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Formation of 4,4′-methylene-bis(2-chloroaniline)-DNA adducts in yeast expressing recombinant cytochrome P450s

Y. Endo-Ichikawa, H. Kohno[a], R. Tokunaga[a], Y. Yabusaki[b], T. Sakaki[b], H. Ohkawa[c] and S. Taketani[a]

Departments of Public Health and [a]Hygiene, Kansai Medical University, Moriguchi, Osaka 570, Fax +81 6 992 5194, [b]Takarazuka Research Center, Sumitomo Chemical Co., Ltd., Takatsukasa, Takarazuka, Hyogo 665, and [c]Department of Plant Protection, Kobe University, Nada, Kobe 657 (Japan)
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Abstract. *N*-Oxidation of 4,4′-methylene-bis(2-chloroaniline) (MBOCA) may lead to formation of DNA adducts. To determine if cytochrome P450s are involved in the formation of MBOCA derived-DNA adducts, yeast strains expressing rodent P450s were exposed to MBOCA, and ^{32}P-postlabelling of nucleotides from yeast genomic DNA was done. Chromatographic analysis on PEI cellulose showed that, upon exposure to MBOCA for 1 h, nine DNA adducts were formed in yeast expressing phenobarbital-inducible rabbit P450 2B5. With a 4-h-exposure, all adducts increased in parallel. In cell-free experiments, the incubation of MBOCA with phenobarbital-induced rat microsomal fraction followed by incubation with thymus DNA, led to the formation of more than ten DNA adducts. When yeast expressing 3-methylcholanthrene-inducible rat P450 1A1 was exposed to MBOCA, one major and two minor adducts were formed. No adducts were detected in control yeast. These results show that recombinant rabbit P450 2B5 exhibits a potential activation of MBOCA and that rat P450 1A1 has some effect. The use of yeast expressing recombinant P450s and the technique of ^{32}P-postlabelling facilitates a simple search for chemicals with carcinogenic potential.
Key words. 4,4′-Methylene-bis(2-chloroaniline); MBOCA-DNA adduct; P450 2B5; P450 1A1; yeast.

Cytochrome P450s play important roles in metabolic activation and detoxication of chemical carcinogens[1]. The multiple forms of cytochrome P450s present in liver microsomes contribute to a variety of reactions, producing a number of metabolites. Some metabolites may be active intermediates in the carcinogenic process.

The process of chemically-induced carcinogenesis is generally divided into initiation and promotion[2]. DNA adduct formation in target tissues is considered to be the first step of initiation; therefore, detection of adducts would indicate the risk of cancer from exposure to compounds. ^{32}P-Postlabelling analysis, which can detect a very low level of adducts in DNA, was developed by Randerath et al.[3] and is a useful way of detecting this step in the initiation of cancer.

4,4′-Methylene-bis(2-chloroaniline) (MBOCA) is an aromatic diamine widely used in the plastics and rubber industry as a cross-linking agent for the synthesis of products such as polyurethanes and epoxy resins[4]. This compound is carcinogenic in animals[5], inducing lung and liver cancers in rats[6,7], and bladder tumors in dogs[8]. MBOCA metabolites activated by rat liver microsomes exhibited genotoxicity in the Ames test[9]. The metabolism of MBOCA has been studied in vivo and in vitro and *N*-hydroxylation proved to be the first process of activation. *N*-Hydroxylation of MBOCA in rat is predominantly catalyzed by phenobarbital-inducible P450 isozymes (the CYP 2B family)[1], while other carcinogenic aromatic amines are activated by the CYP 1A family. *N*-Hydroxy-MBOCA is considered to be the final carcinogenic form.

Heterologous expression of human and rodent P450s in mammalian cells and yeast has been reported. The expression of human P450 3A subtype in yeast resulted in *N*-hydroxylation of MBOCA[10]. Yeast contains intracellular organelles similar to mammalian cells and can be used as a simple model of eukaryotic cells. Although yeast microsomes possess endogenous P450s, the levels are extremely low under aerobic culture conditions[11]. We developed a method to measure P450-mediated metabolism of chemicals to DNA-binding derivatives, using the yeast expression system, and we report here the formation of DNA adducts derived from MBOCA in yeast expressing recombinant P450s (2B5 and 1A1).

Materials and methods

Yeast strains AH22/pACCD-1 expressing rat P450 (CYP 1A1)[12] and AH22/pABO-1 expressing rabbit P450 (CYP 2B5)[13,14] were used. The yeast strains were grown in synthetic minimal medium (SD) to a density of 5×10^6 cells/ml, as described in the report by Oeda et al.[11]. MBOCA (Tokyo Kasei Co., Tokyo, Japan) was purified by HPLC as described previously[15]. The purified MBOCA (99% pure) was dissolved in ethanol, and added to cultures at a final concentration of 120 μM. After incubation at 30 °C for the indicated period, the cells were collected by centrifugation at

1000 × *g* for 10 min, and were then treated with Zymolyase (Seikagaku Co., Tokyo, Japan)[11]. DNA was isolated on an Applied Biosystems 341 Nucleic Acid Purification System (GENEPURE). The DNA thus obtained was dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA (TE solution), and used for ^{32}P-postlabelling experiments.

Determination of cytochrome P-450 hemoprotein was done by reduced CO-difference spectra[11], and 7-ethoxycoumarin *O*-deethylation activity using the yeast microsomal fractions was measured as described by Sakaki et al.[16], showing specific activities of 9.0 and 0.59 nmol of 7-hydroxycoumarin formed/nmol of P450/min in pACCD-1 and pABO-1 strains, respectively.

For activation of MBOCA in a cell-free system, phenobarbital and 5,6-benzoflavone-treated rat liver 9000 × *g* supernatant (S-9) (Oriental Yeast Co., Tokyo, Japan) was used[17]. Twenty microliters of ethanol containing MBOCA (60 mM) were added to the reaction mixture (480 µl) containing 100 µl of S-9, 80 µM $MgCl_2$, 20 mM glucose 6-phosphate, 2 mM NADP, and 50 mM potassium phosphate buffer (pH 7.4). After incubation at 37 °C for 1 h, the chemicals were extracted three times with ethylether[18] and the ethylether was evaporated under a stream of N_2. The residue was dissolved in 20 µl of ethanol and mixed with 50 µg of calf thymus DNA (Type 1, Sigma Co.) in TE solution. After incubation at 37 °C for 4 h, the chemicals were removed with ethylether after which the DNA was precipitated with ice-cold ethanol. The DNA was washed twice with 75% ethanol and dissolved in TE solution[18]. DNA concentration was estimated spectrophotometrically at 260 mm (1 A_{260} = 50 µg/ml).

DNA (10 µg) was digested to 3′-dNMP with 0.42 units of micrococcal endonuclease (Sigma Co.) and 0.004 units of spleen phosphodiesterase (Sigma Co.) in 20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0, at 37 °C for 1.5 h[19]. To concentrate the adducted dNMP, 1-butanol extraction was done, according to the method of Gupta[20].

The adducted dNMP prepared from 10 µg of DNA was labelled using 50 Ci of [γ-^{32}P]ATP (6000 Ci/mmol) (Amersham, USA) and 10 units of T4-polynucleotide kinase at 37 °C for 30 min, then unreacted ^{32}P-ATP was digested with 0.1 units of apyrase (grade I, Sigma Co.)[21]. Fingerprints of ^{32}P-labelled adducts were made by two-directional development using 2 µl of the labelled solution on a PEI-cellulose sheet (12 × 10 cm) (Macherey-Nagel, Doren, Germany). The sheet was preconditioned, according to the method by Gupta and Randerath[21]. The chromatographic solvents for PEI-cellulose TLC were, 2.7 M lithium formate and 6.4 M urea (pH 3.5) for D_1, 0.68 M LiCl, 0.43 M Tris-HCl buffer (pH 8.0) and 7.2 M urea, followed by 1.7 M sodium phosphate buffer (pH 6.0) for D_2. To calculate the relative adduct level (RAL), 1 µg of digested normal nucleotide was also ^{32}P-labelled and diluted 1000-fold. Two microliters of the diluted solution was mapped in one-directional development using 0.27 M ammonium sulfate[21]. After being air-dried, the sheet was exposed to X-ray film at −80 °C for 4–5 days. The determination of the DNA adducts formed was carried out using a Fuji Film Image Analyzer BAS2000. The relative adduct level (RAL) was calculated according to Gupta and Randerath[21], and was expressed as cpm in adduct nucleotides/cpm in total nucleotides.

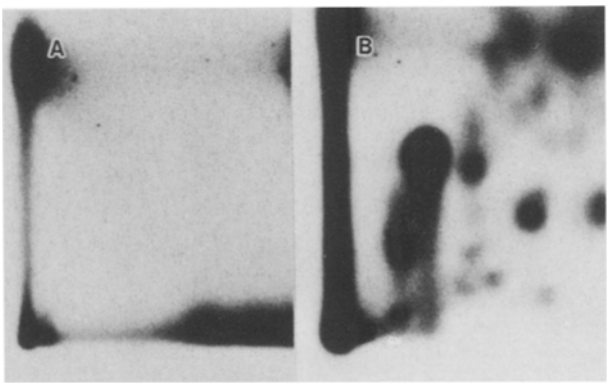

Figure 1. ^{32}P-Postlabelling analysis of DNA adducts after treatment. MBOCA was treated without (*A*) or with S-9 (*B*) at 37 °C for 1 h. The resulting compounds reacted with calf thymus DNA. DNA digests were postlabelled, spotted onto PEI sheets, and developed in D_1 (from bottom to top) and D_2 (from left to right).

Results

To examine whether P450s are involved in the formation of DNA adduct from MBOCA, MBOCA was incubated with S-9 for 1 h, and the reaction products were extracted and incubated with calf thymus DNA. Figure 1 shows a chromatographic pattern of DNA adducts from MBOCA activated by S-9. The incubation of MBOCA with S-9 resulted in the formation of more than 10 DNA adducts, while no adducts were observed in the sample without S-9. In addition, without glucose 6-phosphate and NADP no adducts were found (data not shown). These results are consistent with in vivo observations that several DNA adducts were formed in the rat liver after the administration of MBOCA[22] and in human urothelium exposed to MBOCA[23]. This also suggests that conversion of MBOCA to the corresponding metabolites, mediated by P450, is necessary for the formation of DNA adducts.

Using the ^{32}P-postlabelling method, we next examined the formation of DNA adducts in yeast expressing recombinant P450s. Of numerous P450s, phenobarbital-inducible b and d types of rodent P450s[1] and 3A4 form of human P450[10] are considered to be most effective in catalyzing the *N*-hydroxylation of MBOCA. In this study, the yeast AH22/pABO1 strain expressing rabbit phenobarbital-inducible liver cytochrome P450 (CYP

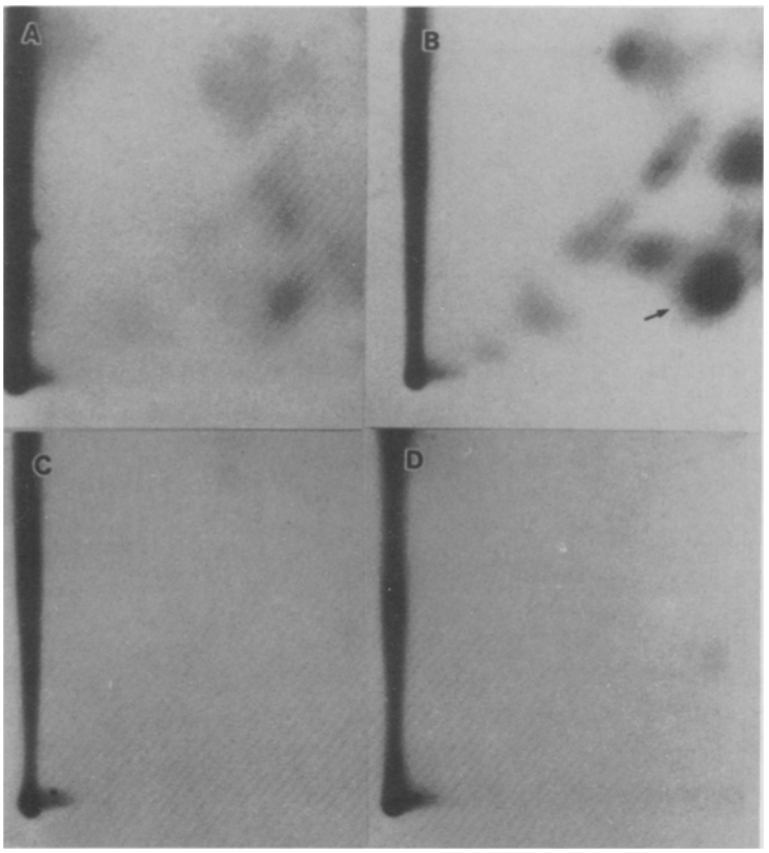

Figure 2. Detection of MBOCA-DNA adducts in AH22/pABO-1 expressing P450 2B5. Yeast AH22/pABO-1 cells were exposed to 120 μM MBOCA for 1 h (*A*) or for 4 h (*B*). DNA was also isolated from untreated AH22/pABO-1 (*C*) or from control yeast AH22 cells treated with 120 μM MBOCA for 4 h (*D*). An arrow points to the most prominent adduct formed. The extent of the DNA modification was 8 adducts/10^6 nucleotides.

2B5) was exposed to 120 μM MBOCA, and the genomic DNA was isolated and digested to mononucleotides. The adducted mononucleotides were ^{32}P-postlabelled and analyzed on a PEI-cellulose sheet, as described above. As shown in figure 2, nine adducts were observed in AH22/pABO-1 upon exposure to MBOCA for 1 h. The level of each adduct increased over a 4-h incubation. No adducts were detected in AH22/pABO-1 without MBOCA or in control yeast treated with MBOCA for 4 h (fig. 2, C and D). The RAL of the most prominent DNA adduct in yeast AH22/pABO-1 exposed to 120 μM MBOCA for 4 h was calculated to be 8×10^{-6}.

To examine the involvement of another form of P450 in the formation of MBOCA-DNA adducts, yeast AH22/pACCD-1 cells expressing rat 3-methylcholanthrene-inducible cytochrome P450 (CYP 1A1) were also exposed to 120 μM MBOCA. After isolating DNA, DNA adducts were analyzed as described above. As shown in figure 3, one major and two minor DNA adducts were formed in yeast exposed to MBOCA for 4 h. No DNA adducts were detected in yeast AH22/pACCD-1 without MBOCA exposure. The RAL of the major adduct in yeast AH22/pACCD-1 upon exposure to 120 μM MBOCA for 4 h was 2×10^{-6}. These results clearly show that recombinant CYP 1A1 or 2B5 activates MBOCA to the compounds which are directly bound to yeast genomic DNA. The potency of the formation of DNA adducts by CYP 2B5 was much greater than that by CYP 1A1.

Benzo(a)pyrene (B(a)P), a polycyclic aromatic hydrocarbon, is well known as an environmental carcinogen. B(a)P is activated by some types of P450s, although the activation process of B(a)P is considered to differ from that of aryl amine or MBOCA[24]. We next examined the formation of DNA adducts from B(a)P. When yeast AH22/pACCD-1 or AH22/pABO-1 was exposed to 100 μM B(a)P for 4 h, neither yeast AH22/pACCD-1 nor AH22/pABO-1 formed DNA adducts from B(a)P (data not shown). These results confirmed previous observations[24] that P450s alone cannot produce the final carcinogenic form of B(a)P, and that the contribution of epoxide hydrolase is required for the activation.

Discussion

We now have demonstrated for the first time DNA adduct formation in yeast expressing rodent P450s. In

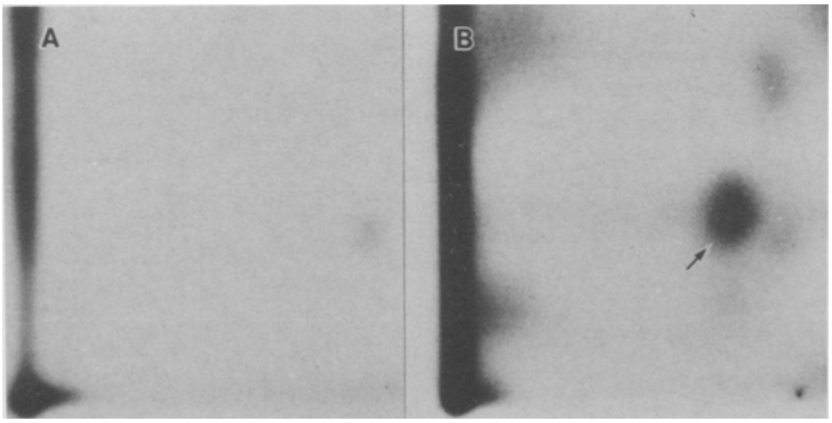

Figure 3. Detection of MBOCA-DNA adducts in yeast AH22/pACCD1 expressing P450 1A1. The genomic DNA was isolated from the yeast treated without (*A*) or with 120 μM MBOCA for 4 h (*B*). ^{32}P-Postlabelled adducts were analyzed. An arrow points to the major MBOCA-DNA adduct. The extent of the DNA modification is 2 adducts/10^6 nucleotides.

yeast AH22/pABO-1 expressing rabbit phenobarbital inducible P450 2B5, 9 MBOCA-derived DNA adducts were detected upon exposure to 120 μM MBOCA, thereby indicating that recombinant P450 2B5 is involved in the formation of multiple MBOCA-DNA adducts. MBOCA bound covalently to yeast genomic DNA in a time-dependent manner. The RAL in yeast AH22/pABO-1 upon exposure to MBOCA showed a value of 8×10^{-6}, a value that was much higher than that seen in the case of rats and dogs given MBOCA ($<10^{-7}$)[22]. Therefore, the yeast expression system is sufficiently sensitive to detect adduct formation. Silk et al.[22] reported that synthesized *N*-hydroxy derivatives of MBOCA directly reacted with DNA in vitro, after which the adduct, *N*-(deoxyadenosine-8-yl)4-amino-3-chlorobenzyl alcohol was formed. This study showed that incubation of MBOCA with S-9, followed by incubation with thymus DNA, led to the formation of more than 10 distinct adducts (fig. 1). The number of DNA adducts observed upon incubation of MBOCA with S-9 exceeds that seen in yeast AH22/pABO-1 exposed to MBOCA. Thus, several isoforms of P450s in S-9 may contribute to the activation of MBOCA. The structures of the modified nucleotides are the subject of ongoing investigations.

Exposure of yeast AH22/pACCD-1 expressing rat P450 1A1 to MBOCA also led to the formation of MBOCA-DNA adducts. The potential of P450 1A1 to form adducts is lower than that of P450 2B5. These findings support the observations of Butler et al.[1] that rat P450 1A subtypes, which catalyze the hydroxylation of various aryl amines, contribute less to the *N*-hydroxylation of MBOCA than do P450 2B subtypes. Yun et al.[10] showed that human P450 3A4 makes a major contribution to *N*-hydroxylation of MBOCA and that P450 2A6 has a minor role. We cannot compare catalytic properties of human and rodent P450s, but rabbit P450 2B5 does appear to play an important role in the activation of MBOCA.

Exposure of yeast AH22/pABO-1 or AH22/pACCD-1 to B(a)P did not lead to the formation of DNA adducts. Other investigators[25] have shown that three reactions involving P450s and microsomal epoxide hydrolase produce 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-B(a)P, which is the putative final mutagen. Although transfection of P450 1A1 into monkey kidney COS 1 cells led to the formation of B(a)P metabolite-DNA adducts[26], yeast cannot produce either a putative final carcinogen or a DNA-B(a)P metabolite adduct because yeast lacks epoxide hydrolase[24]. Transfection of human P450 2A2 and microsomal epoxide hydrolase cDNAs into human lymphoblastoid cells with high levels of native P450 1A1 activity was reported to make these cells more sensitive to the mutagenic effects of B(a)P[27]. Coexpression of P450 1A1 and epoxide hydrolase in yeast has been demonstrated, and the transformed cells exhibited an increased rate of formation of B(a)P-dihydrodiol[24]. Therefore, it will be interesting to determine whether DNA adducts from B(a)P are formed in the yeast coexpression system.

Expression of multiple P450s in yeast might be extremely valuable in defining the catalytic specificities of individual P450 forms and in the evaluation of chemical carcinogenesis. Since many other research groups have expressed heterologous P450s in yeast, there are many more types of P450s available in principle for such postlabelling studies. Based on the fact that the ^{32}P-postlabelling method permits the detection of distinct populations of carcinogen-DNA adducts, use of the postlabelling technique and the yeast P450 expression system will be valuable in the design of studies in molecular epidemiology, to examine the role of P450s in cancer susceptibility, and to develop P450-based tests for chemical carcinogenic potential. Establishment of these methods will to some extent obviate the need to use animals for such studies.

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