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Metal-mediated oxidative DNA damage induced by methylene blue

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ABSTRACT

Background: Methylene blue (MB) is used for various clinical purposes, including chromoendoscopy and methemoglobinemia treatment. However, MB induces tumors of pancreatic islets and small intestine in experimental animals. This finding raises a possibility that MB induces carcinogenicity in these organs via light-independent mechanisms, although MB is known to cause light-dependent DNA damage.
Methods: We investigated the mechanism of MB-induced DNA damage using ^{32}P-5′-end-labeled DNA fragments of human tumor-relevant genes. We investigated the redox reaction of MB by UV–visible spectrometry.
Results: MB induced DNA damage at the 5′-ACG-3′ sequence, a hot spot of the *p53* gene, in the presence of NADH and Cu(II). DNA damage was inhibited by catalase and bathocuproine, a Cu(I)-specific chelator. MB induced DNA damage at every nucleotide in the presence of NADH and Fe(III)-ethylenediaminetetraacetic acid, which was inhibited by •OH scavengers and catalase. MB significantly increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine, an oxidative DNA lesion, in the presence of NADH and metal ions. UV–visible spectrometry revealed that the absorbance of oxidized form of MB at 668 nm was decreased by NADH, and the addition of metal ions attenuated the spectral change.
Conclusions: MB undergoes NADH-dependent reduction followed by metal ion-mediated reoxidation. Reduced metal ions [Cu(I) and Fe(II)] interact with H_2O_2, generated during the redox reaction, to produce Cu(I)OOH and •OH that cause DNA damage, respectively. These findings suggest that metal-mediated DNA damage contributes to MB-mediated carcinogenesis.
General significance: This study would provide an insight into the mechanism of MB-induced carcinogenesis and its safety assurance for clinical use.

1. Introduction

Methylene blue (MB) has been clinically used to reverse methemoglobinemia caused by genetic deficiencies and metabolic poisoning [1]. MB is widely used as a very efficient dye for chromoendoscopy, which optimizes the evaluation of premalignant gastric lesions [2]. MB has been introduced in sentinel lymph node mapping of gastrointestinal and breast cancer [3,4]. Recent animal experiment revealed that MB photodynamic therapy induced a significant decrease in tumor volume and weight in a mouse model [5]. Moreover, clinical applications of MB for other purposes have been reported [6,7].

It is known that MB exposed to ultraviolet light (UV) or visible light causes guanine-specific DNA damage via the generation of singlet oxygen ($^1O_2$) [8–10]. Actually, the amount of oxidative DNA lesions was increased in Barrett's mucosa after chromoendoscopy, due to the presence of MB and endoscopic white light [11]. Recently, National Toxicology Program (NTP) has reported that there was some evidence for the carcinogenic activity of MB in experimental animals. Oral administration of MB increased the incidences of pancreatic islet cell adenoma or carcinoma in male rats and carcinoma in the small intestine in male mice [12,13]. This finding raises a possibility that light-independent DNA damage participates in MB-induced carcinogenesis in these abdominal organs. In addition, MB was mutagenic in some strains of *Salmonella typhimurium* and *Escherichia coli* with and without liver S9 [12]. It has been reported that MB is reduced by endogenous reductants, including NAD(P)H, and then accumulates in cells, although a precise mechanism remains to be clarified [14]. We have demonstrated that a wide variety of carcinogenic chemicals are reduced by NADH and induce DNA damage in the presence of metal ions, which catalyze the generation of reactive oxygen species (ROS) [15,16]. These findings led us to an idea that MB may induce metal-dependent oxidative DNA damage, which contributes to carcinogenesis.

Abbreviations: MB, methylene blue; UV, ultraviolet light; $^1O_2$, singlet oxygen; NTP, National Toxicology Program; ROS, reactive oxygen species; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; HPLC–ECD, an electrochemical detector coupled to HPLC; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase; Fpg, *E. coli* formamidopyrimidine-DNA glycosylase; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; $O_2^{\bullet -}$, superoxide; H_2O_2, hydrogen peroxide; •OH, hydroxyl free radical

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In this study, we examined the mechanism of MB-induced DNA damage using ^{32}P-5′-end-labeled DNA fragments obtained from the human c-Ha-*ras* protooncogene and *p53* tumor suppressor gene in the presence of NADH and endogenous metal ions. We also quantified the formation of an oxidative DNA lesion, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), using an electrochemical detector coupled to HPLC (HPLC–ECD). 8-OxodG is a mutagenic DNA lesion, which causes DNA misreplication and resulting G → T transversions [17,18]. We also performed UV–visible spectrometry to investigate NADH- and metal-mediated redox reaction of MB and ROS generation.

2. Materials and methods

2.1. Materials

[γ-^{32}P]-ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA, USA). Restriction enzymes (*Ava*I, *Xba*I *Pst*I and *Hind*III) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). Restriction enzymes (*Eco*RI and *Apa*I) and calf intestine phosphatase were from Roche (Mannheim, Germany). MB and nuclease P_1 were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA, superoxide dismutase (CuZn-SOD, 3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co. (St. Louis, MO, USA). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was from Trevigen Co. (Gaithersburg, MD, USA). Deferoxamine was purchased from Novartis Pharma (Tokyo, Japan).

2.2. Preparation of ^{32}P-5′-end-labeled DNA fragments

DNA fragments were obtained from the human *p53* tumor suppressor gene [19]. The 5′-end-labeled 650-base pair fragment (*Hind*III* 13972–*Eco*RI* 14621, asterisk indicates ^{32}P-labeling) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase as described previously [20]. This fragment was digested with *Apa*I to obtain a singly labeled 443-base pair (*Apa*I 14179–*Eco*RI* 14621) DNA fragment. DNA fragments were also prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene [21,22]. A singly labeled 261-base pair fragment (*Ava*I* 1645–*Xba*I 1905) and a 337-base pair fragment (*Pst*I 2345–*Ava*I* 2681) were obtained as described previously [21,22]. Nucleotide numbering starts with the *Bam*HI site [23].

2.3. Detection of MB-induced damage to ^{32}P-labeled DNA fragments

The standard reaction mixtures in microtubes (1.5-ml Eppendorf) contained MB, 100 μM NADH, 20 μM $CuCl_2$ or Fe(III)-ethylenediaminetetraacetic acid (EDTA), ^{32}P-labeled DNA fragment and 10 μM/base calf thymus DNA in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were covered with aluminum foil to avoid light exposure and incubated for 1 h at 37 °C. In a certain experiment, the mixture (without NADH and metal ion) was exposed to 10 J/cm^2 UVA (365 nm) as described previously [24]. After ethanol precipitation, the DNA fragments were heated in 1 M piperidine for 20 min at 90 °C or treated with 5 units of Fpg for 2 h at 37 °C. Fpg protein catalyzes the excision of 8-oxodG as well as Fapy residues [25]. Subsequently, DNA fragments were denatured by heating for 1 min at 90 °C followed by chilling on ice and electrophoresed on an 8% polyacrylamide/8 M urea gel. Then the autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [26] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia LKB Biotechnology, Uppsala, Sweden). A laser densitometer (Personal Densitometer SI, Amarsham Biosciences, Uppsala, Sweden) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

2.4. Measurement of 8-oxodG formation induced by MB

The amount of 8-oxodG was measured by a modified method of Kasai et al. [27]. The reaction mixtures containing 100 μM/base calf thymus DNA, MB, 100 μM NADH and 20 μM $CuCl_2$ or Fe(III)EDTA in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 1 h at 37 °C. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed with an HPLC–ECD as described previously [28]. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test using an IBM SPSS Statistics software version 20 for Macintosh. p values less than 0.05 were considered to be statistically significant.

2.5. UV–visible spectra of MB plus NADH and metal ions

UV–visible spectra were measured with a UV–visible spectrometer (UV-2500PC, Shimadzu, Kyoto, Japan). The reaction mixtures contained 10 μM MB, 100 μM NADH and 20 μM $CuCl_2$ or Fe(III)EDTA in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The spectra of the mixture were measured repeatedly at 37 °C every 5 min for 30 min, and the absorbance at 668 nm, the maximum absorption of oxidized form of MB, was traced.

2.6. Superoxide (O_2•$^-$) generation during the reaction of MB with NADH and metal ions

To examine MB-mediated O_2•$^-$ generation, a maximum absorption at 550 nm due to ferrocytochrome c formed by ferricytochrome c reduction was measured at 37 °C with a UV–visible spectrophotometer (UV-2500PC, Shimadzu). The reaction mixtures contained 10 μM MB, 100 μM NADH, no or 20 μM metal ions [$CuCl_2$ or Fe(III)EDTA] and 50 μM cytochrome c with or without 150 units/mL SOD in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The spectral tracing was repeated every 5 min for 30 min at 37 °C. The actual amount of O_2•$^-$ generation was calculated by subtracting the absorbance with SOD from that without SOD at 550 nm ($\varepsilon = 21.1 \times 10^3\ M^{-1}\ cm^{-1}$) [29].

3. Results

3.1. MB-induced damage to ^{32}P-labeled DNA fragments

Fig. 1 shows an autoradiogram of DNA fragments treated with MB in the presence of NADH and metal ions. Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. MB caused DNA damage in the presence of NADH and Cu(II) (Fig. 1A) or Fe(III)EDTA (Fig. 1B) in a dose-dependent manner. Cu(II)-mediated DNA damage was stronger than that mediated by Fe(III)EDTA. MB did not cause DNA damage in the absence of NADH or metal ions.

3.2. Effects of scavengers and metal chelators on MB-induced DNA damage

The effects of scavengers and metal chelators on MB-induced DNA damage are shown in Fig. 2. MB-induced DNA damage in the presence of NADH and Cu(II) was inhibited by catalase and bathocuproine, a Cu(I) chelator, suggesting that hydrogen peroxide (H_2O_2) and Cu(I) were involved (Fig. 2A). Typical hydroxyl free radical (•OH) scavengers (ethanol, mannitol, sodium formate and methional) did not inhibit the DNA damage (Fig. 2A). MB-induced DNA damage in the presence of