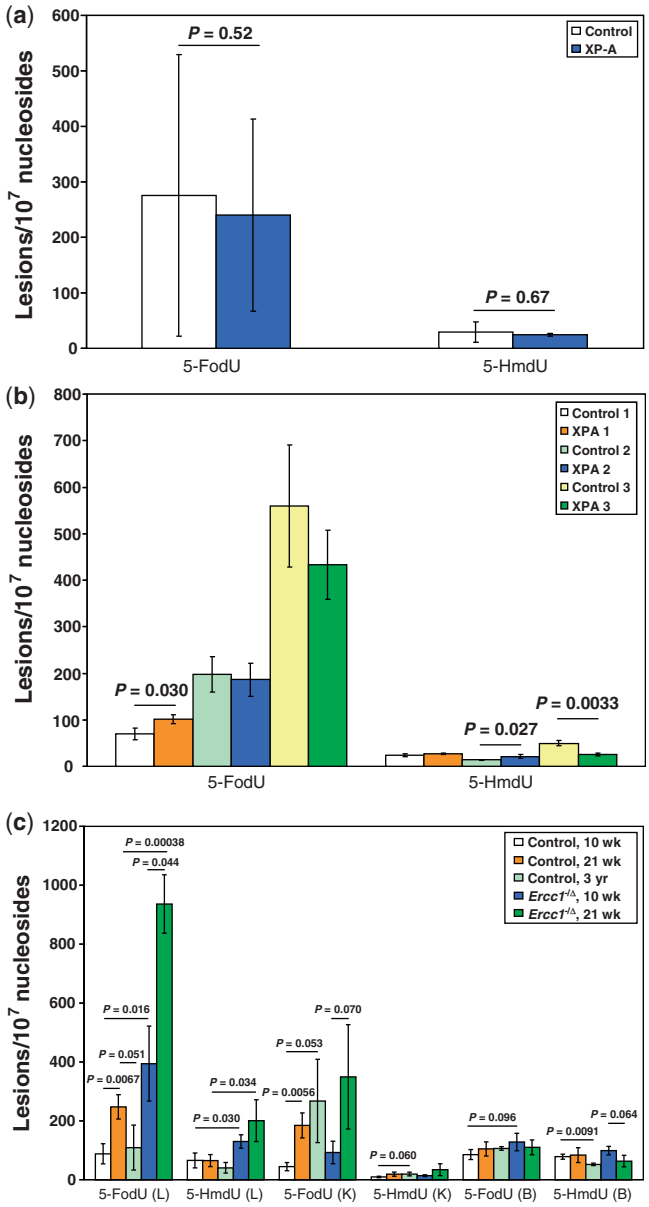

Figure 4. (**a**) Levels of 5-FodU and 5-HmdU in DNA from the brain of XPA patients and paired XPA-proficient individuals. For human brain samples, the data represent the mean ± SD of results from three pairs of age- and gender-matched human brain tissues; for mouse samples, the values represent mean ± SD of results obtained from two $Xpa^{-/-}$ mice and three wild-type littermates. (**b**) Levels of 5-FodU and 5-HmdU in DNA from individual XPA and the corresponding age- and gender-matched human brain samples. The data for each brain tissue sample represent the mean ± SD of results from three independent enzymatic digestion, offline HPLC enrichment and LC–MS/MS/MS measurements. (**c**) Levels of 5-FodU and 5-HmdU in DNA isolated from the liver, kidney and brain tissues of $Ercc1^{-/\Delta}$ mice ($n = 3$) and the age-matched control littermates ($n = 3$). The values represent the mean ± SD of results obtained from three mice per group. All the *P*-values were calculated by using unpaired two-tailed *t*-test except the *P*-values for the human brain data shown in (a) which were calculated using paired two-tailed *t*-test.

d(G[8-5 m]T) could be readily detected in DNA isolated from tissues of humans and laboratory animals (Figures 2 and 3). Moreover, the quantification data for these lesions in LEA and LEC rat tissues demonstrated that elevated generation of endogenous ROS arising from aberrant accumulation of transition metal ions (i.e. Cu^{2+}) leads to higher levels of d(G[8-5 m]T) in tissues (Figure 2).

It was observed previously that *E. coli* UvrABC nuclease could recognize and excise d(G[8-5 m]T) *in vitro* (22,23), with the relative excision activity for this lesion falling between those for *cis-syn* cyclobutane pyrimidine dimer and pyrimidine(6-4)pyrimidone photoproducts formed at TT sites, i.e. d(T[*c*,*s*]T) and d(T[6-4]T) (22), suggesting that d(G[8-5 m]T) might constitute an NER substrate *in vivo*. Our results obtained from XPA-deficient human brain and mouse liver, various tissues of ERCC1-deficient mice, and the normal controls (Figure 3) demonstrate that NER is indeed required for repairing d(G[8-5 m]T) *in vivo*.

In contrast to the d(G[8-5 m]T) intrastrand cross-link lesion, deficiency in XPA did not lead to increased accumulation of the two single-nucleobase lesions, 5-FodU and 5-HmdU, in human brain (Figure 4), showing that NER is specifically required for the repair of d(G[8-5 m]T). This was further strengthened by the lack of compromised repair of 5-FodU and 5-HmdU in ERCC1-deficient mouse brain and kidney (Figure 4). In addition, while the brain of control humans, mice and rats had similar levels of 5-FodU and 5-HmdU, the level of d(G[8-5 m]T) in the brain was approximately an order of magnitude lower in mice compared to the other two species; further investigation will be required to determine the origin of this difference.

ROS-induced bulky DNA lesions may have important implications in human health, including aging and genetic diseases associated with inherited defects in NER or aberrant accumulation of transition metal ions. The need for NER in repairing these bulky DNA lesions (22,23,42,43) and the lack of NER activity in terminally differentiated cells (44,45), particularly in neurons, give rise to accumulation of bulky DNA lesions in these cells, as demonstrated for d(G[8-5 m]T) in the present study. Such accumulation events may contribute to pathological conditions in patients bearing genetic diseases with deficiency in NER, e.g. xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne's syndrome (46–48).

XP neurologic disease is observed in approximately 20% of XP patients (49). This disease is characterized at the macroscopic level by the atrophy of the brain, spinal cord and peripheral nervous system, and at the microscopic level by neuron loss in different regions of the brain (50). Since UV light cannot reach human brain, it was hypothesized that XP neurologic disease is caused by transcription-blocking endogenous DNA damage that is specifically repaired by NER but not other repair pathways (51,52). In this context, it is worth noting that other endogenously induced and oxidatively generated DNA lesions, including thymine glycol and 8-oxo-7,8-dihydroguanine, were found to be substrates for NER *in vitro* (53–55). These lesions, however, are also substrates for DNA glycosylase-initiated BER, which is the primary pathway for the repair of these lesions *in vivo* (56). Therefore, the physiological significance of NER in repairing the oxidatively induced single-nucleobase lesions remains unclear.

While the levels of d(G[8-5 m]T) lesions in XPA human brain samples are higher than in age-matched controls, the magnitude of the increase is not drastic relative to variation among samples from XPA or control human brain. This could be attributed to a variety of other uncontrollable factors as noted above. In relation to XP neurological disease, it should be emphasized that we analyzed the cerebellum, a brain region predominantly comprised of small granule cell neurons that do not undergo severe degeneration in XP patients (57). While neurodegeneration is much more severe in other brain regions of XP patients, such as the cerebral cortex and striatum, the neuronal loss and resulting proliferation of glial cells would make it difficult to interpret lesion levels in these areas.

The d(G[8-5 m]T) is an intrastrand cross-link and therefore structurally similar to the d(T[c,s]T), which is a strong block to transcription (58). Specifically, access to the active site of RNA polymerase II requires a dramatic separation of adjacent template nucleotides at a nearly 90° angle (59), which would be strongly inhibited by the additional covalent bond in d(G[8-5 m]T), thereby rendering this lesion likely to be a strong block to transcription by a mechanism analogous to the d(T[c,s]T) (60). Therefore, we conclude that d(G[8-5 m]T) has the characteristics of a causative endogenous lesion for XP neurologic disease.

In conclusion, the results from the present study demonstrate that the oxidatively induced d(G[8-5 m]T) lesion is present in tissues of healthy animals and humans and is a substrate for mammalian nucleotide excision repair *in vivo*. Future studies about how this and other endogenously induced intrastrand cross-link lesions perturb the efficiency and fidelity of DNA replication and transcription in mammalian cells will help better understand the biological consequences of these lesions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–6.

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Conflict of interest statement. None declared.

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