Free Rad. Res., Vol. 29, pp. 197–205
Reprints available directly from the publisher
Photocopying permitted by license only

DNA Damage Induced by *m*-Phenylenediamine and its Derivative in the Presence of Copper Ion

FANG CHEN[a], MARIKO MURATA[a], YUSUKE HIRAKU[b], NARUTO YAMASHITA[a], SHINJI OIKAWA[a] and SHOSUKE KAWANISHI[a,*]

[a]*Department of Hygiene, Mie University School of Medicine, Tsu, Mie 514-8507, Japan;*
[b]*Department of Public Health, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan*

Accepted by Prof. M. Dizdaroglu

(Received 2 March 1998; In revised form 27 April 1998)

To clarify the mechanism of carcinogenesis by hair dyes, we compared the extent of DNA damage induced by mutagenic *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine, using ^{32}P-5′-end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. Carcinogenic 4-methoxy-*m*-phenylenediamine caused DNA damage at thymine and cytosine residues in the presence of Cu(II). Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited 4-methoxy-*m*-phenylenediamine-induced DNA damage, suggesting the involvement of H_2O_2 and Cu(I). Superoxide dismutase (SOD) enhanced the DNA damage. Formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) was induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). UV-visible spectroscopic studies have shown that Cu(II) mediated autoxidation of 4-methoxy-*m*-phenylenediamine and SOD accelerated the autoxidation. On the other hand, non-carcinogenic *m*-phenylenediamine did not cause clear DNA damage and significant autoxidation even in the presence of Cu(II). These results suggest that carcinogenicity of *m*-phenylenediamines is associated with ability to cause oxidative DNA damage rather than bacterial mutagenicity.

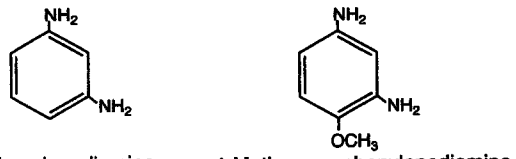
Keywords: 4-Methoxy-*m*-phenylenediamine, *m*-phenylenediamine, DNA damage, copper, H_2O_2, SOD

Abbreviations: 8-OH-dG, 8-hydroxy-2′-deoxyguanosine (and also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine); HPLC-ECD, an electrochemical detector coupled to a high performance liquid chromatograph; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; PCR, polymerase chain reaction

INTRODUCTION

Epidemiologic studies have revealed an association between occupational exposure to hair dyes and incidence of cancers.[1,2] *m*-Phenylenediamine and its derivatives are used for the production of many dyes, and are also used directly as color-yielding compounds which include hair and fabric dyes. It has been reported that increased incidence of bladder cancer was observed among workers in the manufacturing industry of 4-methoxy-*m*-phenylenediamine.[3] *m*-Phenylenediamine and 4-methoxy-*m*-phenylenediamine (Scheme 1) have been tested for

* Corresponding author. Tel./Fax: +81-59-231-5011. E-mail: kawanisi@doc.medic.mie-u.ac.jp.



SCHEME 1 Chemical structures of *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine.

the evaluation of their carcinogenicity using experimental animals. It has been reported that 4-methoxy-*m*-phenylenediamine is carcinogenic,[3,4] whereas *m*-phenylenediamine is not.[4,5] 4-Methoxy-*m*-phenylenediamine caused carcinomas of thyroid glands and skin in rats and mice.[3,4] However, the mutagenicity tests revealed that both *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine are mutagenic with metabolic activation.[5–8] *m*-Phenylenediamine induced reverse mutations in *Salmonella typhimurium* TA 1538 in the presence of a rat liver post-mitochondrial supernatant fraction.[5] Peroxidase can activate *m*-phenylenediamine into an agent mutagenic in *Salmonella typhimurium* TA 98 and YG 1024.[6] 4-Methoxy-*m*-phenylenediamine induced mutations in *Salmonella typhimurium* TA 98 in the presence of S9 mix.[7] These studies suggest that carcinogenicity of *m*-phenylenediamines is not consistent with their mutagenicity in bacterial systems.

To clarify the mechanism of carcinogenesis by *m*-phenylenediamines, we examined DNA damage induced by *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine in the presence of Cu(II), using ^{32}P-5′-end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 protooncogene and the *p53* tumor suppressor gene. These two genes are known to be the targets for chemical carcinogens, and the damaged sites may lead to mutational hotspots.[9] In addition, we measured the content of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a marker of oxidative DNA damage, in calf thymus DNA with an electrochemical detector coupled to a high performance liquid chromatograph (HPLC-ECD). It has been reported that 8-OH-dG formation can lead to DNA misreplication resulting in mutation and cancer.[10,11] Furthermore, to clarify the mechanism of the DNA damage, we examined the $O_2^{\bullet -}$ generation from *m*-phenylenediamines by cytochrome *c* reduction method and the spectral changes, using UV-visible spectrophotometry.

MATERIALS AND METHODS

Materials Restriction enzymes (*Sma* I, *Eco* RI, *Sty* I, *Apa* I, *Xba* I, *Ava* I and *Hind* III) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). A human *p53* Amplimer Panel was from Clontech lab. (Palo Alto, CA, USA). The primers designed for the use in the polymerase chain reaction (PCR) process for the amplification of *p53* are contained in this product (kit). [γ-^{32}P]-ATP (222 TBq/mmol) was from ICN biomedicals Inc. (Costa Mesa, CA, USA). *m*-Phenylenediamine dihydrochloride and 4-methoxy-*m*-phenylenediamine sulfate were from Tokyo Kasei Co (Tokyo, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Superoxide dismutase (SOD, 3,000 units/mg from bovine erythrocytes), catalase (45,000 units/mg from bovine liver) and cytochrome *c* were from Sigma Chemical Co. (St. Louis, MO, USA).

Detection of damage to ^{32}P-5′-end-labeled DNA fragments obtained from the p53 and the c-Ha-ras-1 genes DNA fragment was obtained from the human *p53* tumor suppressor gene[12] and the human c-Ha-*ras*-1 protooncogene.[13] DNA fragments from the *p53* gene containing exons (exon 5, 13055–13238; exon 6, 13320–13432; exon 7, 14000–14109; exon 8, 14452–14588) were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with *Sma* I and ligated into

Sma I-cleaved pUC18 plasmid, and then transferred to *Escherichia coli* JM109. The plasmid pUC18 was digested with *Eco* RI and *Hind* III, and the resulting DNA fragments were fractionated by electrophoresis on a 2% agarose gel. The 5′-end-labeled 650-base pair fragment (*Hind* III*13972–*Eco* RI*14621) and 460-base pair fragment (*Hind* III*13038–*Eco* RI*13507) were prepared by dephosphorylation with calf intestine phosphatase and rephosphorylation with T_4 polynucleotide kinase using [γ-^{32}P] ATP (the asterisk indicates ^{32}P-labeling). The 650-base pair fragment was further digested using *Apa* I to obtain a singly labeled 443-base pair fragment (*Apa* I 14179–*Eco* RI*14621) and a 211-base pair fragment (*Apa* I 13972–*Hind* III*14182), and the 460-base pair fragment was further digested with *Sty* I to obtain a singly labeled 348-base pair fragment (*Sty* I 13160–*Eco* RI*13507) and a 118-base pair fragment (*Hind* III*13038–*Sty* I 13155), as described previously.[14] DNA fragment was also obtained from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene.[13] The 5′-end-labeled 602-base pair fragment (*Ava*I* 1645–*Ava*I* 2246) was prepared according to the method described previously[15,16] and digested using *Xba*I to obtain a singly labeled 261-base pair (*Ava*I* 1645–*Xba*I 1905) fragment. The nucleotide numbering starts with the *Bam* HI site.[13]

The standard reaction mixture in a 1.5-mL microtube contained *m*-phenylenediamine or 4-methoxy-*m*-phenylenediamine, 20 μM $CuCl_2$, the ^{32}P-labeled DNA fragment and sonicated calf thymus DNA (20 μM/base) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C, the DNA fragment was heated at 90°C in 1 M piperidine for 20 min and treated as described previously.[15,16] 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[17] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Measurement of 8-OH-dG formation The amount of 8-OH-dG was measured by a modified method of Kasai *et al.*[18] Calf thymus DNA fragments (100 μM) were incubated with *m*-phenylenediamine or 4-methoxy-*m*-phenylenediamine and 20 μM $CuCl_2$ for 2 h at 37°C. For the experiment with denatured DNA, calf thymus DNA was heated at 90°C for 5 min and quickly chilled before the reaction. After ethanol precipitation, DNA fragments were digested to the nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed with an HPLC-ECD as described previously.[19]

UV-visible spectra measurement during autoxidation of m-phenylenediamines UV-visible spectra of *m*-phenylenediamines were measured with a UV-2500PC spectrometer (Shimadzu, Kyoto, Japan). The reaction mixture contained 200 μM *m*-phenylenediamines and 20 μM $CuCl_2$ in 10 mM phosphate buffer (pH 7.8). Where indicated, SOD (150 units/mL) was added to the reaction mixtures. The spectra of the mixture were measured every 10 min for 90 min at 37°C.

Detection of $O_2^{\bullet-}$ formation during autoxidation of m-phenylenediamines The amounts of $O_2^{\bullet-}$ formation by the reactions of 4-methoxy-*m*-phenylenediamine in the presence and absence of Cu(II) were determined by measuring cytochrome *c* reduction. The mixtures containing 100 μM ferricytochrome *c*, 200 μM 4-methoxy-*m*-phenylenediamine, 20 μM $CuCl_2$ and 2.5 μM DTPA in 1 mL of 10 mM sodium phosphate buffer(pH 7.8) were then incubated at 37°C. The absorption at 550 nm recorded every 2 min for 20 min using a UV-visible spectrophotometer. Where indicated, SOD (90 units/mL) was added to the reaction mixtures.

RESULTS

Damage to ^{32}P-labeled DNA fragments induced by m-phenylenediamines in the presence of metal ions Figure 1 shows the autoradiogram of DNA damage induced by *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine. As shown in Figure 1, 4-methoxy-*m*-phenylenediamine (lanes 9–12) caused DNA damage in the presence of Cu(II), whereas *m*-phenylenediamine did not clearly (lanes 4–7). The intensity of DNA damage increased depending on concentrations of 4-methoxy-*m*-phenylenediamine (lanes 9–12), and incubation time (data not shown). DNA damage by 4-methoxy-*m*-phenylenediamine was enhanced by piperidine treatment, suggesting that 4-methoxy-*m*-phenylenediamine caused not only DNA strand breakage but also base modification and liberation (data not shown). However, 4-methoxy-*m*-phenylenediamine did not cause DNA damage in the presence of Mn(II), Fe(II) or Fe(III) (data not shown).

Effects of scavengers and bathocuproine on DNA damage The effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage by 4-methoxy-*m*-phenylenediamine were investigated and the results are shown in Figure 2. Typical $^{\bullet}$OH scavengers, ethanol

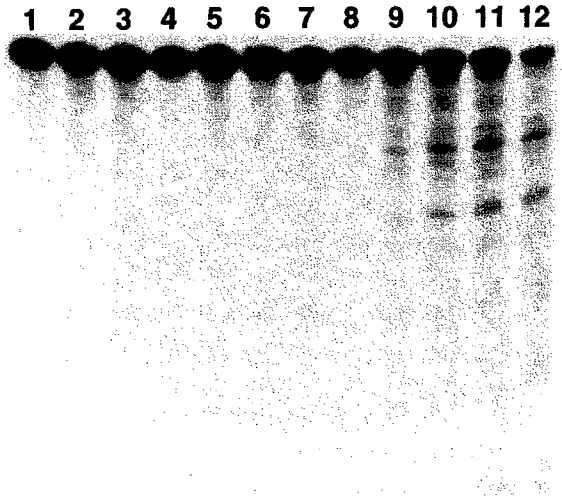


FIGURE 1 Autoradiogram of DNA fragments incubated with *m*-phenylenediamines. The reaction mixture contained the ^{32}P-5′-end-labeled 261-base pair DNA fragment, 20 μM/base of sonicated calf thymus DNA, indicated concentration of *m*-phenylenediamine or 4-methoxy-*m*-phenylenediamine and 20 μM $CuCl_2$ in 200 μL of 10 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated for 2 h at 37°C. The DNA fragments were treated with 1 M piperidine for 20 min at 90°C, and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. Lane 1, control; lane 2, Cu(II) alone; lane 3, 500 μM *m*-phenylenediamine alone; lane 4, Cu(II) + 50 μM *m*-phenylenediamine; lane 5, Cu(II) + 100 μM *m*-phenylenediamine; lane 6, Cu(II) + 200 μM *m*-phenylenediamine; lane 7, Cu(II) + 500 μM *m*-phenylenediamine; lane 8, 500 μM 4-methoxy-*m*-phenylenediamine alone; lane 9, Cu(II) + 50 μM 4-methoxy-*m*-phenylenediamine; lane 10, Cu(II) + 100 μM 4-methoxy-*m*-phenylenediamine; lane 11, Cu(II) + 200 μM 4-methoxy-*m*-phenylenediamine; lane 12, Cu(II) + 500 μM 4-methoxy-*m*-phenylenediamine.

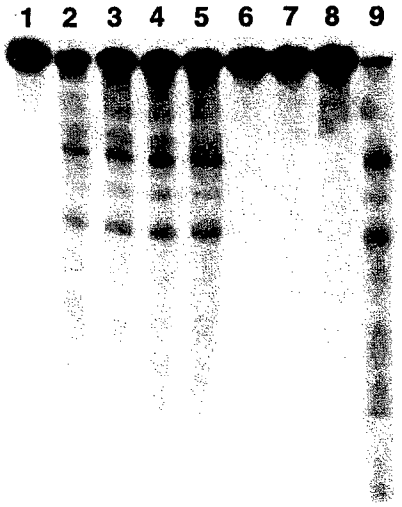


FIGURE 2 Effects of scavengers and bathocuproine on DNA damage by 4-methoxy-*m*-phenylenediamine. The reaction mixture contained the ^{32}P-5′-end-labeled 261-base pair DNA fragment, 20 μM/base of sonicated calf thymus DNA, 200 μM 4-methoxy-*m*-phenylenediamine, and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated for 2 h at 37°C. The DNA fragments were treated with 1 M piperidine for 20 min at 90°C, and analyzed by the method described in the legend to Figure 1. Control (lane 1) contained neither *m*-phenylenediamines nor $CuCl_2$. Scavenger or bathocuproine was added as follows: lane 2, no scavenger; lane 3, 1 M ethanol; lane 4, 0.1 M mannitol; lane 5, 0.1 M sodium formate; lane 6, 0.1 M methional; lane 7, 150 units/mL catalase; lane 8, 50 μM bathocuproine; lane 9, 50 units/mL SOD.

(lane 3), mannitol (lane 4) and sodium formate (lane 5), did not inhibit DNA damage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II), whereas methional inhibited the DNA damage (lane 6). Catalase (lane 7) and bathocuproine (lane 8) inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). SOD showed an enhancing effect on the DNA damage (lane 9).

Site specificity of DNA damage by 4-methoxy-m-phenylenediamine Figures 3 and 4 show the site specificity of DNA damage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). 4-Methoxy-*m*-phenylenediamine could cause DNA damage at certain thymine, cytosine and guanine residues, although there remains a possibility that base damage might be over- or under-represented, depending on their sensitivity to piperidine treatment. 4-Methoxy-*m*-phenylenediamine caused cleavages at guanine residues of the GGC sequence (Figure 3B) and the ACG sequence (Figure 4B). It is interesting that the ACG sequence is complementary to codon 273 (a known hotspot)[9,20] in exon 8 of the *p53* gene. The other predominant cleavage sites were thymine residue of the 5′-CTG-3′ sequence (Figure 3A) and cytosine residues located 5′ or 3′ to cytosine (Figures 3B and 4A).

Formation of 8-OH-dG in calf thymus DNA by m-phenylenediamines in the presence of Cu(II) We measured 8-OH-dG contents in calf thymus DNA treated with *m*-phenylenediamines plus Cu(II) using an HPLC-ECD (Figure 5). The amount of 8-OH-dG increased with increasing concentrations of 4-methoxy-*m*-phenylenediamine. The content of 8-OH-dG formed was larger, when DNA was denatured before incubation. On the other hand, *m*-phenylenediamine generated little 8-OH-dG in the presence of Cu(II).

UV-visible spectroscopic studies on the autoxidation of m-phenylenediamines Figure 6 shows changes in UV-visible spectra of *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). Both of the spectra of *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine did not change in the absence of Cu(II). The spectrum of *m*-phenylenediamine

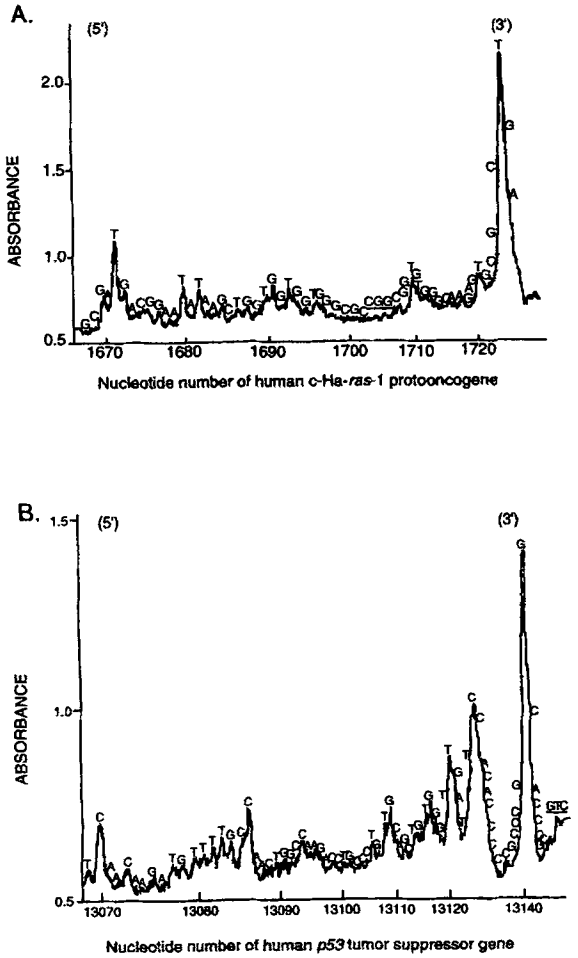


FIGURE 3 Site specificity of DNA cleavage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end labeled 261-base pair (*Ava* I* 1645–*Xba* I 1905) (A) or 118-base pair (*Hind* III*13038–*Sty* I 13155) (B) DNA fragment, 20 μM/base of sonicated calf thymus DNA, 500 μM 4-methoxy-*m*-phenylenediamine and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated for 2 h at 37°C. After piperidine treatment, DNA cleavage sites were determined as described under Materials and Methods. The horizontal axis shows the nucleotide number of the human c-Ha-*ras*-1 protooncogene (A) and the *p53* tumor suppressor gene (B). *Underscoring*, codon 12 of the human c-Ha-*ras*-1 protooncogene and codon 157 of the *p53* tumor suppressor gene.

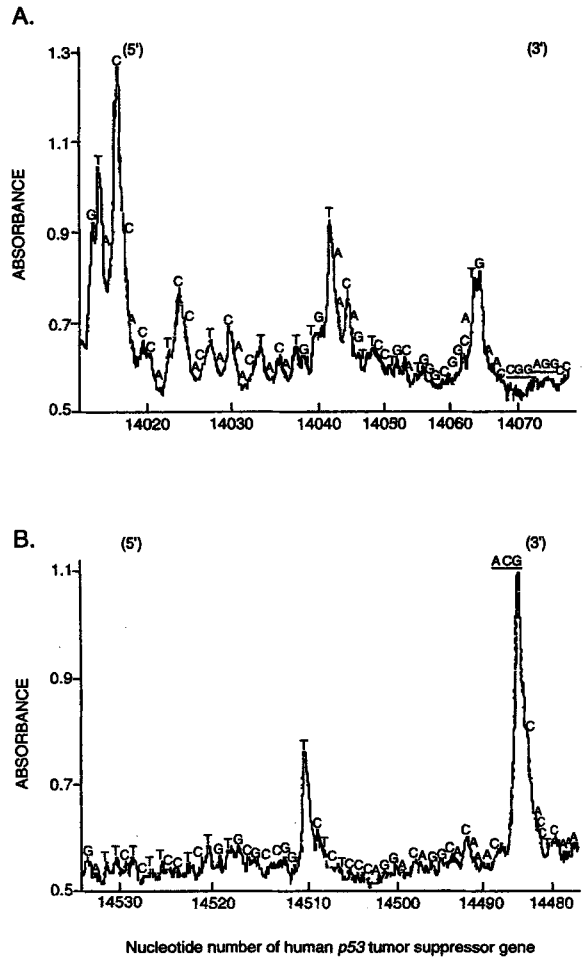


FIGURE 4 Site specificity of DNA cleavage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end labeled 211-base pair (*Apa* I 13972-*Hind* III*14182) (A) or 443-base pair (*Apa* I 14179-*Eco* RI*14621) (B) DNA fragment, 20 μM/base of sonicated calf thymus DNA, 500 μM 4-methoxy-*m*-phenylenediamine and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated for 2 h at 37°C. After piperidine treatment, DNA cleavage sites were determined as described under Materials and Methods. The horizontal axis shows the nucleotide number of the *p53* tumor suppressor gene. *Underscoring*, (A) codons 248 and 249 of the *p53* tumor suppressor gene, (B) complementary sequence to codon 273 of the *p53* tumor suppressor gene.

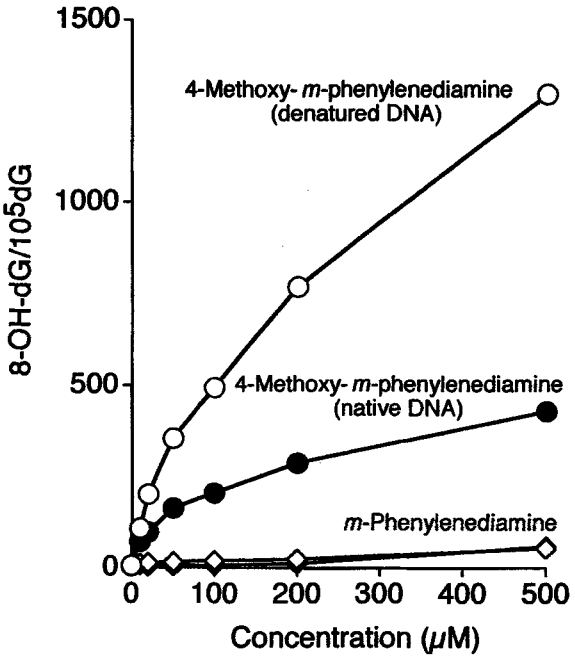


FIGURE 5 Formation of 8-OH-dG by *m*-phenylenediamines in the presence of Cu(II). The reaction mixture contained calf thymus DNA (100 μM/base) and 20 μM $CuCl_2$, indicated concentrations of 4-methoxy-*m*-phenylenediamine (circle) or *m*-phenylenediamine (square) in 400 μL of 4 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Native DNA (closed) and denatured DNA (open) were used. After incubation for 2 h at 37°C, 0.2 mM DTPA was added to stop the reaction, and then the DNA was precipitated in ethanol. The DNA fragment was enzymatically digested into nucleosides, and 8-OH-dG formation was measured with an HPLC-ECD as described in Materials and Methods.

(Figure 6A) changed only a little, suggesting very slow autoxidation in the buffer solution at pH 7.8, even in the presence of Cu(II). 4-Methoxy-*m*-phenylenediamine plus Cu(II) showed an increase in the absorbance at about 350 nm (Figure 6B). The increase of the absorbance at about 350 nm (Figure 6C) was enhanced by the addition of SOD, suggesting that SOD enhanced the rate of Cu(II)-mediated autoxidation of 4-methoxy-*m*-phenylenediamine.

Generation of $O_2^{\bullet-}$ *by 4-methoxy-m-phenylenediamine* Cytochrome *c* reduction by 4-methoxy-*m*-phenylenediamine was measured in the presence and absence of Cu(II). 4-Methoxy-*m*-phenylenediamine reduced cytochrome *c* in the presence Cu(II) and the reduction was partially inhibited by the addition of SOD, suggesting $O_2^{\bullet-}$ generation. Even in the absence of Cu(II), $O_2^{\bullet-}$ generation was observed in this system.

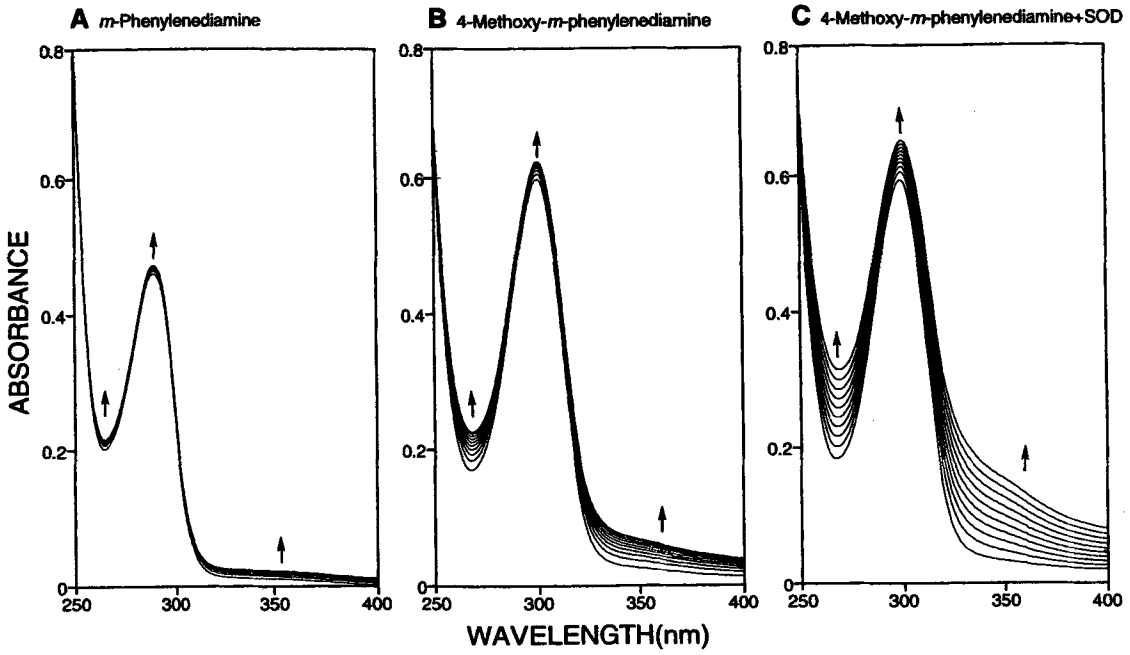


FIGURE 6 Changes in UV-visible spectra of *m*-phenylenediamines. (A) 200 μM *m*-phenylenediamine plus 20 μM $CuCl_2$ (B) 200 μM 4-methoxy-*m*-phenylenediamine plus 20 μM $CuCl_2$ (C) sample (B) plus 150 units/mL SOD. The spectra were measured every 10 min for 90 min at 37°C.

DISCUSSION

In the present study, carcinogenic 4-methoxy-*m*-phenylenediamine caused site-specific DNA damage, and non-carcinogenic *m*-phenylenediamine could not induce DNA damage under the condition used. Both *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine are mutagenic in the bacterial test system.[5–8] However, 4-methoxy-*m*-phenylenediamine is carcinogenic in rodents and possibly in humans,[3,4] whereas *m*-phenylenediamine is not.[4,5] Our results showed that carcinogenicity of *m*-phenylenediamines might be associated with an ability to cause oxidative DNA damage rather than bacterial mutagenicity.

Site-specific and characteristic mutations were found in human cancers as molecular mutational fingerprints associated with chemical carcinogens.[21] We showed that 4-methoxy-*m*-phenylenediamine could induce Cu(II)-mediated DNA damage at certain thymine residues e.g. the 5′-CTG-3′ in the human c-Ha-*ras*-1 protooncogene, at cytosine residues located 3′ or 5′ to cytosine in the human *p53* tumor suppressor gene. It is noteworthy that the guanine residue of the ACG sequence complementary to codon 273 (a known hotspot[9,20]) in the *p53* gene was damaged intensively.

To clarify the kinds of active species participating in site-specific DNA damage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II), the effects of scavengers and bathocuproine on the DNA damage were examined. The DNA damage was inhibited by both catalase and bathocuproine, suggesting the involvement of H_2O_2 and Cu(I) in the DNA damage. UV-visible spectroscopic studies on the autoxidation of 4-methoxy-*m*-phenylenediamine showed that the autoxidation occurred only in the presence of Cu(II), but not in the presence of Fe(II), Fe(III) or Mn(II). This suggested that only Cu(II) might

catalyze the autoxidation, and explained the reason why 4-methoxy-*m*-phenylenediamine did not cause DNA damage in the presence of Fe(II), Fe(III) or Mn(II). Spectroscopic studies using cytochrome *c* and SOD have shown the generation of $O_2^{\bullet-}$ during the autoxidation of 4-methoxy-*m*-phenylenediamine. On the basis of our results, a possible mechanism of DNA damage induced by 4-methoxy-*m*-phenylenediamine in the presence of Cu(II) could be explained as follows: 4-methoxy-*m*-phenylenediamine undergoes Cu(II)-mediated autoxidation to generate phenylenediamine-derived radicals and Cu(I). $O_2^{\bullet-}$ is generated probably by the reaction of O_2 with the radicals and/or Cu(I), and then is dismutated to H_2O_2. H_2O_2 interacts with Cu(I) to form the metal–oxygen complex, such as Cu(I)–OOH, capable of causing DNA damage. Typical $^{\bullet}OH$ scavengers showed no inhibitory effects on DNA damage by 4-methoxy-*m*-phenylenediamine, suggesting that $^{\bullet}OH$ does not play an important role in the DNA damage. Little involvement of $^{\bullet}OH$ is supported by the site-specific DNA damage observed with 4-methoxy-*m*-phenylenediamine, because $^{\bullet}OH$ causes DNA cleavage at any nucleotides with little site specificity.[22,23] Relevantly, Youngman and Elster proposed a concept of crypto-hydroxyl radical on the basis of reactions that were much less sensitive to the inhibition by traditional $^{\bullet}OH$ scavengers e.g. ethanol and mannitol, and the crypto-hydroxyl radical was reported to be reactive to sulfur compounds like methionine and methional.[24] The inhibitory effect of methional on the DNA damage can be explained by assuming that sulfur compounds are reactive to the metal–oxygen complex, such as Cu(I)–OOH.

It is noteworthy to find that SOD, which should protect organelle from oxidative stress, enhanced both the DNA damage and the autoxidation of 4-methoxy-*m*-phenylenediamine in the presence of Cu(II). SOD accelarated the process of $O_2^{\bullet-}$ generation by removing $O_2^{\bullet-}$. As the result, H_2O_2 generated by $O_2^{\bullet-}$ dismutation, participated in the formation of the copper–oxygen complex responsible for the DNA damage. It has been reported that copper occurs in the mammalian cell nucleus,[25] and copper mediates production of active oxygen species and DNA damage.[26] Therefore, it is concluded that SOD and Cu(II)-mediated oxidative DNA damage may have an important role in carcinogenesis of 4-methoxy-*m*-phenylenediamine.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] C. La Vecchia and A. Tavani (1995) Epidemiologic evidence on hair dyes and the risk of cancer in humans. *European Journal of Cancer Prevention*, **4**, 31–43.
[2] F. Rodstein, C.H. Hennekens, G.A. Colditz, D.J. Hunter and M.J. Stampfer (1994) A prospective study of permanent hair dye use and hematopoietic cancer. *Journal of the National Cancer Institute*, **86**, 1466–1470.
[3] NCI (1978) Bioassay of 2,4-diaminoanisole sulfate for possible carcinogenicity. In *NCI Carcinogenesis Technical Report Series*. No. 84, DHEW Publication No. (NIH) 78–1334.
[4] H.A. Milman and C. Peterson (1984) Apparent correlation between structure and carcinogenicity of phenylenediamines and related compounds. *Environmental Health Perspectives*, **56**, 261–273.
[5] IARC Working Group (1987) Some industrial chemicals and dyestuffs. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*. IARC, Lyon, Vol. 16, 111–124.
[6] M.I. Plewa, E.D. Wagner, T.-W. Yu and D. Anderson (1995) Genotoxicity of *m*-phenylenediamine and 2-aminofluorene in Salmonella typhimurium and human lymphocytes with and without plant activation. *Environmental and Molecular Mutagenesis*, **26**, 171–177.
[7] S. Parodi, P. Taningher, R. Russo, M. Pala, M. Tamaro and C. Monti-Bragadin (1981) DNA-damaging activity *in vivo* and bacterial mutagenicity of sixteen aromatic amines and azo-derivatives, as related quantitatively to their carcinogenicity. *Carcinogenesis*, **2**, 1317–1326.
[8] T. Gichner, G.C. Lopez, E.D. Wagner and M.J. Plewa (1994) Induction of somatic mutations in Tradescatia clone 4430 by three phenylenediamine isomers and the antimutagenic mechanisms of diethyldithiocarbamate and ammonium meta-vanadate. *Mutation Research*, **306**, 165–172.
[9] M.F. Denissenko, A. Pao, M. Tang and G.P. Pfeifer (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in *p53*. *Science*, **274**, 430–432.
[10] R.A. Floyd (1990) The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis*, **11**, 1447–1450.

[11] S. Shibutani, M. Takeshita and A.P. Grollman (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*, **349**, 431–434.
[12] P. Chumakov (1990) *EMBL Data Library*, accession number X54156.
[13] D.J. Capon, E.Y. Chen, A.D. Levinson, P.H. Seeburg and D.V. Goeddel (1983) Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature*, **302**, 33–37.
[14] N. Yamashita, M. Murata, S. Inoue, Y. Hiraku, T. Yoshinaga and S. Kawanishi (1998) Superoxide formation and DNA damage induced by a fragrant furanone in the presence of copper(II). *Mutation Research*, **397**, 191–201.
[15] K. Yamamoto and S. Kawanishi (1991) Site-specific DNA damage induced by hydrazine in the presence of manganese and copper ions: The role of hydroxyl radical and hydrogen atom. *Journal of Biological Chemistry*, **266**, 1509–1515.
[16] S. Kawanishi and K. Yamamoto (1991) Mechanism of site-specific DNA damage induced by methylhydrazines in the presence of copper(II) or manganese(III). *Biochemistry*, **30**, 3069–3075.
[17] A.M. Maxam and W. Gilbert (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods in Enzymology*, **65**, 499–560.
[18] H. Kasai, P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama and H. Tanooka (1986) Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis*, **7**, 1849–1851.
[19] K. Ito, S. Inoue, K. Yamamoto and S. Kawanishi (1993) 8-Hydroxydeoxyguanosine formation at the 5′-site of 5′-GG-3′ sequences in double-stranded DNA by UV radiation with riboflavin. *Journal of Biological Chemistry*, **268**, 13221–13227.
[20] M. Hollstein, D. Sidransky, B. Vogelstein and C.C. Harris (1991) *p53* Mutations in human cancers. *Science*, **253**, 49–53.
[21] M. Toyota, T. Ushijima, H. Akiuchi, F. Canzian, M. Watanabe, K. Imai, T. Sugimura and M. Nagao (1996) Genetic alterations in rat colon tumors induced by heterocyclic amines. *Cancer*, **77**, 1593–1597.
[22] S. Kawanishi, S. Inoue and S. Sano (1986) Mechanism of DNA cleavage induced by sodium chromate(VI) in the presence of hydrogen peroxide. *Journal of Biological Chemistry*, **261**, 5952–5958.
[23] D.W. Celander and T.R. Cech (1990) Iron(II)-ethylenediaminetetraacetic acid catalyzed cleavage of RNA and DNA oligonucleotides: Similar reactivity toward single- and double-stranded forms. *Biochemistry*, **29**, 1355–1361.
[24] R.J. Youngman and E.F. Elstner (1981) Oxygen species in paraquat toxicity: the crypto-OH radical. *FEBS Letters*, **129**, 265–268.
[25] M.J. Burkitt (1994) Copper-DNA adducts. *Methods in Enzymology*, **234**, 66–79.
[26] Y. Li and M.A. Trush (1994) Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. *Cancer Research*, **54**, 1895s–1898s.