Replication of DNA templates containing 5-formyluracil, a major oxidative lesion of thymine in DNA

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ABSTRACT

5-Formyluracil (5-foU) is a major lesion of thymine produced in DNA by ionizing radiation and various chemical oxidants. To assess its biochemical effects on DNA replication, 22mer oligonucleotide templates containing an internal 5-foU at defined sites were synthesized by the phosphoramidite method and examined for ability to serve as a template for various DNA polymerases in vitro. Klenow fragments with and without 3′→5′ exonuclease of DNA polymerase I, Thermus thermophilus DNA polymerase (exonuclease-deficient) and Pyrococcus furiosus DNA polymerase (exonuclease-proficient) read through the site of 5-foU in the template. Primer extension assays revealed that the 5-foU directed not only incorporation of dAMP but also dCMP opposite the lesion during DNA synthesis. Misincorporation opposite 5-foU was unaffected by 3′→5′ exonuclease activity. DNA polymerases had different dissociation rates from a dCMP/T mispair and from a dCMP/5-foU mispair. The incorporation of an ‘incorrect’ nucleotide was dependent on the sequence context and DNA polymerase used. These results suggest that 5-foU produced in DNA has mutagenic potential leading to T→G transversions during DNA synthesis.

INTRODUCTION

Active oxygen species are generated in living cells by normal metabolism and by exogenous sources such as ionizing radiation and various chemical oxidants (1–3). They modify the base and sugar moieties in DNA (4–7). Thymine glycols, 8-hydroxyguanine and 5-hydroxypyrimidines are formed in DNA by exposure to such oxidizing agents (5–8). 5-Formyluracil (5-foU) is a novel type of oxidatively modified thymine in DNA (9,10). The methyl group of thymine is vulnerable to hydroxyl radical attack and it produces 5-hydroperoxymethyluracil, which is spontaneously decomposed to form 5-hydroxymethyluracil and 5-foU (4,11). 5-foU is formed in a yield comparable with that of thymine glycols and 8-hydroxyguanine by ionizing radiation (4,9,12) and quinone-sensitized UVA photooxidation (13,14). Bjelland et al. (15,16) and Zhang et al. (12) have shown that Escherichia coli and mammalian cells have DNA glycosylase activity that removes 5-foU from DNA exposed to ionizing radiation. 5-Formyl-2′-deoxyuridine is mutagenic to Salmonella TA102 when added to the culture medium (9). However, there are no direct indications regarding the biological consequences of 5-foU formed in DNA.

Recent developments in the chemical synthesis of oligonucleotides have allowed modified bases to be introduced into the oligonucleotide templates at defined sites (17–21). This is a useful means of predicting their lethal and mutagenic consequences based on the effect on DNA synthesis in vitro. In this study we synthesized oligonucleotide templates with one 5-foU at various sites using phosphoramidite chemistry (22) and examined their interaction with various DNA polymerases in vitro. The present experiments demonstrate that 5-foU directed not only incorporation of dAMP but also dCMP during DNA synthesis, suggesting that it is a potent mutagenic lesion leading to T→G transversions.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase was purchased from TOYOBO Co. Klenow fragments of DNA polymerase I with and without 3′→5′ exonuclease (KF+ and KF−, respectively) (23) were from TOYOBO Co. and Ambion Inc., respectively. Thermus thermophilus (Tth) DNA polymerase came from Epicentre Technologies Corp. and Pyrococcus furiosus (Pfu) DNA polymerase from Stratagene. Four normal HPLC-grade 2′-deoxyribonucleotide 5′-triphosphates (dNTPs) were purchased from Takara Shuzo. [γ-32P]ATP (>259 TBq/mmol) was the product of ICN Biomedicals Inc. Dithiothreitol (DTT) and nuclease-free BSA were from Wako Pure Chemicals.
In vitro DNA synthesis

To assess the overall effect of 5-foU on DNA synthesis, templates 1 or 3/primer 2 (Fig. 1) (0.1 pmol as the primer) in a reaction buffer (10 mM Tris–HCl pH 7.5, 5 mM MgCl2, 7.5 mM DTT and 200 µg/ml BSA) were incubated with 0.1 U of KF+ or KF− in the presence of four dNTPs (100 µM) at 25°C. One unit of KF+ and KF− is the amount of enzyme activity that incorporates 10 nmol of dNTP into acid-insoluble materials in 30 min at 25°C. The reaction with Tth DNA polymerase and Pfu DNA polymerase was carried out at 74°C. One unit of the thermostable DNA polymerases converts 10 nmol of dNTP into acid-insoluble materials in 30 min at 74°C.

To determine the nucleotides incorporated opposite 5-foU during DNA synthesis, the complementary pairs template 1 or 3/primer 2 (Fig. 1) (50 fmol) in 10 µl reaction mixture were incubated with various DNA polymerases. The reaction proceeded during DNA synthesis were done under the conditions described above using 0.01–500 µM dNTP and 0.2 U DNA polymerase. The complementary pairs template 1 or 3/primer 2 (50 fmol) in 10 µl reaction mixture were incubated at 25°C (KF+ or KF−) or at 74°C (Tth and Pfu DNA polymerases) for 5 min. The Michaelis constant (Km) and the maximal velocity of the reaction (Vmax) were obtained from Lineweaver–Burk plots of the kinetic experimental results. The kcat/Km value was calculated according to Dong et al. (24).

Gel electrophoresis

The reaction mixtures were heated at 95°C for 5 min, cooled and loaded onto 20% polyacrylamide gels in the presence of 7 M urea. The reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. The results with KF+ are shown in Figure 2. After incubation for >5 min full-length DNA (22mer) was synthesized with the templates containing thymine and 5-foU. Termination bands due to pausing of DNA synthesis were not observed 1 nt prior to and opposite the modified base in the template. Similar results were obtained with KF+, Tth DNA polymerase and Pfu DNA polymerase (data not shown). These results indicate that DNA polymerases read through the site of 5-foU.

RESULTS

DNA synthesis on the templates containing 5-foU

To examine the effects of 5-foU on DNA synthesis in vitro, a 22mer template containing one 5-foU at position 17 from the 3′-end (template 3) was primed with a 13mer primer (primer 2) and replicated with various DNA polymerases in the presence of four dNTPs. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. The results with KF+ are shown in Figure 3. The extension of primer 3 annealed to template 3 was extended by various DNA polymerases in the presence of a single dNTP. The extension of primer 3 annealed to template 3 by KF+ was analyzed by 7 M urea–20% polyacrylamide gel electrophoresis. The results are shown in Figure 3. KF+ incorporated dCMP in addition to dAMP opposite thymine and 5-foU. dGMP was slightly incorporated. Some incorporation of dA and dT opposite 5-foU was observed. Some incorporation of 5-foU was unaffected by exonuclease activity (Fig. 4). KF+ also inserted dCMP opposite the lesion as well as dAMP. The ratios of dCMP/dAMP incorporated opposite 5-foU were determined using 0.01–500 µM dNTP and 0.2 U DNA polymerase. The complementary pairs template 1 or 3/primer 2 (50 fmol) in 10 µl reaction mixture were incubated at 25°C (KF+ or KF−) or at 74°C (Tth and Pfu DNA polymerases) for 5 min. The Michaelis constant (Km) and the maximal velocity of the reaction (Vmax) were obtained from Lineweaver–Burk plots of the kinetic experimental results. The kcat/Km value was calculated according to Dong et al. (24).
Figure 2. Time course of in vitro DNA synthesis catalyzed by KF + . Template 1 (lanes 2–9) or template 3 (lanes 10–17) was annealed with primer 2 (0.1 pmoI) and incubated with 0.1 U KF + in 10 mM Tris–HCl, pH 7.5, containing 5 mM MgCl₂, 7.5 mM DTT and 200 µg/ml BSA in the presence of four dNTPs (100 µM) at 25°C for 0 (lanes 2 and 10), 1 (lanes 3 and 11), 2 (lanes 4 and 12), 5 (lanes 5 and 13), 10 (lanes 6 and 14), 20 (lanes 7 and 15), 40 (lanes 8 and 16) or 80 min (lanes 9 and 17). The reaction was stopped by adding termination solution (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 20 mM EDTA), followed by denaturing polyacrylamide gel electrophoresis at a constant voltage of 1800 V. Lane 1, size marker.

Figure 3. Primer extension assay to identify the nucleotides incorporated opposite 5-foU by KF – . Template 1 or 3/primer 3 (50 fmol) in a 10 µl reaction mixture was incubated with KF – in the presence of a single dNTP at 25°C for 5 min. The reaction was stopped by adding termination solution, followed by denaturing polyacrylamide gel electrophoresis. Template 1, lanes 1–4; template 3, lanes 5–8. Lanes 1 and 5, dGTP; lanes 2 and 6, dATP; lanes 3 and 7, dTTP; lanes 4 and 8, dCTP.

Figure 4. Primer extension assay to identify the nucleotides incorporated opposite 5-foU by KF + and KF + enzymes. Template 1 or 3/primer 3 (50 fmol) in a 10 µl reaction mixture was incubated with KF + (lanes 1–8) and KF + (lanes 9–16) in the presence of a single dNTP at 25°C for 10 min. Template 1, lanes 1–4 and 9–12; template 3, lanes 5–8 and 13–16. Lanes 1, 5, 9 and 13, dGTP; lanes 2, 6, 10 and 14, dATP; lanes 3, 7, 11 and 15, dTTP; lanes 4, 8, 12 and 16, dCTP.

Table 1. Kinetic parameters for incorporation of dAMP and dCMP opposite thymine and 5-formyluracil by KF –

<table>
<thead>
<tr>
<th>Missincorporation</th>
<th>Kₘ (µM)</th>
<th>Vₘₐₓ (%/min)</th>
<th>kₗₑₜ (per s)</th>
<th>kₗₑₜ/Kₘ (per µM/s)</th>
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<tr>
<td>dAMP–T</td>
<td>1.1</td>
<td>26.4</td>
<td>0.34</td>
<td>0.31</td>
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<tr>
<td>dCMP–T</td>
<td>242</td>
<td>5.6</td>
<td>0.073</td>
<td>0.0003</td>
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<tr>
<td>dAMP–5-foU</td>
<td>3.9</td>
<td>21</td>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>dCMP–5-foU</td>
<td>8.8</td>
<td>14</td>
<td>0.18</td>
<td>0.02</td>
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</table>

The kinetic constants Kₘ and Vₘₐₓ were determined for KF +, KF +, Tth and Pfu DNA polymerases. The specificity of a substrate was defined as kₗₑₜ/Kₘ (24). The values of Kₘ, Vₘₐₓ and kₗₑₜ/Kₘ for incorporation of dAMP and dCMP opposite thymine and 5-foU by KF – are presented in Table 1. The kₗₑₜ/Kₘ values were 0.06 and 0.02/µM/s for dAMP and dCMP incorporation respectively.

Primer 3 annealed to template 3 was extended by Tth and Pfu DNA polymerases in the presence of a single dNTP. Tth DNA polymerase is devoid of exonuclease activity (25). On the other hand, Pfu DNA polymerase possesses an associated 3′→5′ exonuclease (proofreading) activity (26). These polymerases incorporated only dAMP opposite 5-foU in this sequence context. We identified the nucleotides incorporated opposite the lesion in template 2 primed by primer 1 (9mer) during DNA synthesis. As seen in Figure 5, in the presence of dCTP 11mer DNA was opposite 5-foU and 0.31 and 3 × 10⁻³/µM/s for dAMP and dCMP incorporation respectively opposite thymine. The kₗₑₜ/Kₘ values were also determined with KF + (Table 2). The values were 0.43 and 5 × 10⁻⁴/µM/s for dAMP and dCMP incorporation opposite thymine and 0.09 and 0.04/µM/s for dAMP and dCMP opposite 5-foU respectively. Dissociation rates of KF + from a dCMP-T mispair and from a dCMP-5-foU mispair were different. The ratio dCMP–T/dAMP–T for KF – (3 × 10⁻⁴/0.31) was lowered ~10-fold by 3′→5′ exonuclease activity (5 × 10⁻⁵/0.43), whereas the ratio dCMP–5-foU/dAMP–5-foU was unaffected (0.02/0.06, 0.04/0.09). These results also indicated that the 5-foU–dCMP mispair could be proofread.
Table 3. The specificity of nucleotide incorporation opposite 5-foU by KF

<table>
<thead>
<tr>
<th>Primer</th>
<th>Template</th>
<th>Percentage dAmp incorporated</th>
<th>dTMP</th>
<th>dCMP</th>
<th>dGMP</th>
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<tr>
<td>3</td>
<td>1</td>
<td>91.5</td>
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<td>6.8</td>
<td>&lt;0.3</td>
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<tr>
<td>3</td>
<td>3</td>
<td>67.6</td>
<td>&lt;0.3</td>
<td>30.8</td>
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<tr>
<td>1</td>
<td>1</td>
<td>98.2</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>98.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
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</tbody>
</table>

**DISCUSSION**

When cellular DNA is exposed to oxidizing agents, several thymine hydroperoxides are formed. The major products are cis-6-hydroperoxy-5-hydroxy-5,6-dihydrothymine and cis-5-hydroperoxy-6-hydroxy-5,6-dihydrothymine, which gradually undergo secondary reactions to form thymine glycols (4,11). On the other hand, 5-hydroperoxymethyluracil spontaneously decomposes to produce 5-hydroxymethyluracil and 5-foU (4,11). Because 5-foU is readily formed in DNA by exposure to ionizing radiation and active oxygen species (4,9,12–14), it is important to assess its mutagenic potential. Kasai et al. (9) have reported that 5-formyl-2′-deoxyuridine is mutagenic to *Salmonella* TA102 when added to the culture medium. However, the biological consequences of 5-foU formed in DNA remain unknown.

Many types of DNA damage have been identified in cells exposed to ionizing radiation and oxidizing agents (4–8). If left unrepaired these could have lethal and mutagenic consequences. Recent developments in the chemical synthesis of oligonucleotides have made it possible to introduce a modified base into oligonucleotide templates at defined sites (17–21). This is a useful means of predicting its lethal and mutagenic consequences based on the effects on DNA synthesis in *vitro*. In the present study oligonucleotide templates containing one 5-foU were synthesized by phosphoramidite chemistry (22). It is noteworthy that these oligonucleotides are stable to heat and alkali (data not shown).

Replication of the templates containing 5-foU with KF+ or KF− was not arrested 1 nt prior to or opposite the lesion (Fig. 2). Either a correct or an incorrect nucleotide other than dAMP could be incorporated opposite 5-foU. If the latter occurs *in vivo* it would result in base substitution mutations. In the presence of a single dNTP, KF+ incorporated dCMP in addition to dAMP opposite the site of 5-foU. KF− also inserted dCMP and dAMP opposite 5-foU. The ratios of dCMP to dAMP incorporated opposite the 5-foU during reactions catalyzed by KF+ and KF− were 0.3 and 0.32 respectively. Therefore, the 5-foU–dCMP mispair is stable to proofreading activity. This argument was supported by the results obtained with the Tth (exonuclease-deficient) and Pfu (proofreading exonuclease-proficient) DNA polymerases. These results of this study predict that T→G transversions are induced by 5-foU.

The mutation spectrum will potentially be sequence context dependent, since the dCMP incorporation frequency opposite 5-foU was affected by the nearest neighbour base pair. If this is true *in vivo*, the mutagenic effect of 5-foU needs to be examined in association with nearest neighbour influence. The precise mechanisms for the sequence context-dependent manner of dCMP misincorporation opposite 5-foU remain unsolved. Nearest neighbour base stacking interactions appear to have
different effects on the $K_{in}$ and $V_{max}$ of DNA polymerase reactions.

Powerful blocking lesions, such as urea residues and β-ureidobutyric acid, are also mutagenic (27). Although these lesions direct specific misincorporations that are influenced by sequence context (27), their mutagenic activities might also be SOS dependent. Although 5-foU is not a blocker of DNA synthesis sequence context (27), their mutagenic activities might also be lesions direct specific misincorporations that are influenced by (23,28–30). 5-foU in DNA has recently been identified as a lesions are usually removed by cellular DNA repair enzymes incorporation of incorrect nucleotides. Lethal and mutagenic DNA constitutes a lethal and mutagenic lesion by directing mis-

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REFERENCES