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Ionizing radiation and tritium transmutation both cause formation of 5-hydroxymethyl-2′-deoxyuridine in cellular DNA

([^{3}H]thymidine/hydroxyl radicals/HPLC/DNA-protein crosslinks/DNA repair)

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ABSTRACT **HeLa cells grown in the presence of [*methyl*-^{3}H]thymidine contained large amounts of 5-hydroxymethyl-2′-deoxyuridine (HMdU) in their DNA. When the cells were grown in [*6*-^{3}H]thymidine and their DNA was labeled to the same specific activity, no HMdU was present. When such [*6*-^{3}H]thymidine-labeled cells were exposed to increasing amounts of γ-radiation, small but increasing amounts of HMdU were formed in their DNA. This indicates that HMdU can be formed in DNA by two distinct mechanisms. The first is the result of the transmutation of ^{3}H to ^{3}He (β decay) in the methyl group of thymidine, leading to formation of a carbocation. This short-lived ion reacts with hydroxide ions of water, yielding the hydroxymethyl group. HMdU that is formed by this mechanism is formed at the rate of β decay of ^{3}H. It appears only in [*methyl*-^{3}H]thymidine residues and is present in the DNA of both nonirradiated and γ-irradiated cells. The second mechanism is the result of the radiolysis of water caused by ionizing radiation. The resultant radical species, particularly hydroxyl radicals, may react with many sites on DNA. When the methyl group of thymine is attacked by hydroxyl radicals, the hydroxymethyl group is formed. The formation of HMdU by this mechanism was detected only when [*6*-^{3}H]thymidine-labeled cells were used, since transmutation of ^{3}H in position 6 of thymine cannot yield HMdU.**

Ionizing radiation causes the formation of strand breaks in DNA, release of bases from the DNA backbone, and modification of the bases themselves (1–5). This modification is thought to be effected primarily through the reaction of radical species derived from the radiolysis of water (6–10) with the bases, of which thymine (Thy) is the most reactive (9, 10). To study the radiation-induced formation of derivatives of thymine in human cells, we used HPLC to separate 2′-deoxyribonucleosides derived from enzymatically digested DNA of γ-irradiated HeLa cells grown in the presence of [*methyl*-^{3}H]thymidine (11, 12). The major thymine derivative proved to be 5-hydroxymethyl-2′-deoxyuridine (HMdU) (13). This was surprising because 5-hydroxymethyluracil (HMUra) and HMdU had been found only as minor products of irradiated solutions of thymine and thymidine at neutral pH (7, 8), as compared to ring-saturated derivatives such as 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol, TG). This result had been attributed to the greater susceptibility of the 5,6-ethylenic bond of thymine to hydroxyl radical attack than that of its methyl group (9). Equally surprising was our finding that the HMdU content of the DNA of nonirradiated cells was only slightly less than that of irradiated cells (11–13). The HMdU content of nonirradiated DNA was 0.15% of the total ^{3}H applied to the HPLC column. Thirty-six kilorads (krad; 1 krad = 10 gray) of externally applied γ-radiation increased the HMdU content by only one-third to a total of 0.20% (11–13). This suggested that the effective dose of radiation to the cell nucleus from incorporated [^{3}H]thymidine was equivalent to 108 krad (3 × 36) of γ-radiation. However, the actual dose of radiation from ^{3}H was calculated to be less than 0.5 krad.† Therefore, it seemed as if ionizing radiation from [*methyl*-^{3}H]thymidine was 250 times more effective in forming HMdU than was externally applied γ-radiation. We report here that this discrepancy between radiation dose and formation of HMdU is due to its formation primarily through transmutation of ^{3}H to ^{3}He in the methyl group of thymine and not through ionizing radiation. This is a demonstration of a unique transmutation product of a ^{3}H-labeled radionuclide in cellular DNA. However, in this report we show that HMdU can also be formed in cellular DNA through the action of externally applied γ-radiation and we cite evidence that the formation of HMdU by this mechanism may contribute to the cytotoxic effects of ionizing radiation.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq), [*6*-^{3}H]thymidine (16.2 Ci/mmol), and [*methyl*-^{14}C]thymine (496 mCi/mmol) were purchased from New England Nuclear. HMdU was obtained from Sigma. Cell growth medium, horse serum, and L-glutamine were purchased from GIBCO. Acetone and acetonitrile, both HPLC grade, were obtained from Fisher.

Growth and Labeling of HeLa Cells. HeLa cells were grown in suspension culture in Joklik-modified minimal essential medium supplemented with 10% horse serum and L-glutamine (0.002 M) in the presence of either [*methyl*-^{3}H]thymidine or [*6*-^{3}H]thymidine at 1.25 μCi/ml of medium, at 37°C for 24 hr. Because the specific activity of [*6*-^{3}H]thymidine was 2.4 times higher than that of [*methyl*-^{3}H]thymidine it was diluted with unlabeled thymidine to achieve the same final thymidine concentration. The radioactive medium was removed and cells were grown for an additional 2 hr in nonradioactive thymidine (10 μM).

γ-Irradiation of Cells and Isolation of DNA. Cells were washed once with cold phosphate-buffered saline, resuspended in phosphate-buffered saline, and divided into two tubes. One tube (control nonirradiated HeLa cells) was placed in an ice-water bath, while the other, also in an ice-

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Abbreviations: HMdU, 5-hydroxymethyl-2′-deoxyuridine; HMUra, 5-hydroxymethyluracil; TG, thymine glycol (5,6-dihydroxy-5,6-dihydrothymine); dTG, thymidine glycol (5,6-dihydroxy-5,6-dihydrothymidine); HMH, 5-hydroxy-5-methylhydantoin.

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†This estimate is obtained by the following calculation: one tritium decay (*d*) equals 0.27 rad delivered to the cell nucleus (14). The specific activity of the DNA after a 24-hr labeling period was 1.5×10^5 *d*/min per μg of DNA, and the mean DNA content of a HeLa cell nucleus is 15.7×10^{-6} μg (15). Thus, 1.5×10^5 *d*/min per μg of DNA × 15.7×10^{-6} μg of DNA per cell nucleus × 0.27 rad/*d* × 60 min/hr × 24/2 hr = 458 rad per cell nucleus.

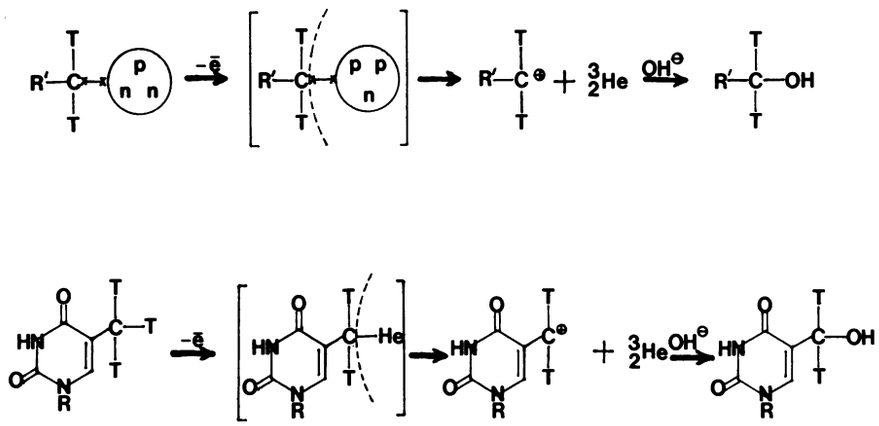

FIG. 1. Proposed mechanism of formation of HMdU from [*methyl*-^{3}H]thymidine as a result of the transmutation of ^{3}H to ^{3}He. p, Proton; n, neutron; ē, electron; T, tritium; R = H or 2′-deoxyribose; R′ = alkyl or aryl moiety.

water bath, was irradiated from a ^{137}Cs source at a rate of 600 rad/min. The cells from both tubes were harvested and washed and the DNA was extracted (11). The specific activity of the DNA was between 1.2 and 1.5 × 10^5 dpm/μg, assuming that A_{260} of 1 = 50 μg/ml.

Enzymatic Hydrolysis of Control and Irradiated DNA. DNA isolated from nonirradiated and γ-irradiated cells grown in the presence of either [*methyl*-^{3}H]thymidine or [*6*-^{3}H]thymidine was dissolved in 0.1 M NaCl/0.01 M Tris·HCl, pH 7.5, and enzymatically digested to 2′-deoxyribonucleosides (11). The hydrolysates were precipitated with 5 vol of acetone and centrifuged (Sorvall RC-2 with SS-35 rotor) at 7,000 rpm. Supernatants were evaporated, and the residues were dissolved in water, filtered through a Millipore 0.22-μm-pore-diameter filter, mixed with ^{14}C-containing and UV-absorbing markers, and analyzed by HPLC.

HPLC Analysis. ^{3}H-containing enzymatic hydrolysates (10^7 cpm each) purified as above were chromatographed together with the ^{14}C-labeled marker compounds TG, 5-hydroxy-5-methylhydantoin (HMH), 5,6-dihydroxy-5,6-dihydrothymidine (dTG), and thymine and unlabeled marker HMdU on a 5-μm Ultrasphere-ODS column (Altex; 1 × 25 cm) with water as eluent, using a Beckman HPLC model 330, equipped with a gradient elution accessory and a model 421 microprocessor–controller. The flow rate was 2 ml/min. Fractions were collected at 0.5-min intervals (1 ml/0.5 min per fraction) and, starting with fraction 71, at 1-min intervals. ^{14}C-containing TG, HMH, and dTG were synthesized in this laboratory according to refs. 11–13. The small difference in retention times between ^{3}H- and ^{14}C-labeled derivatives is due to an isotopic effect (11–13).

Acetylation. HPLC fractions containing [^{3}H]HMdU and unlabeled HMdU were evaporated to dryness and acetylated in dry pyridine with acetic anhydride at room temperature for 18 hr. The reaction was stopped by the addition of water, and the mixture was concentrated under reduced pressure at 30°C, extracted with acetone, and analyzed by HPLC on the ODS column with acetonitrile/water (22:78, vol/vol) as eluent (13).

RESULTS

A proposed mechanism for the formation of HMdU through ^{3}H transmutation is depicted in Fig. 1. In compounds in which ^{3}H is linked to carbon, the emission of an electron (β particle) leads to the formation of an unstable intermediate containing ^{3}He linked to carbon. Such a C–^{3}He bond has a very short half-life (10^{-4} to 10^{-5} sec), and neutral ^{3}He is released by abstracting an electron from carbon, leaving a reactive carbocation (16). If the carbocation is formed in the methyl group of thymidine, reaction with a hydroxide ion of water will yield HMdU as the stable product. Since the [*methyl*-^{3}H]thymidine we used contained three ^{3}H atoms per methyl group,‡ [^{3}H]HMdU in DNA could be detected.

Fig. 2 shows the HPLC analysis of the enzymatic hydrolysate of nonirradiated HeLa cell DNA labeled with [*methyl*-^{3}H]thymidine. The majority of the ^{3}H eluted with marker thymidine at fraction 110 and is not depicted. In all such analyses there was a peak of radioactivity in fractions 30–50 that constituted a residual 0.1–0.5% of total ^{3}H (including thymidine), indicating that enzymatic digestion of the DNA to 2′-deoxyribonucleosides was generally greater than 99.5%. Some of the smaller ^{3}H-containing peaks were coincident with marker TG, its rearrangement product HMH, and thymine (11–13). This indicates that both modified and normal free bases were released from the DNA backbone during the overnight incubation period of the enzymatic digestion of the DNA. The spontaneous release of bases from irradiated DNA has been reported (2). The ^{3}H-containing peak between fractions 91 and 98 constituted 0.15% of the total ^{3}H applied to the column and was coincident with UV marker HMdU. The identification of this peak as [^{3}H]HMdU was confirmed by its acetylation and HPLC analysis of the derivative. That transmutation of ^{3}H in [*methyl*-^{3}H]thymidine leads to formation of [^{3}H]HMdU can be proven by analysis of [*6*-^{3}H]thymidine-labeled DNA, which should not contain [^{3}H]HMdU because transmutation of ^{3}H in position 6 of thymidine could not yield [^{3}H]HMdU. Fig. 3 shows the HPLC analysis of the enzymatic hydrolysate of nonirradiated HeLa cell DNA labeled with [*6*-^{3}H]thymidine. The HPLC profile is similar to that of Fig. 2, with the notable difference that virtually no [^{3}H]HMdU is present. This DNA was of the same specific activity as that of Fig. 2 to ensure that the dose of ionizing radiation from incorporated [*6*-^{3}H]thymidine was the same as that from [*methyl*-^{3}H]thymidine. Since the same amount of DNA-derived ^{3}H was analyzed in Fig. 3 as in Fig. 2, it can be concluded that the large amount of [^{3}H]HMdU present in [*methyl*-^{3}H]thymidine-labeled DNA was formed not as the result of internal ionizing radiation from incorporated ^{3}H but rather through the transmutation of ^{3}H to ^{3}He. Further confirmation of this conclusion was obtained through the analysis of solutions of both radionuclides as received from the manufacturer. The solution of [*methyl*-^{3}H]thymidine contained [^{3}H]HMdU while that of [*6*-^{3}H]thymidine did not.

Since [*6*-^{3}H]thymidine-labeled DNA contained virtually no HMdU, we used cells labeled with this radionuclide to determine whether HMdU could also be formed in cellular

‡[*methyl*-^{3}H]Thymidine is manufactured carrier-free (—C^3H_3; 87 Ci/mmol) and then diluted with unlabeled thymidine (—C^1H_3) to the appropriate specific activity (K. J. O'Brien, New England Nuclear; personal communication).

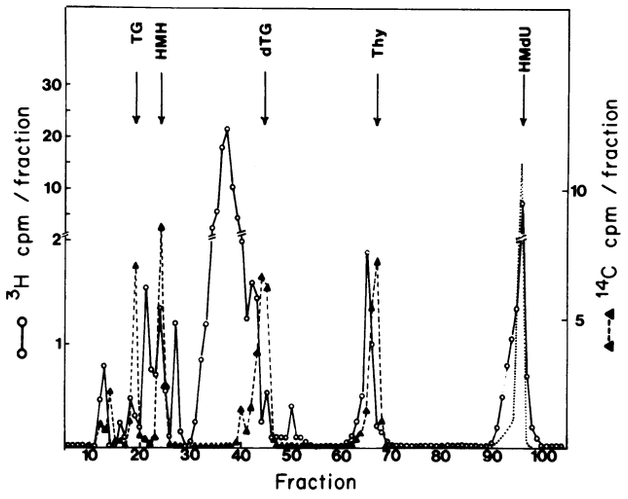

FIG. 2. HPLC analysis of an enzymatic hydrolysate (2'-deoxyribonucleosides) of [*methyl*-^{3}H]thymidine-labeled DNA (○) isolated from nonirradiated HeLa cells in the presence of marker compounds [^{14}C]TG, [^{14}C]HMH, [^{14}C]dTG, and [^{14}C]thymine (▲) and unlabeled HMdU (···).

DNA through the action of ionizing radiation. Fig. 4 shows the HPLC analysis of the enzymatic hydrolysate of HeLa cell DNA after exposure of the cells to 18 krad of γ-radiation. There is a small ^{3}H-containing peak coincident with marker HMdU, the identity of which was confirmed by acetylation and subsequent HPLC analysis. This HMdU peak constituted 0.002% of the total ^{3}H applied to the column. After 27 krad of radiation, the HMdU content increased to 0.004% of total ^{3}H.

DISCUSSION

We now understand that the apparent increase in [^{3}H]HMdU content of 0.05% of the total ^{3}H analyzed we previously observed after 36 krad of γ-radiation (11–13) was primarily due

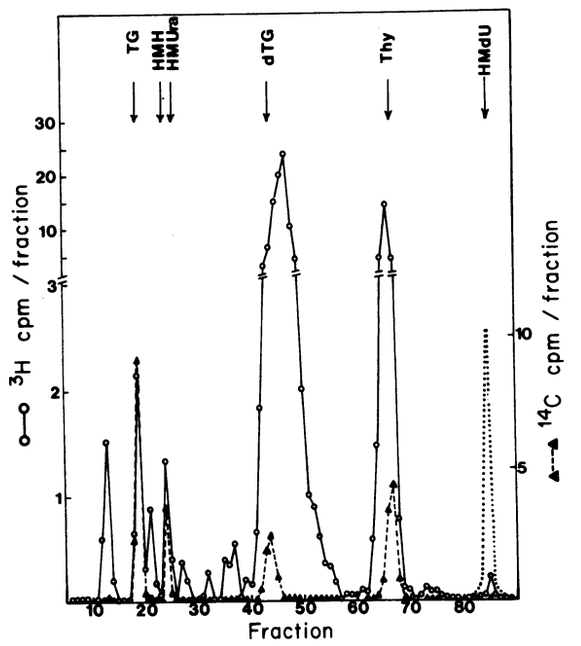

FIG. 3. HPLC analysis of an enzymatic hydrolysate (2'-deoxyribonucleosides) of [6-^{3}H]thymidine-labeled DNA (○) isolated from nonirradiated HeLa cells in the presence of marker compounds [^{14}C]TG, [^{14}C]HMH, [^{14}C]dTG, and [^{14}C]thymine (▲) and unlabeled HMdU (···).

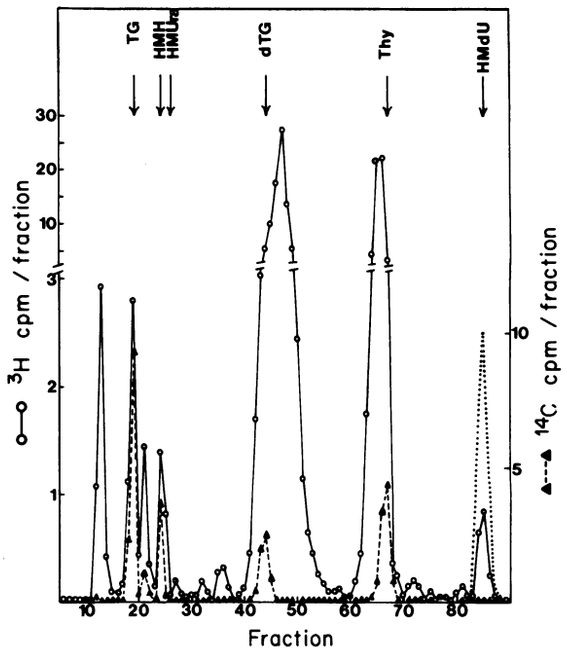

FIG. 4. HPLC analysis of an enzymatic hydrolysate (2'-deoxyribonucleosides) of [6-^{3}H]thymidine-labeled DNA (○) isolated from γ-irradiated (18 krad) HeLa cells in the presence of marker compounds [^{14}C]TG, [^{14}C]HMH, [^{14}C]dTG, and [^{14}C]thymine (▲) and unlabeled HMdU (···).

to transmutation. This was also the cause of the high [^{3}H]HMdU content of the DNA of nonirradiated cells labeled with [*methyl*-^{3}H]thymidine. The formation of [^{3}H]HMdU through transmutation of [*methyl*-^{3}H]thymidine occurs at the rate of β decay, which is 0.017% per day (^{3}H half-life is 12.26 yr). A 72-hr difference in time of analysis of nonirradiated and γ-irradiated DNA led to an increase in [^{3}H]HMdU content of 0.05% (3 × 0.017). The high [^{3}H]HMdU content (0.15%) of nonirradiated DNA was due to the time needed to label cells, irradiate (or mock-irradiate), extract, digest, and analyze the DNA. Further proof of this interpretation was obtained from the analysis of [*methyl*-^{3}H]thymidine-containing DNA that had been stored for 17 months after preparation and contained 8% of its ^{3}H as HMdU, the exact amount expected from the rate of ^{3}H decay. It must be emphasized that these percentages are not the actual HMdU content of the DNA but the percentage of radioactive molecules converted to the transmutation product. In contrast, the experiments in which [6-^{3}H]thymidine was used are true tracer experiments and offer a correct estimate of the extent of formation of HMdU in cellular DNA through the action of ionizing radiation.

The demonstration that ^{3}H transmutation can cause formation of a unique thymine derivative in DNA complements the observations of Person *et al.* (17) and Cleaver (18), who showed that labeling the DNA of *Escherichia coli* (17) and Chinese hamster ovary cells (18) with [6-^{3}H]thymidine caused more mutations than did labeling with [*methyl*-^{3}H]thymidine. Indeed, Cleaver suggested that a radioactive decay on the pyrimidine ring would be more disruptive than on the methyl group. It is possible that the formation of a carbocation on the 6 carbon of thymine leads to formation of a transmutation product(s) that is (are) more mutagenic than HMdU. Such transmutation products may contribute to the known carcinogenic effects of [^{3}H]thymidine and [^{3}H]water (19–21) and may constitute a cumulative biologic hazard when incorporated into the DNA of long-lived cells.

The mechanisms by which ionizing radiation exerts cytotoxic and mutagenic effects are not fully understood, but it

has been suggested that base modification contributes to these effects (1, 22). The formation of HMdU through the action of ionizing radiation, albeit in small amounts, may lend additional support to this hypothesis. Our finding that about 1–1.5 HMdU molecules were formed per 10^5 thymidine residues per 10 krad is in agreement with data of Roti Roti and Cerutti (1, 23). By measuring formation of ^3H_2O after γ-irradiation of [*methyl*-^{3}H]thymidine-labeled Chinese hamster ovary cells, they estimated that the methyl groups of 0.4 thymidine moiety per 10^5 thymidine residues were modified per 10 krad.

Although HMdU replaces thymidine in the DNA of some phages of *Bacillus subtilis* (24–26), it was cytostatic when added to growth medium of baby hamster kidney, mouse L, and Ehrlich ascites cells and human embryonic fibroblasts (27). HeLa cells were resistant. When given parenterally to mice, HMdU caused diarrhea and leukopenia, indicating it was also cytostatic *in vivo* (27). The DNA of Ehrlich ascites cells grown in the presence of HMdU could not be readily dissociated from nucleoprotein, and the authors of that report suggested that the HMdU incorporated into DNA might act as a weak alkylating agent, leading to covalent binding with chromatin proteins (28). Indeed, crosslinks between DNA and nonhistone proteins have been demonstrated after *in vitro* γ-irradiation of chromatin (29).

In contrast to other radiation-induced thymine derivatives such as TG (30), HMUra may not be enzymatically removed when present in cellular DNA. Extracts of *E. coli* and *B. subtilis* did not effect removal of HMUra from SP01 phage DNA, which contains HMUra in place of thymine, although the same extracts did remove uracil from PBS phage DNA, which contains uracil in place of thymine (31). If HMdU in cellular DNA is indeed not repaired, it may prove to be a useful marker of exposure to ionizing radiation.

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