Analysis of 8-hydroxyguanine in rat kidney genomic DNA after administration of a renal carcinogen, ferric nitrilotriacetate

Minoru Nomoto, Raizo Yamaguchi, Masaru Kawamura, Kimitoshi Kohno and Hiroshi Kasai

Department of Molecular Biology and 1Department of Biology, School of Medicine and 2Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health Japan, Kitakyushu 807, Japan

1To whom correspondence should be addressed
Email: m-nomoto@mail.med.uoeh-u.ac.jp

Introduction

Reactive oxygen species (ROS) induce several kinds of DNA damage, for example single-strand breaks, double-strand breaks, base modifications, including apurinic and apyrimidinic sites, and DNA–protein crosslinking (1–3). Such damage is an important intermediate in the pathogenesis of cancer and in aging, because many ROS-induced base modifications are promutagenic (4–13). 8-Hydroxyguanine (8-OH-Gua) is a major form of oxidative DNA damage (14,15), which induces mainly GC→TA transversions in Escherichia coli and mammalian cells (16). The 8-OH-Gua level in cellular DNA can be measured with high sensitivity (17,18). However, the relationship between the induced DNA damage and mutation spectra has not been elucidated, because a suitable method for detecting damaged nucleotides in DNA in vivo has yet to be established.

Recently, an application of ligation-mediated PCR (LM-PCR) brought about a breakthrough in the detection of damaged nucleotides at the nucleotide level of resolution (19). LM-PCR was initially developed for genomic sequencing, including the detection of CpG methylation sites, and also for in vivo footprinting (20–22). Based on our experience with LM-PCR (23,24), we tried to elucidate the distribution of 8-OH-Gua within the genome; whether 8-OH-Gua was scattered in the whole genome with almost equal frequency or had a restricted distribution in particular genes or regions. The ultimate goal of this project was to determine the target gene(s) of this chemical carcinogenesis system.

In the present study, we observed a time-dependent alteration of hot piperidine-cleaved sites, corresponding to 8-OH-Gua formation and repair, in the upstream region of the Na,K-ATPase α1 subunit gene, whereas no time-dependent cleavage was observed in the p53 or HSP70-1 genes. Moreover, a similar cleavage pattern in the promoter region of the Na,K-ATPase α1 gene was observed in formamidopyrimidine-DNA glycosylase (Fpg)-digested genomic DNA. These results suggest that oxidative damage, such as 8-OH-Gua, preferentially occurred in restricted genes or regions.

Materials and methods

Animals

Six-week-old male Wistar rats were purchased from Seiwa Experimental Animal (Fukuoka, Japan). They were provided with commercial rat chow (Clea, Tokyo, Japan) and tap water ad libitum and were used after 4 days of acclimatization.

Chemicals and Fpg

Fe(NO₃)₃, Na₂NTA and the DNA Extractor WB Kit were purchased from Wako Biochemicals (Osaka, Japan). Ferric nitriloacetate (Fe-NTA) solution was prepared immediately before use by the method of Awai et al. (25), with a slight modification. Briefly, Fe(NO₃)₃ and Na₂NTA were each dissolved in Milli-Q water and then mixed at a molar ratio of 1:4. The pH was adjusted to 7.4 with NaHCO₃. Fpg was purchased from Trevigen (catalog no. 4040-100-01). Fpg digestion of genomic DNA was performed according to the manufacturer’s instructions.

Protocol of Fe-NTA treatment

A total of 30 animals were divided into either the Fe-NTA or control groups. In the Fe-NTA group, they were killed 1, 6, 24, 72 and 120 h after injection of Fe-NTA (15 mg Fe/kg body wt i.p.). In the control group, they were killed without any treatment. Each subgroup contained five animals. The animals were killed under ether anesthesia. The kidneys were immediately removed and then used for the experiments. A part of the organ was frozen and kept at –80°C.

Ligation-mediated PCR (LM-PCR)

DNA was extracted from the rat kidneys (100–200 mg) with the DNA Extractor WB Kit according to the method of Nakae et al. (26). The extracted DNA was cleaved with 1 M piperidine at 90°C for 30 min (27,28). As a control guanine ladder, an aliquot of genomic DNA from control animals was reacted with DMS and cleaved with piperidine as described above. LM-PCR was performed as described previously (23,24). The nucleotide sequences of...
Table I. Quantitative results of major cleavage signals in Figure 2A and B

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Control</th>
<th>1 h</th>
<th>6 h</th>
<th>24 h</th>
<th>72 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non–transcribed strand (Figure 2A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–143</td>
<td>(650) 1.00</td>
<td>2.39</td>
<td>2.42</td>
<td>1.28</td>
<td>1.18</td>
<td>1.24</td>
</tr>
<tr>
<td>–129</td>
<td>(1350) 1.00</td>
<td>1.95</td>
<td>2.08</td>
<td>1.29</td>
<td>1.14</td>
<td>1.17</td>
</tr>
<tr>
<td>–95 (C)</td>
<td>(5780) 1.00</td>
<td>1.35</td>
<td>1.60</td>
<td>1.00</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>–79 (C)</td>
<td>(3610) 1.00</td>
<td>1.47</td>
<td>1.60</td>
<td>0.96</td>
<td>0.92</td>
<td>0.94</td>
</tr>
<tr>
<td>–13</td>
<td>(940) 1.00</td>
<td>1.26</td>
<td>1.73</td>
<td>1.13</td>
<td>1.07</td>
<td>1.07</td>
</tr>
<tr>
<td>–4</td>
<td>(1910) 1.00</td>
<td>1.31</td>
<td>1.36</td>
<td>1.14</td>
<td>1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>–3</td>
<td>(4410) 1.00</td>
<td>1.28</td>
<td>1.09</td>
<td>0.96</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>+13</td>
<td>(5160) 1.00</td>
<td>1.43</td>
<td>1.17</td>
<td>1.00</td>
<td>0.85</td>
<td>0.87</td>
</tr>
<tr>
<td>Transcribed strand (Figure 2B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+13 (C)</td>
<td>(1240) 1.00</td>
<td>1.10</td>
<td>1.12</td>
<td>1.72</td>
<td>0.95</td>
<td>1.02</td>
</tr>
<tr>
<td>–13 (C)</td>
<td>(770) 1.00</td>
<td>1.27</td>
<td>1.69</td>
<td>2.60</td>
<td>0.68</td>
<td>0.81</td>
</tr>
<tr>
<td>–94 (C)</td>
<td>(3730) 1.00</td>
<td>1.17</td>
<td>1.76</td>
<td>1.35</td>
<td>0.76</td>
<td>0.89</td>
</tr>
<tr>
<td>–95</td>
<td>(2230) 1.00</td>
<td>1.04</td>
<td>1.73</td>
<td>1.57</td>
<td>0.82</td>
<td>0.80</td>
</tr>
<tr>
<td>–138</td>
<td>(9000) 1.00</td>
<td>1.04</td>
<td>1.29</td>
<td>1.37</td>
<td>0.89</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Each value is expressed as an average of four independent reactions. Values in parentheses are absolute activities (counts) of cleavage signals in the control sample. Other values are expressed as ratios with respect to the signal intensities of the control sample at the nucleotide position indicated. Italics denote the maximum levels of signal intensities.

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Results

Main hotspot codons of p53 gene mutation have been observed in exon 5 (codons 154, 157, 175 and 179), exon 7 (codons 245, 248 and 249) and exon 8 (codons 267, 273, 282 and 306) in human (31,32). In the case of the rat genome, exon 6 of the p53 gene corresponds to human p53 exons 6 and 7 with a fused exon organization (29). No marked cleavage site with a time-dependent fluctuation in signal intensity was observed in exons 5, 6 or 7. The results for exon 6 are shown in Figure 1A, as a representative analysis. Some background signals, not corresponding to guanine residues, were observed. Although a few signals in guanine positions were detected, for example the second letter of codon 249, a time-dependent fluctuation could not be observed. A similar situation was observed for the HSP70-1 gene, as shown in Figure 1B.

On the other hand, time-dependent cleaved signals at guanine residues were detected at –143, –129, –93, –13, –4, –3 and +13 in the upper strand (non-transcribed strand) of the Na,K-ATPase α1 subunit gene, as shown in Figure 2A. Such cleaved guanine signals were also observed at –95 and –138 in the lower strand (transcribed strand) of the gene (Figure 2B). These cleavage sites were found within particular GpG and GpGpG sequences or their penultimate nucleotides. The location of GpG and GpGpG sequences are shown to the left of each figure and potential target sites are indicated by capital letters (Figure 2A and B). Interestingly, some time-dependent...
Analysis of 8-OH-Gua in rat kidney DNA \textit{in vivo}

Fig. 2. Piperidine cleavage profiles of the promoter region of the Na,K-ATPase $\alpha_1$ gene. Cleavage profiles for the non-transcribed strand (A) and transcribed strand (B) are shown. Cleavage sites with time-dependent fluctuations in signal intensity are marked at the right side of each panel with nucleotide numbers. Positions of GpG and GpGpG sequences are indicated at the left side of each panel, in capital (potential reactive site) or lower case (inert site) letters.

cleavage signals, not corresponding to guanine residues, could be seen at $-95$ (cytosine, C) and $-79$ (C) in the upper strand and at $+13$ (C), $-13$ (C) and $-94$ (C) in the lower strand. These additional cleaved sites located at bases (+13, –13 and –95) opposite guanine residues were detected as time-dependent cleaved signals in the complementary strand. Quantitative results of the cleavage signals in question are summarized in Table I. These cleavage profiles were reproducibly obtained even in different batches of genomic DNA preparations (data not shown). To confirm that these cleavage signals are indeed derived from 8-OH-Gua, we digested the Fe-NTA-treated genomic DNA with Fpg. A similar cleavage pattern was obtained, as shown in Figure 3, and the results of quantitative analyses are listed in Table II. These results strongly suggest that 8-OH-Gua formation was highly restricted in the genome. Although we detected some cleavage signals in the promoter region of the Na,K-ATPase $\alpha_1$ gene (Figures 2 and 4), it is not easy to perform a similar analysis on the coding region of the Na,K-ATPase $\alpha_1$ gene because of other $\alpha$ subunit genes which encode highly homologous amino acid sequences. Based on the nucleotide sequences of particular regions encoding clustered amino acid substitutions between $\alpha$ subunits, we analyzed the piperidine cleavage sites in the coding region of the Na,K-ATPase $\alpha_1$ subunit gene (the transcribed strand of exon 12 and the non-transcribed strand of exon 10). However, significant time-dependent signals were not detected (data not shown).

Fig. 3. Comparison of cleavage profiles on piperidine treatment and Fpg digestion. The distribution of cleavage sites of genomic DNA after digestion by Fpg or without digestion (No Enz.) were compared with that of piperidine treatment (Pip.). Cleavage profiles for the non-transcribed strand of the Na,K-ATPase $\alpha_1$ gene promoter region are shown.

Discussion

In order to analyze 8-OH-Gua formation \textit{in vivo}, we used hot piperidine-treated genomic DNA, since 8-OH-Gua is known to be cleaved upon piperidine treatment (28). The genomic DNA fragments were subjected to LM-PCR. We chose three genes for the analysis, the tumor suppressor gene $p53$, the $HSP70$-1 gene and the Na,K-ATPase $\alpha_1$ subunit gene. The $p53$ gene is a negative control for 8-OH-Gua formation, because mutation of the $p53$ gene is not found in this Fe-NTA chemical carcinogenesis system (33). The $HSP70$-1 gene is not induced under non-stressed conditions (34). The Na,K-ATPase $\alpha_1$ subunit gene is active in kidney, although its expression in other tissues is relatively low (35,36). As shown in Figure 1, cleavage signals were observed in positions not corresponding to guanine bases and/or they did not show any time-dependent alteration in signal intensity. Because these signals were not observed in piperidine-untreated genomic DNA samples (data not shown), with the exception of the Na,K-ATPase $\alpha_1$ subunit gene promoter (Figure 3), they are probably derived from base modifications which could be cleaved by piperidine. Indeed, such additional cleavage signals were significantly reduced in intensity in Fpg-digested samples (Figure 3).

Conspicuous signals with time-dependent fluctuations in signal intensity were observed at $-93$ and $-95$ in the non-transcribed strand and $-95$ in the transcribed strand of the Na,K-ATPase $\alpha_1$ subunit gene. The signal intensity of these
Fe-NTA

\[
\begin{array}{cccccccc}
-129 & -100 & -80 & -60 \\
T\underline{T}A\underline{A}GCGGAGCGCGGCCCACTGAGCGGTGTCAGGTTGCTCCGTATTACGAGTGACGTCAGCAGTGCGGAGCGCAATGTCAGACGGCACCAGCCGCTCGGT \\
\end{array}
\]

DMS (Dimethyl Sulfate)

\[
\begin{array}{cccccccc}
-129 & -100 & -80 & -60 \\
T\underline{T}A\underline{A}GCGGAGCGCGGCCCACTGAGCGGTGTCAGGTTGCTCCGTATTACGAGTGACGTCAGCAGTGCGGAGCGCAATGTCAGACGGCACCAGCCGCTCGGT \\
\end{array}
\]

Fig. 4. Comparison of distribution of piperidine cleavage sites and DMS-hypersensitive sites. Piperidine cleavage sites on the core promoter region of the Na,K-ATPase α1 gene are summarized in the upper part of the figure. The closed and open arrows denote the cleavage site at guanine and at other nucleotide residues, respectively. Sites hypersensitive to DMS are shown with a closed arrow in the lower part of the figure. The distribution of hypersensitive sites is taken from our previous report (23).

Table II. Quantitative results of major cleavage signals in Figure 3

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>No Fpg enzyme</th>
<th>Fpg digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 h</td>
<td>6 h</td>
</tr>
<tr>
<td>–129</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>–95 (C)</td>
<td>0.37</td>
<td>0.47</td>
</tr>
<tr>
<td>–93</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>–79 (C)</td>
<td>0.40</td>
<td>0.35</td>
</tr>
<tr>
<td>–13</td>
<td>0.39</td>
<td>0.46</td>
</tr>
<tr>
<td>–4</td>
<td>0.45</td>
<td>0.51</td>
</tr>
<tr>
<td>–3</td>
<td>0.44</td>
<td>0.49</td>
</tr>
<tr>
<td>±13</td>
<td>0.37</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Each value is an average of duplicates. Values in parentheses are absolute activities (counts) of cleavage signals in the control sample. Other values are expressed as ratios with respect to the signal intensities of the control sample at the nucleotide position indicated. Italics denote the maximum levels of signal intensities during the time course.

positions was relatively high even in Fe-NTA-untreated ‘control’ samples (Figure 2). Since faint signals at –93 and –95 were observed even in undigested DNA samples (Figure 3), these sites might be highly reactive with very poor repair efficiency. Thus, a portion of DNA seems to maintain a nicked state (Tables I and II). Although the overall 8-OH-Gua level within the whole genome increased several-fold at 1 h after Fe-NTA treatment, slightly decreased at 6 h and then returned to the control level at 24 h after Fe-NTA treatment (37), the time points showing maximum intensity in our analysis differed slightly among cleaved positions (Figures 2 and 3 and Tables I and II). These observations suggest that for each nucleotide in question, the equilibrium constant for formation and repair of modification differed. As described above, reduced levels of background signals were observed in Fpg-digested DNA samples (Figure 3) and maximum intensity values with respect to controls in Fpg digests were higher than the peak values in piperidine-treated DNA samples (Table II versus Table I). This suggests that the piperidine cleavage sites in question could be targets not only for hydroxylation but also for some other modifications being reactive to piperidine. Although we observed some cleavage signals at cytosine residues, including –94 in the transcribed strand and –95 in the non-transcribed strand, we do not know the chemical entity of the modification at present.

As summarized in Figure 4, the distribution of 8-OH-Gua sites is highly homologous to that of DMS-hypersensitive sites reported previously (23). This observation suggests that the region could be accessible to both hydroxy radical and DMS attacks. The accessibility might be derived from an altered or open structure in chromatin. Our observations demonstrate that preferential adduct formation in the non-transcribed strand by transcription coupled repair (19,38,39) is not the case in oxidative DNA damage in the promoter region of the Na,K-ATPase α1 gene.

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References

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