Endogenous Apurinic/Apyrimidinic Sites in Genomic DNA of Mammalian Tissues

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Abstract

Apurinic/apyrimidinic (AP) sites are one of the most frequent lesions in DNA. Using a highly sensitive slot blot assay, we determined the number and condition of endogenous AP sites in normal tissues of rats and human liver. The number of AP sites (50,000–200,000 per mammalian cell) was greatest in brain, followed by colon and heart, and then liver, lung, and kidney. The majority of endogenous AP sites were cleaved 5' to the AP site. These data suggest that removal of the deoxyribose phosphate moiety is the rate-limiting step in base excision and AP site repair in vivo.

Introduction

Cellular DNA is continuously exposed to insults from exposure to endogenous and exogenous alkylating agents, ionizing radiation, and oxidative stress. It has been proposed that DNA base damage generated by these agents is repaired mainly by base excision repair pathways (1). This repair process is initiated by spontaneous depurination or DNA-glycosylase-mediated hydrolytic cleavage at the N-glycosyl bond between the base and deoxyribose, resulting in the formation of an AP site. The spontaneous depurination rate was estimated at 10,000 bases per cell per day under physiological conditions by extrapolation of the data obtained upon release of radiolabeled bases from DNA at 70°C (2). Recently, using a combination of an ARP and slot blot techniques (ASB assay), we directly demonstrated the spontaneous depurination rate under physiological conditions to be 1.5 AP sites per 10⁶ nucleotides per day, which corresponds to ~9000 AP sites per cell per day (3). These experiments suggest that spontaneous depurination is one of the most frequent promutagenic events in genomic DNA. Furthermore, direct release of bases is also induced by free radical attack of the deoxyribose (4). These AP sites have to be repaired efficiently because of their potential cytotoxicity and mutagenicity (5). To date, the kinetics of AP site repair have been studied in detail only in vitro experiments with oligonucleotides and plasmids. The steady-state number of AP sites in genomic DNA has not been characterized because of technical problems related to sensitivity and specificity of existing methods. To better understand the steady state of AP sites at basal levels of base excision repair in vivo, we measured the amount of endogenous AP sites in genomic DNA extracted from various tissues of adult rats and human liver using the ASB assay. In addition, the endogenous AP sites were further characterized by the determination of cleavage sites to the AP sites in these genomic DNAs.

Materials and Methods

DNA Extraction

Six male F344 rats were obtained from Charles River Laboratories and maintained until they were ~7.5 months old. Rats were sacrificed after being anesthetized with metofane. Tissues and organs were quickly harvested and frozen at ~80°C until DNA extraction. Eight human liver samples were provided by Dr. C. J. Omiecinski (University of Washington, Seattle, WA; Ref. 6). DNA was extracted by a procedure slightly modified from the method reported by Nakamura et al. (3). Briefly, frozen tissues were thawed and homogenized in PBS with a Tehran homogenizer (Wheaton Instruments, Millville, NJ). After centrifugation at 2,000 × g for 10 min, the nuclear pellets were incubated in lysis buffer (Applied Biosystems) overnight at 4°C with protease K (500 mg/ml; Applied Biosystems). DNA was then extracted twice with a mixture of phenol, chloroform, and water and once with Sepag, followed by ethanol precipitation. The extracted DNA was incubated in PBS (pH 7.4) with a mixture of RNase and RNase A. After DNA precipitation with cold ethanol, the DNA pellet was resuspended in sterilized distilled water. The DNA solution was stored at ~80°C for the ASB assay. The DNA extraction method used in this study was unlikely to have modified the original number of AP sites in genomic DNA from intact tissues, based on reextraction data of DNA highly exposed to methylmethane sulfonate.

ASB Assay

The AP site assay was performed by a procedure slightly modified from the method reported by Nakamura et al. (3). Briefly, 15 μg of DNA in 150 μl of PBS were incubated with 1 mM ARP (Dojindo Laboratories, Kumamoto, Japan) at 37°C for 10 min. After precipitation using cold ethanol, DNA was resuspended in Tris-EDTA buffer. The DNA concentration was measured by a spectrophotometer, and the DNA solution was then prepared at 1.5 μg per 100 μl of Tris-EDTA buffer. Heat-denatured DNA was then immobilized on a NC membrane. The NC membrane was soaked with 5 × SSC and then baked in a vacuum oven. The membrane was preincubated with 10 μl of Tris-HCl buffer containing BSA. The NC filter was then incubated in the same solution containing streptavidin-conjugated horseradish peroxidase (BioGenix) at room temperature for 40 min. After the NC membrane was rinsed, the enzymatic activity on the membrane was visualized by the use of enhanced chemiluminescence reagents (Amersham Corp.). The NC filter was then exposed to X-ray film, and the developed film was analyzed using an Ultrascan XL scanning densitometer.

Heat/Acid Treatment of Calf Thymus DNA

Calf thymus DNA was treated with 100 mM MX in 10 mM Tris-HCl buffer-KOH (pH 7.4) at 37°C for 2 h to reduce the original number of AP sites in calf thymus DNA (Sigma). The DNA was purified twice by ethanol precipitation, followed by resuspension in distilled water. The calf thymus DNA pretreated with MX was incubated with heat/acid buffer as described in a previous paper (3) for various periods of time.

AP Site Cleavage Assay

Regular AP Site Assay. Fifteen μg of DNA in 135 μl of 10 mM Tris-HCl-KOH buffer (pH 7.5) containing 50 mM NaCl and 5 mM MgCl₂ were incubated with 1 mM ARP at 37°C for 10 min, followed by the ASB assay described above.
**5′ Cleavage Assay.** Fifteen μg of DNA and 145 units of *Escherichia coli* Exo III (New England Biolabs) in 135 μl of 10 mM Tris-HCl-KOH buffer as described above were incubated at 37°C for 1 min, immediately followed by the ASB assay. The number of 3′-cleaved AP sites was calculated as the original number of AP sites minus the number of AP sites left after treatment with Exo III alone.

**3′ Cleavage Assay.** Fifteen μg of DNA, 10 mM EDTA, and 100 mM putrescine (Sigma) in 135 μl of 10 mM Tris-HCl-KOH buffer as described above were incubated at 37°C for 30 min, immediately followed by the ASB assay. The number of 5′-cleaved AP sites was calculated as the original number of AP sites minus the number of AP sites left after treatment with putrescine alone.

**Detection of Residual AP Sites.** Fifteen μg of DNA and 145 units of Exo III in 110 μl of 10 mM Tris-HCl-KOH buffer as described above were incubated at 37°C for 1 min, immediately followed by the addition of a 1/10 volume of 100 mM EDTA. The sample was incubated with 100 mM putrescine in the reaction buffer at 37°C for 30 min, immediately followed by the ASB assay. Residual AP sites showed the number of uncleaved aldehydic lesions after the combination treatment of Exo III and putrescine.

**Depurination Assay**

Fifteen μg of DNA in 110 μl of PBS (pH 7.4) were incubated at 100°C for 20 min, followed by renaturation at room temperature for 1 h. After incubation with 100 mM putrescine, DNA was reacted with ARP, followed by the ASB assay. The number of heat-labile sites was calculated as the number of AP sites in DNA treated with heat buffer followed by putrescine minus the number of AP sites left after treatment with putrescine alone.

**End III-sensitive Assay**

Oxidative pyrimidine bases are repaired by End III, leaving AP sites on DNA backbone (5). *E. coli* End III was kindly provided by Dr. Y. W. Kow (Emory University, Atlanta, GA). Fifteen μg of DNA, 1 mM EDTA, 100 mM NaCl, 100 mM putrescine, and *E. coli* End III (0.1 μg) in 135 μl of 10 mM Tris-HCl-KOH buffer as described above were incubated at 37°C for 30 min, immediately followed by the ASB assay. The number of End III-sensitive sites was calculated as number of AP sites in DNA treated with End III and putrescine minus the number of AP sites left after treatment with putrescine alone.

**Results and Discussion**

To better understand the formation and repair of AP sites resulting from base excision repair and spontaneous depurination/depyrimidination *in vivo*, we measured the number of endogenous AP sites in genomic DNA extracted from various tissues of adult rats and transplant-quality human liver using the ASB assay (3). The number of endogenous AP sites varied widely between tissues (Fig. 1) but not within tissues. The highest number of AP sites was detected in the brain, with 30 AP sites per 10⁶ nucleotides, followed by the heart and colon. In contrast, rat liver, kidney, and lung consistently showed the lowest number of AP sites (8–9 AP sites per 10⁶ nucleotides). The number of endogenous AP sites in human liver was comparable to that in rat liver. These data indicate that AP sites persist at 50,000–200,000 lesions per mammalian cell under normal physiological conditions. The steady-state number of AP sites in genomic DNA should reflect the balance between the formation and repair of the AP sites. Possible interpretations of the higher number of endogenous AP sites in the brain are as follows: (a) higher rate of depurination and/or endogenous DNA adduct formation, resulting in more AP sites; (b) higher activity of DNA glycosylase, inducing more AP sites; (c) lower activity of type II AP endonuclease, causing accumulation of AP sites; and (d) less efficiency of dRp-ase or β-elimination, leaving 5′-nicked AP sites.

The major process in the repair of AP sites is the type II AP endonuclease-/β-pol-dependent pathway (7). Type II AP endonuclease can recognize AP sites and incise the phosphodiester backbone immediately 5′ to the lesions, leaving a 3′-hydroxyl group and 5′-AP site terminus (5). The 5′-dRp is subsequently released, and the single

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**A**

**Endogenous AP sites in tissues of F344 Rats**

<table>
<thead>
<tr>
<th>Tissue</th>
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<tr>
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<tr>
<td>Kidney</td>
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</tr>
<tr>
<td>Brain</td>
<td>0.9</td>
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<tr>
<td>Lung</td>
<td>0.7</td>
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<tr>
<td>Heart</td>
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<tr>
<td>Testis</td>
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<td>Colon</td>
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**B**

**Fig. 1.** Endogenous AP sites in rat and human tissues. DNA was extracted at 4°C from intact rat tissues and normal human livers, and the number of AP sites was measured using the ASB assay. A, typical X-ray film showing endogenous AP sites of rat tissues. DNA (including standard DNA samples) was loaded on a NC membrane (1.5 μg per slot). B, scanning densitometric data of endogenous AP sites in rat and human tissues. Columns, means from duplicate slots of five to eight individual samples; bars, SD.
nucleotide gap is filled. It has been demonstrated that significant amounts of type II AP endonuclease are present in the nucleus of mammalian cells (8). Previously, we showed that the combination of ASB assay and type II AP endonuclease or NaOH induces 3'- or 5'-cleavage of AP sites, respectively (3). To understand the activity of AP endonuclease and dRp-ase in vivo, we further optimized the AP site cleavage assay. In this experiment, we used Exo III as the type II AP endonuclease to identify 3'-cleavage of AP sites and putrescine to detect 5'-nicks (Fig. 2). To characterize this AP site cleavage assay, we incubated DNA that had been pretreated with MX to reduce the number of AP sites to 3.8 ± 0.5 AP sites per 10^6 nucleotides (mean ± SD) with heat and acid buffer. Various periods of heat/acid incubation induced 11, 47, 115, and 320 AP sites per 10^6 nucleotides. A single treatment of Exo III reduced the number of AP sites by 4–10 AP sites per 10^6 nucleotides in each DNA sample, regardless of the initial number present (Fig. 3). This reduction may be due to the combination of enzymatic incision on the 5' side by Exo III and nonspecific 3' cleavage of AP sites during incubation with Exo III. This nonspecific 3' cleavage precluded precise quantitation of the number of 3'-nicked AP sites in DNA with this assay. In contrast, putrescine treatment showed no reduction in the number of AP sites. After incubation with Exo III followed by putrescine, the number of AP sites was reduced to the original number of AP sites in calf thymus DNA pretreated with MX. The cleavage efficiency of AP sites by the combination of Exo III and putrescine was >99%.

We applied this assay to genomic DNA extracted from rat and human tissues to characterize endogenous AP sites. The fractions of intact and cleaved AP sites, and residual aldehydeic lesions are summarized in Fig. 4. Rat and human tissue DNA were incubated with Exo III followed by putrescine to examine whether detected lesions were actual AP sites. After 5' and 3' cleavage of AP sites, 1.5–2.2 residual aldehydeic lesions per 10^6 nucleotides were detected. These data indicate that the ASB assay correctly measures endogenous AP sites. The residual lesions may be due to limitations of enzyme reactions in the AP site cleavage assay as well as to the presence of endogenous aldehydeic base lesions such as formyluracil (10). In addition, a combined fraction of 3'-cleaved and intact AP sites was detected at ~2–3 lesions per 10^6 nucleotides in different tissues, which is ~1/3–1/10 of the total number of endogenous AP sites. To examine the number of 5'-cleaved AP sites, we incubated DNA with putrescine. The reduction of AP sites by putrescine (the original number of AP sites minus the number of AP sites left after putrescine incubation) represented the number of 5'-cleaved AP sites. In contrast to calf thymus DNA pretreated with heat/acid buffer, genomic DNA treated with putrescine had a markedly decreased number of AP sites. These data indicate that approximately two-thirds or more of the endogenous AP sites are already cleaved on the 5' side of AP sites in vivo.

After 5' cleavage of the AP sites by type II AP endonuclease, the incised AP sites subsequently have to be released from the DNA backbone via the excision of 5'-dRp residues. This releasing process is possibly performed by dRp-ase via hydrolytic reaction (11) or β-elimination (12) or via an endonucleolytic enzyme that incises downstream of 5'-dRp moieties (13). It has been demonstrated that Xenopus and human β-pol release 5'-dRp by β-elimination (14). Using repair patch size either in β-pol-null or -proficient cells, it has been demonstrated that a single gap repair pathway was predominant in β-pol-proficient cells but not in β-pol-null cells (15). In addition, the interaction between human AP endonuclease and β-pol accelerates the release of 5'-dRp residues in vitro (16). However, the repair efficiency of 5'-dRp moieties has not been well characterized in cells or in vivo. Using yeast cell-free extracts, the processing of the 5'-dRp moieties has been found to be a rate-limiting step during base excision repair of uracil-containing DNA (17). Furthermore, the catalytic efficiency of β-pol to remove 5'-dRp was examined (18). Interestingly, the $K_{cat}$ of 5'-dRp-ase activity of β-pol was ~100-fold lower than the $K_{cat}$ of AP endonuclease. This study demonstrates a clear persistence of 5'-incised AP sites in rat and human tissues. These data suggest that incision by AP endonuclease and the subsequent release of 5'-dRp residues may not be efficiently linked at a basal level in vivo and that the repair process of 5'-dRp may be one of the rate-limiting steps in base excision repair.

In the previous study (3), we demonstrated that spontaneous depurination occurred at 1.5 AP sites per 10^6 nucleotides per day under physiological conditions. To test whether heat-labile DNA adducts such as N3- and N7-alkyl purines were the source of the higher number of endogenous AP sites in brain, a depurination assay was performed.

### AP Site Cleavage Site Assay

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<td>(I)</td>
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<tr>
<td>(II)</td>
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<tr>
<td>(III)</td>
<td>Exo III</td>
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<tr>
<td>(IV)</td>
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<td>(V)</td>
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<td>(IX)</td>
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<tr>
<td>(X)</td>
<td>Exo III</td>
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<th>Release of AP Sites</th>
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**Fig. 2.** Scheme of AP site cleavage assay. To better understand the activities of type II AP endonuclease, as well as dRp-ase in vivo, we determined the existence of cleavages on the 5’ or 3’ side of AP sites in genomic DNA. Putrescine and Exo III (type II AP endonuclease) leave 3’-cleaved [(III)] and 5’-cleaved [(II)] AP sites, respectively. For intact AP sites [(I)] with no incision at the 3’ and 5’ sides of AP sites, the ASB assay can theoretically detect the original number of AP sites after treatment with either Exo III [(III)] or putrescine [(II)] because AP sites remain on the DNA backbone after incision at either side of the phosphodiester bond adjacent to the AP site. However, the combination of Exo III and putrescine cleaves at both 3’ and 5’ sides of the AP site [(IV)], resulting in release of the AP site from the DNA backbone [(V)]. Such released AP sites are not detected by this assay. On the basis of the number of AP sites left on the DNA backbone after this cleavage reaction, the number of 5’- and 3’-cleaved AP sites can be estimated. If 5’-cleaved AP sites [(VII)] are present in DNA, the putrescine single treatment can release the 5’-nicked AP sites by 3’-excision of the AP sites [(VIII) and (VIII)]. Likewise, 3’-nicked AP sites [(IX)] in DNA can be released by 5’-excision using Exo III [(IX) and (XI)].
performed in brain and liver DNA. However, no difference was observed in brain and liver (data not shown). Therefore, the steady state of endogenous AP sites may not be due to labile base lesions. One of the most important and abundant endogenous DNA lesions is oxidative DNA base damage. It has been reported that the steady state of 5-hydroxycytosine in rat brain is 2-fold higher than that in rat liver (19). Such oxidative pyrimidine bases are repaired by End III, leaving AP sites on the DNA backbone. To examine whether oxidative stress could be related to the steady-state number of AP sites in DNA, End III-sensitive sites were quantitated using the combination of the ASB assay and E. coli End III. The number of End III-sensitive sites was three times higher in brain (14 lesions per 10⁶ nucleotides) compared to other tissues (4–5 lesions per 10⁶ nucleotides). Recently, the human homologue of the End III (hNTH1) gene has been cloned.

Fig. 3. AP site cleavage assay of calf thymus DNA treated with heat/acid buffer. To validate the AP site cleavage assay, we examined the effects of Exo III and putrescine on intact AP sites induced in calf thymus DNA. The original number of AP sites in calf thymus DNA was reduced by MX (CTD/MX). DNA was then incubated in heat/acid buffer for different lengths of time to introduce different numbers of intact AP sites (−/−; Ref. 9). DNA was incubated with Exo III and/or putrescine, and the number of remaining AP sites in calf thymus DNA was measured by the ASB assay [Exo III only (Exo III/−), Exo III plus putrescine (Exo III/P), and putrescine only (−/P)]. Columns, means from duplicate slots of three individual samples; bars, SD. A, AP site cleavage assay of DNA containing 115 AP sites per 10⁶ nucleotides. B, summary of AP site cleavage assay of DNA containing different numbers of AP sites.

Fig. 4. Summary of AP site cleavage assay of rat and human tissue DNA. The original number of AP sites as described in the legend to Fig. 1B consisted of 5′-cleaved, 3′-cleaved, intact AP sites, and residual aldehydic lesions. The number of 5′-cleaved AP sites was calculated as the original number of AP sites minus the number of AP sites left after treatment with putrescine alone. The combined fraction of 3′-cleaved and intact AP sites was the difference between putrescine treatment and the combination treatment of Exo III and putrescine. Residual AP sites showed the number of uncleaved aldehydic lesions.
Acknowledgments

We thank C. J. Omiecinski, C. Hassett, and K. Thummel for providing the human liver samples; Y. W. Kow for providing End III; and A. Lindstrom for providing F344 rats. We also thank E. C. Kidd, P. B. Upton, K. S. McDorman, and N. Scheller for assisting in the preparation of the manuscript and for technical assistance. The critical reading of the manuscript by M. D. Topal, S. A. Leadon, W. K. Kaufmann, A-J. L. Ham, A. P-H. Lin, and D. K. La is acknowledged.

References

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