Analyses of Oxidative DNA Damage and Its Repair Activity in the Livers of 3′-Methyl-4-dimethylaminoazobenzene-treated Rodents

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We measured the levels of 8-hydroxyguanine (8-OH-Gua) and its repair activity in the livers of the Donryu rat, the carcinogen-resistant DRH rat, and the ddy mouse, which were fed a 0.06% 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB)-containing diet. In a short-term rat experiment (maximum 2 months), 3′-MeDAB did not increase the 8-OH-Gua levels in the livers of the two rat strains, although it significantly increased the repair activity in only the Donryu rat liver at 1 and 2 months. After long-term 3′-MeDAB administration to the ddy mouse (8 months), the levels of 8-OH-Gua and its repair activity were increased in the liver by 3.6-fold and 1.6-fold, respectively. These experiments suggest that 3′-MeDAB increases 8-OH-Gua generation in rodent liver DNA and the 8-OH-Gua repair assay is a reliable marker of cellular oxidative stress induced by carcinogens.

Key words: 8-Hydroxyguanine — 3′-MeDAB — 8-Oxoguanine DNA glycosylase 1 — Carcinogen-resistant DRH rat

Some aminoazo dyes, such as N-methyl-4-aminoazobenzene (MAB), N,N-dimethyl-4-aminoazobenzene (DAB), and 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB), are hepatocarcinogenic. A 0.06% 3′-MeDAB-containing diet is reported to induce hepatic tumors in rats† and mice.‡ These reports indicated that a 24-week feeding of 3′-MeDAB induced liver tumors in 94% of Donryu rats, while it did not induce any hepatic tumors in carcinogen-resistant DRH rats (the experiment was started at the age of 7 weeks), and induced hepatomas in CBA mice between 44 and 55 weeks (the experiment was started at the age of 4 weeks). Because aminoazo dyes cause a wide variety of aberrant biochemical and histological features,†‡ the carcinogenic mechanism may be very complex. Among several mechanisms that have been proposed, carcinogen-DNA adduct formation is thought to be the major contributor.

Recently, the carcinogen-resistant DRH rat was developed from the parental Donryu strain rat, which has a low incidence of liver tumor induction by 3′-MeDAB administration,†‡ and it has been used for the study of the mechanisms of chemical carcinogens.†‡ The activities of drug-metabolizing enzymes in the liver of the DRH rat were shown to be less than those in the Donryu rat liver,† and this may be relevant to the differences in susceptibility to carcinogens. However, the overall mechanism of the carcinogen-resistance in DRH rat has not yet been established.

Our recent study using the DRH rat showed that both GADD 45 (growth arrest and DNA damage-inducible) and O6-methylguanine methyltransferase mRNA levels were greatly increased in Donryu rat liver, as compared with DRH rat liver, when the rats were fed a 0.06% 3′-MeDAB-containing diet for 2 months.§ We concluded that DNA damage other than bulky DNA adduct formation was involved in the mechanism of 3′-MeDAB-induced carcinogenesis. We also suggested that the generation of reactive oxygen species occurred as well, because we observed a significant induction of cytochrome P-450 2E1 mRNA (CYP2E1) in the Donryu rat liver as compared to the DRH rat liver. CYP2E1 is an effective catalyst for reductive reactions,§ including reduction of oxygen to superoxide and peroxide. Therefore, oxidative DNA damage may play a role in the carcinogenic mechanism.

To test this hypothesis, we measured the levels of 8-hydroxyguanine (8-OH-Gua) and its repair activity in the livers of rats and mice fed a carcinogen-containing diet. 8-OH-Gua is one of the major forms of oxidative DNA damage,§ and has been well studied because it is a known promutagen.†‡ Recently, the genes for human and murine 8-oxoguanine DNA glycosylase 1 (OGG1), a major enzyme for repair of 8-OH-Gua, were cloned by several researchers.†‡ It is necessary to measure the levels of 8-OH-Gua repair activity and OGG1 induction, as well as 8-OH-Gua generation, for analysis of the role of oxidative DNA damage in the carcinogenic mechanism.

In the present study, we performed two kinds of experiments. First, we measured the levels of 8-OH-Gua and its repair activity in the livers of DRH and Donryu rats fed a
Measurement of 8-OH-Gua levels in the animal liver

**Rat**

0.06% 3′-MeDAB-containing diet for a short term (maximum 2 months). Secondly, we measured the levels of 8-OH-Gua, its repair activity, and the induction of mouse OGG1 mRNA in the livers of ddy mice fed the same diet for a long term (8 months).

MATERIALS AND METHODS

Animals and treatments The experimental protocols are shown in Fig. 1. Five-week-old male DRH and Donryu rats and 3-week-old ddy mice were purchased from Seac Yoshitomi, Ltd. (Fukuoka). They were fed a commercial diet (Clea, Tokyo) and tap water *ad libitum* until the start of the experiments. The rats were fed a diet containing 0.06% 3′-MeDAB when they were 8 weeks old. They were killed at the indicated time points (0, 3, 7, and 10 days and 4 and 8 weeks after the start of the carcinogen-containing diet). The livers were perfused with ice-cold saline, removed, and used for the analyses (Fig. 1A). The mice were maintained on a commercial diet for 2 months and then fed a diet containing 0.06% 3′-MeDAB-containing diet (3′-MeDAB-treatment group) or a normal commercial diet (control group). They were killed and the livers were removed at 8 months after the start of the carcinogen-containing diet.

**Mouse**

Analysis of mouse OGG1 mRNA induction by RT-PCR

Mouse OGG1 mRNA induction was examined by RT-PCR, as described previously. Briefly, the first strand of cDNA was synthesized from isolated mRNA and each cDNA was amplified using primers for the mouse OGG1 and GAPDH genes. The GAPDH mRNA was used as an internal standard. The primers for mouse OGG1 were 5′-CTCTGGTCCCTCCAAACAC-3′ and 5′-GCCAGCATTAAGGTCCTCCAC-3′. The primers for mouse GAPDH were 5′-AACGGGAAGCTCATGGCATG-3′ and 5′-TCACCCACAGTGTG-3′. The amplification protocol for mouse OGG1 consisted of 35 cycles at 94°C (60 s), 61°C (60 s) and 72°C (180 s). The PCR products were separated on a 5% polyacrylamide gel and were visualized with ethidium bromide staining.

Statistical analysis All values in the text represent the means±SD. The statistical significance of differences was calculated using Student’s *t* test. Probability values less than 0.05 were considered to indicate significant differences.

RESULTS

Levels of 8-OH-Gua and its repair activity in the rat liver First, we measured the levels of 8-OH-Gua and its repair activity in the livers of DRH and Donryu rats after 3′-MeDAB administration. There were no significant differences in the 8-OH-Gua levels between these two strains during the observation period (Fig. 2A). However, the repair activity showed a significant increase in the Donryu rat liver at 1 and 2 months (P=0.00073 and 0.013, respectively), while in the DRH rat liver, no significant change was observed during the time frame of this study in comparison to the control level (time 0) (Fig. 2B).

Analysis of 8-OH-Gua repair activity Base excision repair activities were assayed by using a previously described method. Briefly, the tissues were homogenized and centrifuged (12 000g, 30 min) to obtain crude extracts. The extracts were incubated with a 22-mer, fluorescently labeled, synthetic oligonucleotide containing an 8-OH-Gua residue in its sequence. These mixtures were electrophoresed on a 20% denaturing polyacrylamide gel. The excised fragments, generated as a consequence of base excision repair activity, were analyzed by a Pharamcia ALF DNA sequencer (Fragment Manager, Ver. 1.1; Amersham Pharmacia Biotech, Uppsala, Sweden).

Analysis of mouse OGG1 mRNA level in the mouse liver In contrast to the rat experiment, the 8-OH-Gua level in the DNA of the 3′-MeDAB-treated mouse liver was 3.6-fold higher than that in the control mouse livers (Fig. 3A). The 8-OH-Gua repair activity was also significantly increased in the 3′-MeDAB-treated...
mouse liver, by 1.6-fold over that of the control mouse liver (Fig. 3B). We also examined the mRNA level of mouse OGG1 by RT-PCR, but its induction was not observed in the liver after the administration of 3′-MeDAB (Fig. 3C).

**DISCUSSION**

To clarify the mechanism of hepatocarcinogenesis induced by aminoazo dyes, many approaches have been employed. 24–30 At least two electrophilic metabolites of 3′-MeDAB, one that binds to yeast RNA and the other to tissue protein, are known to be produced in a rat liver cell-free system 30 and electron spin resonance has revealed that the liver of the DAB-treated rat contains free radicals. 29 Shimpo et al. reported the effects of vitamin C on hepatocarcinogenesis induced by 3′-MeDAB. 30 They concluded that some vitamin C derivatives had anticancer effects, which were assumed to be due to antioxidant activity. These reports strongly suggest that oxidative stresses play a key role in the mechanism of hepatocarcinogenesis induced by aminoazo dyes. Oxidative stresses are known to generate DNA damage, such as point mutations in genomic DNA, which can be responsible for carcinogenesis. Thus, oxidative DNA damage may be a key factor in the carcinogenesis induced by the aminoazo dyes.

In this study, we analyzed the role of oxidative DNA damage in carcinogenesis by measuring 8-OH-Gua and its repair activity. Recently, the carcinogen-resistant DRH rat was developed and used for the study of chemical carcinogenesis. Sakamoto’s group demonstrated that DRH and Donryu rats might be equally capable of generating oxidative stresses when treated with 3′-MeDAB. 31 Moreover, they indicated that the levels of all of the antioxidants and the activities of all of the scavenger enzymes examined in
the liver of the DRH rats were almost the same as those in the Donryu rats. Based on these findings, although 3′-MeDAB is known to generate oxidative stress, it appears likely that this stress is not responsible for 3′-MeDAB-induced carcinogenesis. In fact, our study shows that 3′-MeDAB does not increase the 8-OH-Gua level in the livers of Donryu and DRH rats (Fig. 2A). However, since the measured levels of oxidative DNA damage depend on the balance between its generation and repair, we cannot exclude the possibility that 3′-MeDAB generated 8-OH-Gua without an analysis of the repair activity. In the present study, 3′-MeDAB increased the repair activity in the liver of the Donryu rat (Fig. 2B). Thus, a reasonable explanation is that the production of 8-OH-Gua was increased in the Donryu rat and that the repair systems were induced, thus maintaining the level of 8-OH-Gua at the control level, while in the DRH rat liver, the production of 8-OH-Gua was not increased, and therefore, the repair activity was also not increased. The difference in cellular oxidative stress seems to be due to differences in the metabolism of 3′-MeDAB in these strains.7) In other words, the resistance of the DRH rat to the carcinogen might be, at least in part, due to the low production of 8-OH-Gua.

Based on the results of the first experiment, we planned and performed a second experiment in which we fed ddy mice a 3′-MeDAB-containing diet for 8 months. We observed a 3.6-fold increase in 8-OH-Gua, and its repair activity was increased by 1.6-fold in the livers of the 3′-MeDAB-treated mice as compared to the control (Fig. 3). These results support our previous finding that DNA damage other than bulky DNA adduct formation is involved in the carcinogenic mechanism of 3′-MeDAB.8)

To obtain further information about the repair capacity, we analyzed the mRNA level of the repair enzyme OGG1 by RT-PCR. The OGG1 protein is an 8-OH-Gua repair enzyme, and has glycosylase/lyase activities. Recently, we reported that rat lung OGG1 mRNA was induced by the inhalation of diesel exhaust particles, which increase the 8-OH-Gua level in rat lung DNA.20) In this study, unexpectedly, no induction was observed, while the 8-OH-Gua repair activity was increased by 1.6-fold. The reason why the OGG1 mRNA was not induced remains unknown. However, the lack of OGG1 mRNA induction suggests the existence of repair systems other than the OGG1 protein.

The present data still do not conclusively show whether or not 8-OH-Gua generation is responsible for the 3′-MeDAB-induced hepatocarcinogenesis. We did not perform histopathological analysis, although we examined the surface of the livers and observed that three of six 3′-MeDAB-treated mouse livers had several tumors while no control mouse livers had any tumors. In order to establish the exact relationship between 8-OH-Gua generation and 3′-MeDAB-induced hepatocarcinogenesis, the time-courses of 8-OH-Gua, its repair activity, and mOGG1 expression should be examined in association with histopathological analyses.

In conclusion, our data suggest that 3′-MeDAB generates 8-OH-Gua in rodent liver DNA and that 8-OH-Gua repair assay is a reliable marker of cellular oxidative stress induced by carcinogens.

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REFERENCES

1) Yoshimoto, F., Masuda, S., Higashi, T., Nishii, T., Takamisawa, I., Tateishi, N. and Sakamoto, Y. Comparison of drug-metabolizing activities in the livers of carcinogen-sensitive parent rats and carcinogen-resistant descendants. Cancer Res., 45, 6155–6158 (1985).

2) Medvedev, Z. A., Crowne, H. M. and Medvedeva, M. N. Age related variations of hepatocarcinogenic effect of azo dye (3′-MDAB) as linked to the level of hepatocyte polyplodization. Mech. Ageing Dev., 46, 159–174 (1988).

3) Tullis, D. L., Dooley, K. L., Miller, D. W., Baetcke, K. P. and Kadlubar, F. F. Characterization and properties of the DNA adducts formed from N-methyl-4-aminoazobenzene in rats during a carcinogenic treatment regimen. Carcinogenesis, 8, 577–583 (1987).

4) Miyamoto, K., Sanae, F., Kohei, K., Nomura, M. and Koshiura, R. Changes in number of α-adrenergic receptor subtypes in hepatocytes from rats fed 3′-methyl-4-dimethylaminoazobenzene. Jpn. J. Cancer Res., 81, 557–559 (1990).

5) Karaki, Y., Munakata, S., Saeki, T., Hirota, S. and Fujimaki, M. Appearance of a carcinoid-like pattern in rat hepatic tumors induced by 3′-methyl-4-dimethylaminoazobenzene. Jpn. J. Cancer Res., 82, 397–402 (1991).

6) Súborová, M., Frei, E., Schmeiser, H. H., Wiessler, M. and Hradec, J. Formation and ³²P-postlabeling of DNA and tRNA adducts derived from peroxidative activation of carcinogenic azo dye N,N-dimethyl-4-aminoazobenzene. Carcinogenesis, 13, 1657–1662 (1992).

7) Yano, M., Higashi, T., Kano, S., Tateishi, N., Kido, Y., Mori, T., Higashi, K. and Sakamoto, Y. Decreased activation of carcinogens in the liver of carcinogen-resistant rats.

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Cancer Res., 49, 5352–5357 (1989).

8) Yan, Y., Higashi, K., Yamamura, K., Fukamachi, Y., Abe, T., Gotoh, S., Sugiura, T., Hirano, T., Higashi, T. and Ichiba, M. Different responses other than the formation of DNA-adducts between the livers of carcinogen-resistant rats (DRH) and carcinogen-sensitive rats (Donryu) to 3′-methyl-4-dimethylaminooazobenzene administration. Jpn. J. Cancer Res., 89, 806–813 (1998).

9) Koop, D. R. Oxidative and reductive metabolism by cytochrome P450 2E1. FASEB J., 6, 724–730 (1992).

10) Kasai, H. and Nishimura, S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. Nucleic Acids Res., 12, 2137–2145 (1984).

11) Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S. and Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. J. Biol. Chem., 267, 166–172 (1992).

12) Lu, R., Nash, H. M. and Verdine, G. L. A mammalian DNA repair enzyme that excises oxidatively damaged guanine molecules to a locus frequently lost in lung cancer. Curr. Biol., 7, 397–407 (1997).

13) Rosenquist, T. A., Zharkov, D. O. and Grollman, A. P. Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc. Natl. Acad. Sci. USA, 94, 7429–7434 (1997).

14) Radicella, J. P., Dherin, C., Desmaze, C., Fox, M. S. and Boiteux, S. Cloning and characterization of hOGG1, a human homolog of the OGG1 gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA, 94, 8010–8015 (1997).

15) Roldán-Arjona, T., Wei, Y. F., Carter, K. C., Klungland, A., Anselmino, C., Wang, R. P., Augustus, M. and Lindahl, T. Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase. Proc. Natl. Acad. Sci. USA, 94, 8016–8020 (1997).

16) Arai, K., Morishita, K., Shinmura, K., Kohn, T., Kim, S. R., Nohmi, T., Taniwaki, M., Ohwada, S. and Yokota, J. Cloning of a human homolog of the yeast OGG1 gene that is involved in the repair of oxidative DNA damage. Oncogene, 14, 2857–2861 (1997).

17) Aburatani, H., Hippo, Y., Ishida, T., Takashima, R., Matsuba, C., Kodama, C., Takao, M., Yasui, A., Yamamoto, K., Asano, M., Fukasawa, K., Yoshinari, T., Inoue, H., Ohtsuka, E. and Nishimura, S. Cloning and characterization of mammalian 8-hydroxyguanine-specific DNA glycosylase/apurinic, apyrimidinic lyase, a functional mutant homologue. Cancer Res., 57, 2151–2156 (1997).

18) Tani, M., Shinmura, K., Kohn, T., Shiroishi, T., Wakana, S., Kim, S. R., Nohmi, T., Kasai, H., Takenoshita, S., Nagamachi, Y. and Yokota, J. Genomic structure and chromosomal localization of the mouse Ogg1 gene that is involved in the repair of 8-hydroxyguanine in DNA damage. Mamm. Genome, 9, 32–37 (1998).

19) Nakae, D., Mizumoto, Y., Kobayashi, E., Naguchi, O. and Konishi, Y. Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. Cancer Lett., 97, 233–239 (1995).

20) Hirano, T., Yamaguchi, R., Asami, S., Iwamoto, N. and Kasai, H. 8-Hydroxyguanine levels in nuclear DNA and its repair activity in rat organs associated with age. J. Gerontol., 51A, B303–B307 (1996).

21) Hirano, T., Yamaguchi, Y., Hirano, H. and Kasai, H. Age-associated change of 8-hydroxyguanine repair activity in cultured human fibroblasts. Biochem. Biophys. Res. Commun., 214, 1157–1162 (1995).

22) Yamaguchi, R., Hirano, T., Ootsuyama, Y., Asami, S., Tsurudome, Y., Fukuda, S., Yamato, H., Tsuda, T., Tanaka, I. and Kasai, H. Increased 8-hydroxyguanine in DNA and its repair activity in hamster and rat lung after intratracheal instillation of crocidolite asbestos. Jpn. J. Cancer Res., 90, 505–509 (1999).

23) Tsurudome, Y., Hirano, T., Yamato, H., Tanaka, L., Sagai, M., Hirano, H., Nagata, N., Itoh, H. and Kasai, H. Changes in levels of 8-hydroxyguanine in DNA, its repair and OGG1 mRNA in rat lungs after intratracheal administration of diesel exhaust particles. Carcinogenesis, 20, 1573–1576 (1999).

24) Roberts, J. J. and Warwick, G. P. The covalent binding of metabolites of dimethylnitrosamine and aniline to nucleic acids in vivo. Int. J. Cancer, 1, 179–196 (1966).

25) Lin, J. K., Miller, J. A. and Miller, E. C. Structures of hepatic nucleic acid-bound dyes in rats given the carcinogen N-methyl-4-aminooazobenzene. Cancer Res., 35, 844–850 (1975).

26) Labuc, G. E. and Blunck, J. M. Metabolic activation of the hepatocarcinogen 3′-methyl-4-dimethylnitrosamine by a rat liver cell-free system. Biochem. Pharmacol., 28, 2367–2373 (1979).

27) Tarpley, W. G., Miller, J. A. and Miller, E. C. Adducts from the reaction of N-benzoyloxy-N-methyl-4-aminooazobenzene with deoxyguanosine or DNA in vitro and from hepatic DNA of mice treated with N-methyl- or N,N-dimethyl-4-aminooazobenzene. Cancer Res., 40, 2493–2499 (1980).

28) Samuels, A. R., Bhargava, M. M. and Levine, W. G. Uptake and hepatobiliary fate of two hepatocarcinogens, N,N-dimethyl-4-aminooazobenzene and 3′-methyl-N,N-dimethyl-4-aminooazobenzene, in the rat. Cancer Res., 43, 4816–4821 (1983).

29) Duchesne, J., Goutier, R., Maréchal, R. and van de Vorst, A. Free radical evolution in rat liver following the ingestion of DAB. Phys. Med. Biol., 20, 305–309 (1975).

30) Shimp, K., Takahashi, H., Tsuda, H., Hibino, T., Kawai, K., Kimura, C., Nagatsu, T. and Fujita, K. Inhibition of hepatocellular carcinoma development and erythrocyte polyamine levels in ODS rats fed on 3′-methyl-4-dimethylnitrosamine by hemicalcium ascorbate, 2-O-octadecylascorbic acid, and ascorbyl palmitate. Cancer Detect. Prev., 20, 137–145 (1996).

31) Hammad, H., Higashi, T., Tateishi, N., Hanatani, M. and Sakamoto, Y. Lipid peroxidation in the liver of carcinogen-resistant rats. Biochim. Biophys. Acta, 1045, 99–106 (1990).