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DNA repair following incorporation of 5-fluorouracil into DNA of mouse bone marrow cells*

John D. Schuetz, Hugh J. Wallace, and Robert B. Diasio

Department of Pharmacology and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Summary. 5-Fluorouracil (FUra) was previously demonstrated to be incorporated into DNA at cytotoxic concentrations in mouse bone marrow cells. Subsequently, we showed that under these conditions FUra exhibited a time-dependent removal from DNA accompanied by a decrease in DNA strand length. In the present study we utilized hydroxyurea to inhibit semiconservative DNA synthesis while monitoring DNA repair by measuring the incorporation of [^{3}H]dThd into double-stranded DNA (DNAds), which can be separated from DNA at the replication fork (DNAss) by benzoylated-naphthoylated-DEAE cellulose. Under these conditions we assessed DNA repair in cells that had previously been exposed for 1 h to varying cytotoxic concentrations of FUra. The results demonstrate an increase in labelling of DNAds with increasing FUra concentrations, with no appreciable increase in incorporation of label into DNAss. In conclusion, this study demonstrates that DNA repair occurs following incorporation of FUra. The failure to repair DNA damage at higher FUra concentrations may have a role in the cytotoxicity of this drug.

Introduction

Recent studies have demonstrated 5-fluorouracil (FUra) incorporation into DNA of murine bone marrow cells [11, 13] as well as tumor cells [3, 5, 9], suggesting that incorporation of FUra into DNA may be a potential mechanism of cytotoxicity [3, 5, 9, 11, 13]. However, it must be noted that the FUra residues detected in DNA are the net result of enzymatic incorporation of FUra nucleotides by polymerase alpha [17] and the subsequent excision of the FUra base by uracil glycosylase [2, 7]. The removal of a misincorporated base from DNA is the first step in an enzymatic process defined as base-excision repair and results in an apurinic/apyrimidinic site in the DNA, which in turn may lead to fragmentation of the DNA [6]. Because FUra incorporation into DNA is associated with disruption of DNA structure and possibly replication [12, 14], DNA repair was examined for further exploration of the basis of FUra-induced DNA damage.

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Offprint requests to: R. B. Diasio

In the present study we utilized hydroxyurea (HU) to inhibit semiconservative DNA synthesis [1, 10]. Under these conditions benzoylated-naphthoylated-DEAE cellulose (BND-cellulose) [4, 15, 16] was used to assess repair in DNA isolated from mouse bone marrow cells that had been previously exposed to varying cytotoxic concentrations of FUra. The results of these studies demonstrated that increased DNA repair was associated with cytotoxic concentrations of FUra.

Materials and methods

Chemicals. [methyl-^{3}H]dThd (50–70 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif). FUra, HU, pronase B, RNase A, and BND-cellulose were obtained from Sigma Chemical Co. (St. Louis, Mo). Isoamyl alcohol and phenol reagent were purchased from Fisher Chemical Co. (Pittsburgh, Pa). All chemicals were of the highest quality available.

Preparation of cells. The studies described below utilized single cell suspensions of marrow cells isolated from the femurs and tibias of male CF_1 mice (21–24 g) as described previously [13]. Cell viability was assessed by trypan blue dye exclusion and was 85% or greater during these experiments. Cells were suspended in an alpha-minimal essential media (alpha MEM, no. 410-2000) containing no deoxyribonucleotides or ribonucleosides purchased from Grand Island Biological Co. (Grand Island, NY).

Inhibition of semiconservative DNA synthesis. To assess repair it was necessary to inhibit semiconservative replication [15]. Freshly isolated bone marrow cells were incubated in alpha-MEM at 37°C containing varying concentrations of HU. Cells were incubated for 30 min in this medium before the addition of [^{3}H]dThd (100 μCi/ml). After the addition of [^{3}H]dThd the incubation was continued for an additional 2 h. The unincorporated isotope was removed by washing the cells with ice-cold alpha-MEM. DNA was purified from these cells as previously described [13]. The purified DNA was quantitated spectrophotometrically by determining the optical density at 260 nm assuming that for double-stranded DNA (DNAds) 1 absorbance unit = 50 μg/ml while for single-stranded DNA, i.e., DNA with single-stranded regions such as DNA at the replication fork (DNAss), 1 absorbance unit = 37 μg/ml. The amount of radioactivity was determined by scintillation counting as previously described [13].

Assessment of DNAds and DNAss by BND-cellulose chromatography. A slurry of BND-cellulose was prepared in a 0.3 *M* buffer containing 0.3 *M* NaCl, 0.1 m*M* EDTA, 0.01 *M* Tris-HCl, pH 7.45. Fines were decanted and allowed to settle to a packed bed volume of 1.5 ml in a 3-ml syringe having a diameter of 0.5 cm. Stepwise elution of DNAds was accomplished with 8.0 ml of a 1.0 *M* buffer containing 1.0 *M* NaCl, 0.1 m*M* EDTA, and 0.01 *M* Tris-HCl, pH 7.45, and elution of DNAss was performed with a caffeine buffer containing 1.0 *M* NaCl, 0.1 m*M* EDTA, 0.01 *M* Tris-HCl, pH 7.45, and 1% (wt/vol) caffeine. Fractions containing DNAds were combined, as were those containing DNAss, followed by overnight dialysis against 200 volumes of 0.15 *M* sodium chloride: 0.015 *M* sodium citrate (pH 7.0).

Evaluation of DNA repair. Freshly isolated bone marrow cells were exposed for 1 h at 37° C to FUra concentrations (0–100 μ*M*) known to be cytotoxic [13] and to produce DNA fragmentation [12, 14]. Subsequently, cells were washed with drug-free alpha-MEM at 37°C and resuspended in 5 m*M* HU for 30 min. To this incubation mixture was added [^{3}H]dThd, and the incubation was continued for an additional 2 h. Isotope incorporation was linear over this interval (data not shown). The unincorporated isotope and HU were removed by washing the cells in ice-cold alpha MEM. DNA was then purified from these cells, and the DNA was fractionated on BND-cellulose as described above.

Results and discussion

Fig. 1 depicts the pattern of inhibition of DNA synthesis with increasing concentrations of HU. It can be seen that above 2.5 m*M* there was essentially no further inhibition of DNA synthesis. These data suggest that semiconservative DNA synthesis was almost completely inhibited. The 4%–5% residual DNA synthesis is consistent with reported values of DNA repair [1, 10]. Similar residual DNA synthesis has also been seen with other inhibitors of DNA synthesis, such as aphidicolin [8].

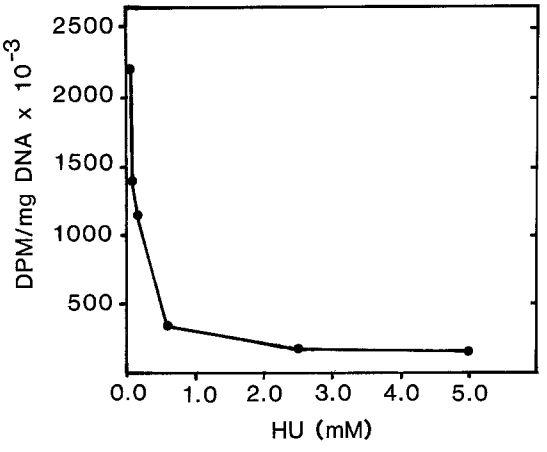

Fig. 1. Effect of HU on semiconservative DNA synthesis. Freshly isolated bone marrow cells were incubated for 30 min in the presence of HU. [^{3}H]dThd was added to the cells and the incubation continued for further 2 h. Cells were washed, DNA purified, and radioactivity measured as described in *Materials and methods*

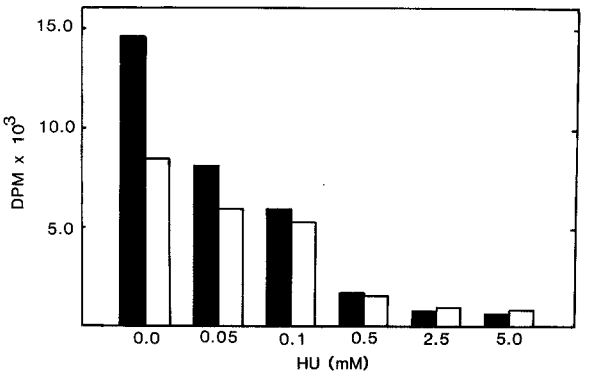

Fig. 2. Effect of HU on the incorporation of [^{3}H]dThd into DNAds and DNAss. Freshly isolated bone marrow cells were incubated with [^{3}H]dThd as described in the legend to Fig. 1, followed by DNA purification. Purified DNA 10 μg was applied to a BND-cellulose column and DNAds and DNAss fractionated as described in *Materials and methods*. Radioactivity eluting as DNAds *(solid bar)* and DNAss *(open bar)* was quantitated. The recovery of radioactivity applied to the column was at least 80% for each analysis

DNA that had been labelled with [^{3}H]dThd in the presence or absence of HU was characterized by BND-cellulose chromatography. This methodology permits separation of DNAds from DNAss [4, 15, 16]. DNAss represents the DNA that has not been ligated. This DNA contains single-stranded regions that bind tightly to the resin, permitting separation from DNAds. Figure 2 illustrates the profile obtained in the presence or absence of HU. In the absence of HU [^{3}H]dThd was incorporated into both DNAds and DNAss, with more radiolabel being detected in DNAds. In the presence of increasing concentrations of HU the incorporation of [^{3}H]dThd into both DNAds and DNAss decreased with incorporation of [^{3}H]dThd into DNAds remaining essentially unchanged above 2.5 m*M* HU. This effect is consistent with HU-induced inhibition of DNA ligation at the replication forks [1, 15].

Using HU to suppress semiconservative DNA synthesis, we assessed DNA repair synthesis in bone marrow cells that had been preincubated with cytotoxic concentrations of FUra (10 μ*M*, LD_{50}; 50 μ*M*, LD_{90}; 100 μ*M*, no detectable viability) by quantitating the radioactivity incorporated into DNAds and DNAss following removal of FUra and incubation of cells with [^{3}H]dThd in presence of the inhibitor. Figure 3 shows the pattern of incorporation of radioactivity into DNAds and DNAss following preincubation in either the absence or the presence of cytotoxic concentrations of FUra. Whereas only a slight (nonsignificant) difference in incorporation of [^{3}H]dThd into DNAss was observed as the concentration of FUra was increased from 0 to 100 μ*M*, there was a marked increase in the incorporation of [^{3}H]dThd into DNAds as the FUra concentration increased, with the incorporation of tritium at 10 μ*M* FUra being significantly different from control (absence of FUra) and the incorporation of tritium at 10 μ*M* FUra being significantly different from that at 100 μ*M* FUra. However, the incorporation of tritium into DNAds did not increase in proportion to the FUra concentration. The minimal increase in incorporation of radioactivity into DNAds as the concentration approached 100 μ*M* may re-

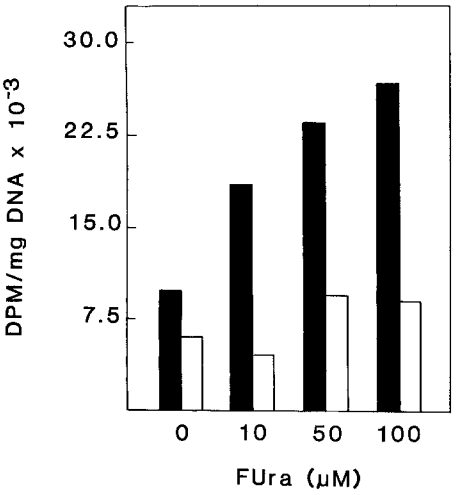

Fig. 3. Assessment of FUra-induced DNA repair. Bone marrow cells were initially incubated with varying FUra concentrations for 1 h. Cells were washed free of FUra and resuspended in HU, followed by the addition of [^{3}H]dThd as described in *Materials and methods*. After the cells had been washed free of radiolabel, DNA was purified and 10 μg DNA was applied to the BND-cellulose column. Radioactivity eluting as DNAds *(solid bar)* and DNAss *(open bar)* was quantitated. The values represent the average of three separate experiments with the standard error of each value being less than 20%

present decreased DNA repair at cytotoxic concentrations or possibly saturation of the repair process.

The results of the present study are best viewed in the context of our previous studies that examined the effects of FUra on DNA in isolated mouse bone marrow cells under similar conditions [12–14]. We demonstrated that following exposure of mouse bone marrow cells to 50 μ*M* FUra for 1 h there was detectable incorporation of FUra into DNA (60 fmol/μg DNA) and a decrease in DNA synthesis (40% of control). Subsequent studies showed that when cells were place in fresh media (after 1 h exposure to 50 μ*M* FUra) there was a decrease in DNA strand length over a 3-h period. Accompanying this was a release of tritiated FUra from DNA, which demonstrated an association between FUra removal and the formation of DNA fragments. In the present study we have utilized HU to block semiconservative DNA synthesis following a 1 h exposure to these same concentrations of FUra. The finding that [^{3}H]dThd is incorporated into DNA (particularly DNAds) demonstrated that the DNA was repaired following removal of FUra from DNA. While this excision-repair process may be effective at relatively low cytotoxic concentrations of FUra, it is likely that it is relatively ineffective at more cytotoxic concentrations, possibly resulting in DNA fragmentation and cell death.

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