c-Ha-ras Containing 8-Hydroxyguanine at Codon 12 Induces Point Mutations at the Modified and Adjacent Positions

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Abstract

To determine the type of mutation induced by 8-hydroxyguanine in a mammalian system, we examined the mutations induced by a synthetic c-Ha-ras protooncogene containing 8-hydroxyguanine in the second position of codon 12 (GGC) in NIH3T3 cells. Transfection of this gene significantly increased the number of transformed foci. The c-Ha-ras gene present in these foci was analyzed by the polymerase chain reaction-restriction enzyme method. Interestingly, sequence analysis revealed random mutations at the modified site (G—>T, G—>A, and G—>C) as well as mutations of the adjacent G on the 5’-side of 8-hydroxyguanine (G—>A and G—>T).

Introduction

8-hGua is one of the DNA lesions produced by oxygen-free radicals generated during cellular respiration, cell injury, and exposure to environmental oxygen radical-forming agents. Formation of 8-hGua in DNA is likely to be involved in mutagenesis and consequently carcinogenesis. An 8-hGua residue in DNA was found to induce exclusively G—>T transversion in vitro in the polymerase reaction (3) and in a prokaryotic system using phage or plasmid with 8-hGua at a specific position (4–6). The DNA sequence of the Escherichia coli mutM mutator locus that generates G—>T transversion is shown to be the same as that of the formamidopyrimidine glycosylase (8-hGua endonuclease) gene (7–9). To determine the type of mutation induced by 8-hGua in a mammalian system, we examined the mutations induced by a synthetic c-Ha-ras protooncogene containing 8-hGua in the second position of codon 12 (GGC) in NIH3T3 cells. We found random mutations at the modified site (G—>T, G—>A, and G—>C) and mutations of the adjacent G on the 5’-side of 8-hydroxyguanine (G—>A and G—>T).

Materials and Methods

Enzymes. Tag DNA polymerase was obtained from Perkin Elmer. BsHII and AatII were purchased from Toyobo. Mscl and protease K were purchased from New England Biolabs. and Boehringer Mannheim, respectively. Other enzymes were obtained from Takara.

Oligonucleotides. The oligonucleotide containing 8-hGua was synthesized by the phosphoramidite method in an Applied Biosystems Model 380A DNA synthesizer. The procedures for phosphorylation of N2-acetyl-8-methoxy-5’-O-monomethoxytrityl-2’-deoxyguanosine and purification and deprotection of the oligonucleotide will be reported elsewhere. The other oligonucleotides were synthesized and purified as reported previously (10).

Results and Discussion

Fig. 1 shows a DNA cassette containing 8-hGua in the second position of codon 12 of the synthetic c-Ha-ras gene. The nucleotide sequence of this DNA cassette is identical to that of a human c-Ha-ras-1 gene. The cassette was joined with a c-Ha-ras-expressing vector after cleavage with Clal and BsaHII according to the procedure described previously (10), except for use of plasmid pCB (11) instead of pRSV-rgl2 (12) to reduce the background focus formation induced by a normal ras gene. Control vectors containing GGC at codon 12 (normal) and GAC at codon 12 (activated) were also prepared by the same procedure. These vectors expressing c-Ha-ras were transfected into NIH3T3 cells by the calcium phosphate method (12, 13) for analysis of their focus-forming ability. The c-Ha-ras gene containing 8-hGua in the second position of codon 12 produced significantly more foci than the normal c-Ha-ras gene but about 1 to 2% of the number induced by the activated c-Ha-ras gene (GAC at codon 12, Asp-12) (Table 1). An enzyme responsible for removal of 8-hGua from DNA has been found in mammalian cells (14) as well as in E. coli (7), so the relatively low efficiency of transformation induced by the c-Ha-ras gene containing 8-hGua may be partly due to a repair reaction.

The sequence in the region of codon 12 of the c-Ha-ras gene present in the transfomants was analyzed by the polymerase chain reaction-restriction enzyme method described previously (10). After the polymerase chain reaction (16) using each mutagenic primer, the amplified DNA was treated with a specific restriction enzyme such as AatII, SflI, Bbel, SstI,

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2 To whom requests for reprints should be addressed.

3 The abbreviation used is: 8-hGua, 8-hydroxyguanine.

4 Inoue et al., manuscript in preparation.

5 To whom requests for reprints should be addressed.

6 The abbreviation used is: 8-hGua, 8-hydroxyguanine.

3483
Fig. 1. Generation of a synthetic c-Ha-ras gene carrying oh'Gua in the second position of codon 12. The oligonucleotide HRU2 which contains oh'Gua (specified as G with * above) was synthesized by the phosphoramidite method in an Applied Biosystems Model 380A DNA synthesizer. Construction of the DNA cassette and its insertion into the vector using Clal and BssHII sites (indicated by shading) were carried out as described previously (10), except that a new plasmid pCB (11) was used instead of pRSV-rg12 (12).

Mscl, or Apal. The three former enzymes were used to detect mutations to T, A, and C in the second position of codon 12, and the three latter to detect mutations to T, A, and C in the first position of codon 12. The third position (on the 3'-side of oh'Gua) was not analyzed because no mutation in this position would alter the 12th amino acid. The cleavage was examined by polyacrylamide gel electrophoresis. Fig. 2 shows the results for the clone obtained with c-Ha-ras containing oh'Gua in Experiment 3 in Table 1. Cleavage was detected when the amplified DNA obtained with the mutagenic primer was digested with AatII (Lane 1), indicating that dAMP was incorporated into the site opposite the oh'Gua residue, resulting in G→T transversion. Clones containing a c-Ha-ras gene with A or C in the modified position were also detected (Fig. 2, B and C). More strikingly, DNA from the clone obtained with c-Ha-ras containing oh'Gua in Experiment 1 in Table 1 was cleaved by Stul, indicating that dAMP was incorporated into the site opposite the flanking G, next to the oh'Gua residue, resulting in G→T transversion (Fig. 2D, Lane 4). A clone with A in the flanking position was also detected (Fig. 2E). Of 18 clones analyzed, 14 clones were found to have a point mutation. Of these 14 clones, 12 clones had a mutation at the modified site (8 G→T transversions, 3 G→A transitions, and a single G→C transversion), and 2 clones had a mutation in the adjacent position (a single G→A transition and a single G→T transversion). These results were confirmed by nucleotide sequencing of the amplified DNA by Maxam-Gilbert analysis (17) (Fig. 3).

Mutation assay after transfection of c-Ha-ras containing oh'Gua into NIH3T3 cells clearly demonstrated that oh'Gua in DNA induced G→T, A and C mutations in the position of the oh'Gua residue, and also G→T and G→A mutations at the G residue next to oh'Gua. This is in contrast to the findings in in vitro and in vivo systems of only G→T transversion in the position of oh'Gua in E. coli (3-6). It should, however, be noted that the most frequent mutation in the mammalian system was also G→T transversion in the position of the oh'Gua residue. E. coli oh'Gua endonuclease (formamidopyrimidine glycosylase) can remove an oh'Gua residue paired with C, T, or G (9). We recently found that mammalian oh'Gua endonuclease can remove an oh'Gua residue from duplex DNA (18). In contrast to prokaryotic cells, in mammalian cells an oh'Gua:C pair is most preferentially recognized. This may be one reason why only G→T transversion was found in the position of oh'Gua in DNA in E. coli systems.

We previously reported that oh'Gua not only had the poten-

### Table 1: Number of foci induced by c-Ha-ras genes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1*</th>
<th>2*</th>
<th>3*</th>
<th>4*</th>
<th>5*</th>
<th>6*</th>
</tr>
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<tbody>
<tr>
<td>Gly-12'</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>(normal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oh'Gua</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Asp-12</td>
<td>92</td>
<td>303</td>
<td>97</td>
<td>162</td>
<td>304</td>
<td>672</td>
</tr>
<tr>
<td>(activated)</td>
<td></td>
<td></td>
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</table>

* Fifty ng of DNA were used.
* One-hundred fifty ng of DNA were used.
* Fifty ng of DNA were used.
* Gly-12, C-Ha-ras gene have GGC at codon 12; oh'Gua, C-Ha-ras gene having G (oh'Gua) C at codon 12; Asp-12, C-Ha-ras gene having GAC at codon 12.

Fig. 2. Sequence analysis of synthetic c-Ha-ras genes present in the transformed NIH3T3 cells. A to C, analysis of clones with a mutation at the oh'Gua site; D and E, analysis of clones with a mutation in the position adjacent to oh'Gua; F, analysis of a clone obtained by transfection of a normal c-Ha-ras gene. Lanes 1 to 3, analysis of the second position of codon 12, AatII for T (Lane 1), SalI for A (Lane 2), and BseB for C (Lane 3). Lanes 4 to 6, analysis of the first position of codon 12, Stul for T (Lane 4), Mscl for A (Lane 5), and Apal for C (Lane 6). Lane 7, HapII digestion for detection of a normal c-Ha-ras gene. M corresponds to the polymerase chain reaction product without treatment with the restriction enzymes.
ential to pair with any base, but also caused misinsertion at adjacent pyrimidines when DNA containing 8-hydroxyguanine (8-Gua) was used as template for in vitro DNA synthesis with Klenow enzyme (19). This observation was somewhat analogous to the present findings. On the contrary, Shibutani et al. (3) recently reported selective incorporation of dAMP and dCMP into the site opposite 8-hydroxyguanine and no misincorporation at the neighboring site with Klenow enzyme or polymerase α. This discrepancy between our previous findings and those of Shibutani et al. may be due to the use of Klenow fragment and dideoxynucleotides. Exonuclease activity (proofreading activity) of the Klenow fragment may bring misreading of 8-hydroxyguanine and the adjacent positions in the presence of a dideoxynucleotide (3).

The molecular mechanism of random incorporation of deoxynucleoside triphosphates into the site opposite 8-hydroxyguanine and the incorporations of dAMP and dTMP at the G residue adjacent to 8-hydroxyguanine are not fully understood. The base pairings between 8-hydroxyguanine and C and between 8-hydroxyguanine and A were elucidated in nuclear magnetic resonance studies (20, 21). It is interesting that 8-hydroxyguanine can take either an anti- or syn-conformation depending upon the base in the complementary strand. These observations suggest that the conformation of 8-hydroxyguanine in DNA is energetically more mobile than that of guanine. In addition, the electron negativity of 8-hydroxyguanine was found to be quite different from that of normal guanine (22). Possibly the local structure of the DNA near 8-hydroxyguanine is modulated by the difference in mobility and electrostatic charge of 8-hydroxyguanine. The region around codon 12 of the c-Ha-ras gene is highly GC-rich. Modification of a guanine base in a GC-rich sequence might induce local structure alteration such as B→Z transition (23). The present finding may reflect a mutation spectrum of 8-hydroxyguanine in DNA in a non-B-type structure to some extent. Various DNA polymerases and protein components involved in DNA replication in mammalian systems may also influence the fidelity of replication at the site opposite 8-hydroxyguanine in vivo, unlike in an in vitro system with purified polymerase α (3).

Further study would reveal the type of mutation induced by 8-hydroxyguanine in the second position of codon 12 of human c-Ha-ras more precisely. In addition, mutation analysis using human c-Ha-ras with 8-hydroxyguanine in the first position of codon 12 or in the first position of codon 61 in the antisense strand would provide more insight into the mechanism of mutagenesis by 8-hydroxyguanine in mammalian systems. Studies along these lines are in progress.

References
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