32P-Postlabeling Detection of DNA Base Adducts in Mice Induced by 4-(Hydroxymethyl)benzenediazonium Salt, a Carcinogen in Mushroom Agaricus bisporus

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4-(Hydroxymethyl)benzenediazonium salt (HMBD), an ingredient in the edible mushroom Agaricus bisporus, is carcinogenic to mice and mutagenic to bacteria. Formation of carbon-centered radical from HMBD which induces DNA single strand breaks and modifies guanine and adenine moieties of DNA into 8-[4-(hydroxymethyl) phenyl]guanine and 8-[4-(hydroxy methyl)phenyl]adenine has been shown. In the present study HMBD was intraperitoneally administered to mice and in vivo formation of DNA base adducts in liver DNA was investigated by 32P-postlabeling assay. A radioactive spot caused by the modified bases was observed in liver DNA 24 h after the administration which disappeared after 48 h. The base adduct formation may play a role in carcinogenesis of HMBD in mice.

Key words — 4-(hydroxymethyl)benzenediazonium salt; DNA base adduct; 32P-postlabeling; mouse liver

Introduction

4-(Hydroxymethyl)benzenediazonium salt (HMBD) (Fig. 1) is an ingredient of the fresh mushroom Agaricus bisporus.1,2 It has been reported to cause subcutis and skin cancer3 and also gastric cancer4 in mice, and has been shown to be mutagenic to bacteria.5,6 Our previous studies7 demonstrated the activity of HMBD to cleave DNA against naked DNA and intracellular DNA of Escherichia coli, and to induce micronucleated reticulocytes in mouse peripheral blood after intraperitoneal injection. The component modifies deoxyribose and deoxyribonucleosides in vitro.8 The active species in the DNA damage by HMBD was shown to be carbon-centered 4-(hydroxymethyl)phenyl radical generated from the component by elimination of molecular nitrogen.7 The reaction products of HMBD with purine nucleosides and DNA were identified as 8-

Fig. 1. Structure of 4-(Hydroxymethyl)-benzenediazonium Ion (HMBD)
[4-(hydroxymethyl)phenyl]deoxyguanosine (8-HMP-dGuo) and 8-[4-(hydroxymethyl)-phenyl]-deoxyadenosine (8-HMP-dAdo). These 8-aryl adducts were later also observed by Gannet et al. The present study was performed to elucidate the \textit{in vivo} formation of DNA base adducts by HMBD in mice.

\textbf{Materials and Methods}

\textbf{Materials} — HMBD was prepared by diazotization of \(p\)-aminobenzyl alcohol (Fluka Company, Tokyo, Japan) with nitrosyl tetrafluoroborate (Aldrich Chemical Company, Milwaukee, WI, U.S.A.) as described previously. Calf thymus DNA was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Six-week old male ICR mice were obtained from Japan Clea Company (Tokyo). Ribonuclease (RNase) T1, RNase A, micrococcal nuclease, phosphodiesterase I, phosphodiesterase II and apyrase were obtained from Sigma. T4 polynucleotide kinase was from Takara Shuzo Company (Kyoto, Japan). TLC plates were the product of Merck (Darmstadt, Germany). \([\gamma^{32}\text{P}]\text{ATP (222 TBq/mmol)}\) was from Amersham Company (Tokyo). Other reagents and enzymes were obtained from Wako Pure Chemical Company (Osaka, Japan).

\textbf{Reaction of Calf Thymus DNA with HMBD} — Calf thymus DNA at a concentration of 50 \(\mu\text{g/ml}\) was incubated with 0.1 \(\text{m}\) HMBD in 0.1 \(\text{m}\) phosphate buffer (pH 7.4) (10 ml) at 37\(^\circ\text{C}\) for 2 h. The reaction mixture was sequentially extracted with an equal volume of 1-butanol 6 times, phenol—chloroform—isoamyl alcohol (25 : 24 : 1, v/v) 5 times and chloroform—isoamyl alcohol (24 : 1, v/v) twice. The aqueous phase was dialyzed twice against 2 \(\text{l}\) of saline at 4\(^\circ\text{C}\).

\textbf{Treatment of Mice with HMBD and Extraction of Liver DNA} — Mice were administered HMBD at a single dose of 90 \(\text{mg/kg}\) intraperitoneally. At 3, 24 and 48 h after the administration each mouse was killed, and liver was immediately isolated and frozen at \(-80\text{\degree C}\) until extraction of DNA. One g of frozen liver from control or treated mice was homogenized in 10 ml of 1\% sodium dodecylsulfate (SDS)/1 \(\text{mM}\) EDTA. The homogenate was treated with proteinase K (0.8 \(\text{mg/ml}\)) at 37\(^\circ\text{C}\) for 30 min. Tris—HCl buffer (1 \(\text{m}\), pH 7.5, 0.5 ml) was added and the mixture was gently extracted once with an equal volume of phenol—chloroform—isoamyl alcohol (25 : 24 : 1, v/v) and then chloroform—isoamyl alcohol (24 : 1, v/v). The DNA in the aqueous mixture was precipitated by addition of 2 volumes of cold ethanol, rinsed with cold 70\% ethanol and dissolved in 2 ml of 1.5 \(\text{mM}\) \(\text{NaCl—0.15 mM sodium citrate containing 1 mM EDTA}\). After addition of 0.1 ml of 1 \(\text{m}\) Tris—HCl (pH 7.4), the mixture was treated with RNase T1 (50 units/ml) and RNase A (0.1 \(\text{mg/ml}\)) at 37\(^\circ\text{C}\) for 30 min. The mixture was extracted once with chloroform—isoamyl alcohol (24 : 1, v/v) and the DNA was precipitated with cold ethanol after addition of 0.2 ml of 5 \(\text{m}\) \(\text{NaCl}\). The DNA was rinsed with 70\% cold ethanol and dissolved in 1.5 \(\text{mM}\) \(\text{NaCl—0.15 mM sodium citrate containing 1 mM EDTA}\). The concentration of DNA was determined by UV absorption, 50 \(\mu\text{g/ml}\) of DNA giving absorbance 1 at 260 nm. The DNA (0.1 \(\text{mg/ml}\)) was digested with nuclease P1 (1 \(\mu\text{g/ml}\)) in 1.1 ml of 20 \(\text{mM}\) acetate buffer (pH 4.8) at 37\(^\circ\text{C}\) for 30 min. After the addition of Tris (1 \(\text{m}, 72 \mu\text{l}\)) to adjust the pH to 8.8, the mixture
was digested with phosphodiesterase I (10 μg/ml) at 37°C for 60 min. Diluted HCl was then added to adjust the pH to 7.5, and the mixture was dephosphorylated by alkaline phosphatase (1 unit/ml) in 0.1 M Tris–HCl (pH 7.5) at 37°C for 60 min. The resulting nucleosides were analyzed by reversed phase HPLC, and the chromatogram indicated the digest contained deoxyribonucleosides but not ribonucleosides.

**Digestion of DNA and ^32P-Postlabeling** — Digestion of DNA, labeling of 3’-mononucleotide and analysis of modified nucleotides were carried out according to the methods of Reddy and Randerath.10) The DNA (6 μg/ml of calf thymus DNA or 0.5 mg/ml of mouse liver DNA) was digested with micrococcal nuclease (75 units/ml) in 20 μl of 50 mM borate buffer (pH 8.8) containing 5 mM NaCl and 2.5 mM CaCl₂ at 37°C for 30 min. After addition of 3 μl of 50 mM HCl to adjust pH to 6.0, the mixture was further digested with phosphodiesterase II (3 units/ml) in 30 μl of 10 mM succinate buffer (pH 6.0) at 37°C for 3 h. The resulting 3’-mononucleotides were labeled with ^32P using T4 polynucleotide kinase (0.4 unit/ml) and [γ-^32P]ATP (80 mM) in the presence of 0.4 mM unlabeled ATP in 75 μl of 30 mM Tris–HCl (pH 9.5), 10 mM dithiothreitol, 10 mM MgCl₂ and 1 mM spermidine at 37°C for 45 min. The excess ATP was removed by digestion by apyrase (1.5 units/ml) at 37°C for 45 min.

An aliquot of the solution of the 5’-^32P-labeled deoxyribonucleoside 3’,5’-bisphosphate (20 μl, 6 μM nucleotides for the analysis of calf thymus DNA or 0.4 mM nucleotides for that of mouse liver DNA) was spotted on an ODS-TLC sheet (10 × 2 cm) to which filter paper was attached on the top, and the sheet was developed with 0.4 M ammonium formate (pH 6.0) for 16 h (D1 development). After drying, a 1-cm square around the origin was cut out and attached face-to-face to a PEI-cellulose TLC sheet (9.5 × 10 cm) with a magnet 1 cm from the bottom. The sheet was developed with 2% NP-40–1-propanol (1:1, v/v) (D2 development) followed by washing with water, drying and 2-dimensional development (D3 and D4 development). D3 development was performed using 1.1 M lithium formate/2.1 M urea (pH 3.5) and D4 development was performed using 0.5 M lithium chloride/0.25 M Tris–HCl/4.25 M urea (pH 8.0). Prior to the D4 development, the sheet was washed with water and dried, and after the development, radioactive spots were detected by radioluminography (BAS 2000, Fuji Photo Film Company, Tokyo).

**Results and Discussion**

In our previous study8) 8-HMP-dGuo and 8-HMP-dAdo were detected in the reaction of calf thymus DNA with HMBD in vitro. The in vitro HMBD-modified calf thymus DNA was sequentially digested with micrococcal nuclease and phosphodiesterase II, and resulting 3’-mononucleotides were transformed into 5’-^32P-labeled deoxyribonucleoside 3’,5’-bisphosphate using [γ-^32P] ATP and T4 polynucleotide kinase. The labeled nucleotides were analyzed on TLC successively in D1—D4 developments. On the TLC of the D3 and D4 developments of control DNA, only one radioactive spot was observed at the origin (Fig. 2A); this spot was due to native nucleotides. On the TLC of
Fig. 2. Radioluminography of TLC of $^{32}$P-Postlabeled Digests of Control (A) and In Vitro HMDB-Treated Calif Thymus DNA (B)

The directions of D3 and D4 development are indicated by arrows.

Fig. 3. Radioluminography of TLC of $^{32}$P-Postlabeled Digests of Liver DNA from Control (A) or HMDB-Administered Mice Killed at 3 (B), 24 (C) and 48 h (D) after Administration

The directions of D3 and D4 development are indicated by arrows.
HMBD-modified DNA, another intense radioactive spot (spot A) apart from the origin was observed (Fig. 2B), which might have been due to $^{32}$P-labeled nucleoside bisphosphate(s) from 8-HMP-dGuo and/or 8-HMP-dAdo.

Mice were administered HMBD at a single dose of 90 mg/kg and killed 3, 24 or 48 h thereafter. DNA in liver was extracted and analyzed by postlabeling assay. In the DNA from a control mouse (Fig. 3A) and that from a mouse killed 3 h after the administration (Fig. 3B), no radioactive spots were observed except at the origin. In the DNA from a mouse killed 24 h after the administration, radioactivity of a spot corresponding to spot A shown in Fig. 2B was seen (Fig. 3C), however, the radioactivity disappeared in the DNA from a mouse killed 48 h after HMBD administration (Fig. 3D). The adduct causing spot A may be repaired after a longer period in mouse liver. The adduct formation may result from a reaction of DNA with carbon-centered 4-(hydroxy-methyl)phenyl radical generated from HMBD in vivo. Although the adduct in mouse liver DNA took longer to disappear after a single intraperitoneal dose, the adduct formation may play a role in carcinogenesis of HMBD in mice.

References