Mechanism for Exclusion of 5-Fluorouracil from DNA

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ABSTRACT

5-Fluorodeoxyuridine 5′-triphosphate is hydrolyzed by the enzyme deoxyuridine triphosphate diphosphohydrolase (EC 3.6.1.23). Uracil-DNA glycosylase removes 5-fluorouracil (FUra) from FUra-containing DNA similar to its removal of uracil from uracil-containing DNA. The absence of FUra in DNA following exposure of cells to FUra can be explained by the activities of these two enzymes.

INTRODUCTION

Cells incorporate the chemotherapeutic agent, FUra, into RNA but not into DNA (12). Synthesis of the pyrimidine analog-containing RNA occurs via the intracellular nucleoside triphosphate derivative, fluorouridine 5′-triphosphate, and RNA polymerase (4). DNA polymerase can utilize the corresponding deoxynucleoside triphosphate, FdUTP (23); however, although the pathway for its synthesis is present (17), FdUTP has not been found in cells (12). Recent investigations of uracil misincorporation into DNA have shown that, even though cells contain the pathway for conversion of dUMP to dUTP (17), FdUTP has not been found in DNA in vivo.

MATERIALS AND METHODS

Chemicals. Unlabeled nucleosides, nucleotides, and bases were purchased from P-L Biochemicals (Milwaukee, Wis.). FUra and fluorodeoxyuridine were obtained from Sigma Chemical Company (St. Louis, Mo.). [6-3H]FdUMP (specific activity, 20 Ci/mmol) was purchased from Moravek Biochemicals (City of Industry, Calif.). [5-3H]FdUTP (specific activity, 30 Ci/mmol) was prepared from [3H]FdUMP by deamination according to the method described by Lohman (20). Polyethyleneimine cellulose thin-layer plates were from Brinkmann Instruments (Westbury, N. Y.). The Lichrosorb RP-18 reverse-phase chromatography column was from Altex (Berkeley, Calif.).

Preparation of [3H]FdUTP. [3H]FdUTP was prepared enzymatically from [3H]FdUMP using, as the source of deoxynucleoside monophosphate kinase and deoxynucleoside diphosphate kinase, an ammonium sulfate fraction of Escherichia coli extract prepared essentially as described by Hurlbert and Furlong (14) except that an E. coli mutant, deficient in dUTPase activity (6), was used. The ammonium sulfate fraction (0 to 210 mg/ml) from 0.9 g E. coli cell paste was dialyzed against 25 mM Tris-HCl (pH 8.0), 0.25 mM EDTA, 0.5 mM dithiothreitol, and 50% glycerol (final volume, 0.2 ml). The reaction mixture (0.2 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5 mM potassium phosphate, bovine plasma albumin (20 µg/ml), 5 mM ATP, 100 µM [3H]FdUMP (6 Ci/mmol), and 2 µl E. coli ammonium sulfate fraction, and this was incubated for 16 hr at 37°C resulting in conversion of 40 to 50% [3H]FdUMP to [3H]FdUTP. [3H]FdUTP was purified by chromatography on S&S 589 Orange Ribbon-C paper (Schleicher & Schuell, Inc., Keene, N. H.) in isobutyric acid/concentrated NH₄OH/water (66/1/33, v/v/v) and eluted with water. Overall recovery of radioactivity in FdUTP was 20 to 30%.

dUTPase Assay. Measurement of dUTPase activity was carried out in a mixture (20 µl) containing 20 mM Tris-HCl (pH 7.6), 20 mM KCl, 10% glycerol, bovine plasma albumin (0.5 mg/ml), 1 µM [3H]FdUTP (6 µCi/nmol), and 0.28 µg purified dUTPase. The enzyme was purified 70-fold from cultured human lymphoblasts, was free of contaminating phosphatase activities, and had an activity of 5 × 10⁻³ nmol dUMP formed from dUTP per mg protein in 10 min at 37°C (details of purification and characterization of dUTPase will be described elsewhere). The mixture was incubated for 10 min at 37°C, and the reaction was terminated by addition of 1 µl of 1 M EDTA. The mixture was analyzed by polyethyleneimine cellulose thin-layer chromatography (1 M LiCl), paper chromatography using Whatman No. 3MM paper (Clifton, N. J.) in 4.5 M (NH₄)₂SO₄/0.1 M potassium phosphate (pH 6.8)/n-propylalcohol (60/100/2, v/v/v) and by paper electrophoresis in Whatman No. 3MM paper in 0.03 M Na₂B₄O₇ buffer (pH 9.0).

Preparation of Radiolabeled DNA. Radiolabeled DNA substrates were prepared in a mixture (200 µl) containing 50 mM Tris (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, bovine plasma albumin (0.2 mg/ml), 30 mM dCTP, 30 mM dATP, 30 mM dGTP, 5 mM [3H]dUTP (1.56 µCi/nmol), or 1 µM [3H]-FdUTP (6 µCi/nmol), or 5 µM [3H]dTTP (0.45 µCi/nmol), 0.2 mM alkali-denatured E. coli DNA, and 1.6 units purified calf thymus DNA polymerase (8). Incubation for 60 min at 37°C resulted in incorporations of 0.08 nmol [3H]dUMP, 0.02 nmol [3H]FdUMP, and 0.14 nmol [3H]dTMP, respectively. The reaction was terminated by adding 50 mM EDTA and 1% sodium dodecyl sulfate. The mixture was digested with pronase (Calbiochem, San Diego, Calif.) (1 mg/ml) for 16 hr at 37°C and extracted with phenol, and the DNA was recovered by ethanol precipitation. The DNA was freed of residual acid-soluble contaminants by passage through a Sephadex G-75 column (Phar...
for 1 hr at 37° with the cytosol. Incubations were terminated again by ethanol precipitation. 0.2 M LiCl/2 mM EDTA/20 mM Tris-HCl (pH 7.6) and recovered mixture was incubated at 0° for 4 hr with the E. coli enzyme or cosylase is active under these conditions (18). The reaction was included to inhibit nucleases since uracil-DNA glycosylase is active under these conditions (18). The reaction mixture was incubated at 0° for 4 hr with the E. coli enzyme or for 1 hr at 37° with the cytosol. Incubations were terminated by adding 5 μg of carrier salmon sperm DNA, 0.1 volume of 10 M LiCl, and 2 volumes of 95% ethanol. After 1 hr at -20°, the mixture was centrifuged (12,000 x g, 6 min at 4°), and 5 μl of the supernatant were dried on a GF/C glass fiber filter (Whatman, Inc., Clifton, N. J.) and counted.

**HPLC Analysis.** Aliquots of the ethanol-soluble supernatants obtained from the uracil-DNA glycosylase incubation reactions described above were analyzed by an initial separation of base and nucleoside from nucleotides and oligonucleotides on S&S Orange Ribbon paper in n-butyl alcohol/H2O (86/14, v/v). The base and nucleoside were then eluted with H2O, and their identity was established by HPLC using a reverse-phase column (Altex/Lichrosorb RP-18; 10 μm, 4.6 x 250 mm) with 0.02 M potassium phosphate (pH 3.7) at a flow rate of 1.5 ml/min. Fractions of effluent (0.3 ml) were collected and counted in a Triton-X/toluene scintillation mixture.

**RESULTS**

**Susceptibility of FdUTP to dUTPase.** To determine whether FdUTP serves as a substrate for dUTPase, [3H]FdUTP was incubated with dUTPase purified from cultured human lymphoblasts. Three different analytical systems, ion-exchange chromatography, paper chromatography, and paper electrophoresis, were used to analyze the products of this incubation. Chart 1 shows that by all 3 methods of analysis dUTPase converted all radioactivity from FdUTP to FdUMP. In control incubations, FdUTP was quantitatively converted to dUMP, whereas there was less than 1% breakdown of other nucleotides (5-fluorodeoxyuridine monophosphate, UDP, dCTP, dCDP, dTDP, dTTP, 5-bromodeoxyuridine triphosphate) under the same incubation conditions. The Km for FdUTP was 5.5 x 10^{-6} M, which is similar to the value found for dUTP (3.0 x 10^{-6} M).

**Uracil-DNA Glycosylase Activity on DNA containing FUra.** Uracil-DNA glycosylases from 2 different sources, E. coli (27) and cultured human lymphoblasts (10), were tested for activity on [3H]FUra-containing DNA. Table 1 shows that with both enzymes a substantial proportion of radioactivity (>25%) was released from the [3H]FUra-DNA. There was a higher proportion of released counts with [3H]FUra-DNA than with [3H]uracil-DNA after incubation with uracil-DNA glycosylase was found almost exclusively (>95%) in the bases, FUra or uracil (Chart 2). No radioactivity was observed with either 5-fluorodeoxyuridine (retention time, 7.1 min) or deoxyuridine (retention time, 5.4 min). The possibility that FdUMP or dUMP was released and then degraded to FUra or uracil was tested by incubating [3H]FdUMP and [3H]-dUMP in the standard reaction mixture and analyzing by paper chromatography. No breakdown was detected, thus indicating that degradation via the nucleotides was not the source for base that appeared during the incubation. Only a background trace level of radioactivity was released with control incubations containing [3H]thymine-DNA, and none of this (<0.1%) was in the form of the base, thymine.

It is known that the excision of misincorporated uracil can be partially inhibited by adding exogenous uracil to the enzyme reaction (3, 10, 19). FUra (10 μM) and uracil (10 μM) were compared for their inhibitory activity with human lymphoblast uracil-DNA glycosylase (experimental conditions described in “Materials and Methods”). FUra inhibited uracil removal by 40%, whereas uracil inhibited uracil removal by 75%. As expected, thymine was ineffective (<0.1%).

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be present in treated cells, its level sustained in part by mechanisms responsible for the elevation of dUTP, including increased activity of ribonucleotide reductase on 5-fluorouridine diphosphate (and UDP) due to decreased dTTP (21). It is likely, therefore, that FdUTP participates along with uracil in the increased misincorporation leading to excision-repair activities under these circumstances. The past inability to detect intracellular FdUTP or FdUra in DNA is not surprising in view of the difficulties in demonstrating this for uracil (9) and in no way conflicts with what is proposed here.

The relationship between misincorporation-removal of FdUra (and uracil) and the observed cytotoxicity of FdUra is not yet clear. It is worth noting that with combined use of FdUra and methotrexate the misincorporation-removal pathway described here is a site of interaction between the 2 drugs. If this pathway proves to play a role in the cytotoxicity of these drugs, it may also help to explain some of the apparently contradictory observations on the concurrent use of both drugs (1, 16, 25).

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