Jpn. J. Cancer Res. 86, 535–539, June 1995

Altered Expression of Hepatic CYP1A Enzymes in Rat Hepatocarcinogenesis

Masakuni Degawa,[1] Shin-ichi Miura, Kouichi Yoshinari and Yoshiyuki Hashimoto[2]

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aramaki-aza-Aoba, Aoba-ku, Sendai 980-77

Hyperplastic nodules of the liver were induced by treating male F344 rats with a combination of diethylnitrosamine and partial hepatectomy. The livers were examined for the expression of cytochrome P450 (CYP) enzymes, mainly CYP1A1 and CYP1A2; the amount and activity of the enzymes in the microsomes were assessed by enzymatic and immunological methods. Levels of CYP1A mRNAs were assayed by Northern blotting. In the liver bearing hyperplastic nodules, the total amount of microsomal CYP enzymes decreased to about 50% of the control. The microsomal activities for the CYP1A2-mediated activation of carcinogenic heterocyclic amines decreased to about 20% of the corresponding controls, in association with decreases in the levels of mRNA and protein of CYP1A2. Furthermore, the inducibility of CYP1A2 by CYP1A inducers such as 3-methoxy-4-aminoazobenzene and 3-methylcholanthrene was also decreased at the mRNA, protein and activity levels. On the other hand, CYP1A1 enzyme, which was undetectable in control rat liver, appeared in the liver bearing hyperplastic nodules, but its inducibility by a CYP1A inducer decreased slightly. The present findings indicated that individual CYP1A enzymes are differently regulated, and the expression of CYP1A2 is reduced preferentially in the liver bearing hyperplastic nodules.

Key words: Cytochrome P450 — CYP1A — Altered expression — Hepatocarcinogenic process — Rat liver

Carcinogenic susceptibilities of animals to hepatocarcinogenic aromatic amines such as food-derived heterocyclic amines[1–3] and 3-methoxy-4-aminoazobenzene[4] correlate closely with the activity and/or inducibility of CYP1A2[3] in the liver at an early stage or in the initiation phase of carcinogenesis. When the liver manifests premalignant changes, as represented by the development of hyperplastic nodules, the total amount of microsomal CYP enzymes decreases, and the substrate-specificity of the microsomes changes.[5–9] These findings suggest that each CYP enzyme may differently alter its expression level during the hepatocarcinogenic process. Indeed, in liver hyperplastic nodules developed by the method of Solt and Farber (DEN/AAF/PH/AAF),[10] the expression of CYP1A2 decreased, while CYP1A1 appeared.[5, 11] Recently, we have also found that treatment of rats with a hepatotoxic agent such as carbon tetrachloride,[12, 13] or ionic lead[14, 15] suppresses the expression of CYP1A enzymes, mainly CYP1A2, in the liver. These findings led us to propose that decrease in the expression of CYP1A2 is a phenotypical event characterizing liver damage, including (pre)neoplastic lesions.

To test the hypothesis, we produced hyperplastic liver nodules by treating rats with a combination of DEN and PH (DEN/DEN/PH/DEN) and examined the expression of CYP1A enzymes in the liver. We report herein that in the liver bearing hyperplastic nodules, CYP1A2 was decreased at the mRNA and protein levels, as well as in terms of catalytic activity, while CYP1A1 appeared.

MATERIALS AND METHODS

Chemicals Trp-P-2 and Glu-P-1 were kindly donated by Dr. Shudo, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo. DEN and MC were obtained from Wako Pure Chemical Industries, Osaka. 3-MeO-AAB was synthesized in our laboratory by the method of Miller *et al.*[16]

Development of liver hyperplastic nodules Male F344 rats were obtained from Japan SLC (Hamamatsu) and were used at 6 to 8 weeks of age after being kept in an air-conditioned room with free access to the basal diet, CE-2 (CLEA Japan). Rats were given an i.p. injection of DEN (200 mg/kg), and after 2 weeks they were given DEN (100 ppm) in drinking water for 2 weeks and then received PH (one-third of the total liver). After DEN feeding for a further 2 weeks, the rats were killed. Some rats received an i.p. injection of 3-MeO-AAB (0.22 mmol/kg) or MC (0.11 mmol/kg) in corn oil 24 h before they were killed.

[1] To whom correspondence should be addressed.

[2] Emeritus Professor of Tohoku University.

[3] Abbreviations used: CYP1A2, cytochrome P450IA2; CYP, cytochrome P450; DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; PH, partial hepatectomy; 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole hydrochloride; MC, 3-methylcholanthrene; CYP1A1, cytochrome P450IA1.

The liver of individual animals was cut into several blocks and used for the analyses of CYP enzymes at the mRNA, protein and activity levels.

Preparation of liver microsomes Livers were homogenized in 3 volumes (v/w) of 1.15% KCl and the microsomes were sedimented by differential centrifugations as described previously.[17] The microsome pellets were resuspended in 1.15% KCl for use. Amounts of protein and CYP in the microsomal fraction were assayed by the methods of Lowry *et al.*[18] and Omura and Sato,[19] respectively.

Microsomal CYP1A enzyme activity Activities of microsomal CYP1A enzymes were assessed by means of the Ames' bacterial mutation test using *Salmonella typhimurium* TA98 as a tester strain, with Glu-P-1 and Trp-P-2 (each 2 nmol/plate) as substrates for CYP1A enzymes.[12, 17]

Western blot analysis of CYP1A proteins CYP1A proteins in microsome preparations (20 or 40 μg/lane) were assayed by Western blotting with an anti-CYP1A monoclonal antibody, APL-2, which is reactive with both CYP1A1 and CYP1A2.[17]

Northern blot analysis of CYP1A mRNAs Total cellular RNAs of liver tissue, which had been stocked at -80°C, were isolated by homogenization of the tissues in 4 *M* guanidine thiocyanate followed by extraction with phenol/chloroform as described by Chomczynski and Sacchi.[20] The RNAs were denatured in 20 m*M* 3-(N-morpholino)propane sulfonate buffer, pH 7.0, containing 2.2 *M* formaldehyde, 15 m*M* sodium acetate, 1 m*M* EDTA and 50% formamide, at 60°C for 15 min.

Northern blot analyses for the CYP1A1 and CYP1A2 mRNAs were performed by the method of Reval *et al.*[21] using synthetic oligonucleotides, 5′-GAGATGCTGAGGACCAGAAGACCG-3′ and 5′-TTCACTTGGAGAAGCGTGGCCAGGCC-3′ as probes for CYP1A1 and CYP1A2 mRNAs, respectively.

RESULTS

Liver samples Hyperplastic nodules of the liver were induced in all rats treated with a combination of DEN and PH (DEN/DEN/PH/DEN). In the present experiments, we used liver samples without isolation of hyperplastic nodules for the analyses of CYP enzymes, because each hyperplastic nodule was too small to isolate. The hyperplastic nodules (placental-form glutathione S-transferase-positive foci) occupied about 15% of the total area of the liver as seen on histological sections (data not shown).

Activities of microsomal CYP1A enzymes Hepatic microsomes were prepared from normal rats (control; group 1) and the rats bearing hyperplastic nodules of the liver (group 2), and the total amount of CYP enzymes and the activity for the mutagenic activation of heterocyclic aromatic amines, Trp-P-2 and Glu-P-1 were determined. Trp-P-2 and Glu-P-1 were selected as substrates for CYP1A enzymes because they show different characters in CYP-mediated mutagenesis; Trp-P-2 is equally sensitive to both CYP1A1 and CYP1A2, but Glu-P-1 is more sensitive to CYP1A2, while both are relatively insensitive to other CYP enzymes.[12] Therefore, the ratio of microsomal activities for activation of Trp-P-2 and Glu-P-1 can be used as a measure of the relative amounts of CYP1A1 and CYP1A2 in microsomes.

The results of the assay are shown in Table I. In the group 2 rats, the total amount of microsomal CYP enzymes was about 50% of that in the corresponding

Table I. Changes in the Expression and Activity of Hepatic Microsomal CYP Enzymes during the Hepatocarcinogenic Process

Exp. group[a]	CYP inducer	CYP content (nmol/mg protein)	Microsomal activity (ratio to the control)		Substrate specificity (A/B)
			Substrates		
			Trp-P-2 (A)	Glu-P-1 (B)	
1	None (control)	0.57	1.0 (3800)[b]	1.0 (2700)	1.0
2	None	0.31[c]	0.3[d]	0.2[d]	1.5
1	3-MeO-AAB	0.67	14.6	14.1	1.0
2	3-MeO-AAB	0.39[c]	2.1[d]	1.5[d]	1.4
1	MC	0.60	62.0	26.6	2.3
2	MC	0.30[c]	40.6[c]	9.4[c]	4.3

a) Group 1, normal rats; group 2, rats bearing liver hyperplastic nodules (rats treated with a combination of DEN and PH).
b) Values shown in parentheses represent the number of revertant colonies/mg protein (mean of triplicate samples). In all samples, the SEMs were less than 15% of the means.
c, *d*) Significantly different from the corresponding group 1 rats; *c* $P<0.01$; *d* $P<0.001$.

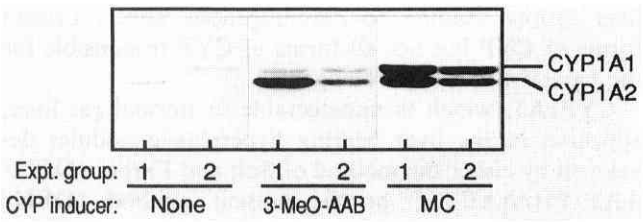

Fig. 1. Analysis of microsomal CYP1A protein in the liver by Western blotting. Microsome preparations were obtained from the pooled livers of three control rats (group 1) and three DEN-treated rats (group 2). An aliquot of the microsomes (20 and 40 μg protein/lane for MC-treated rats and others, respectively) in each group of rats was subjected to Western blotting. CYP1A proteins were immunostained with APL-2 monoclonal antibody reactive with CYP1A proteins.

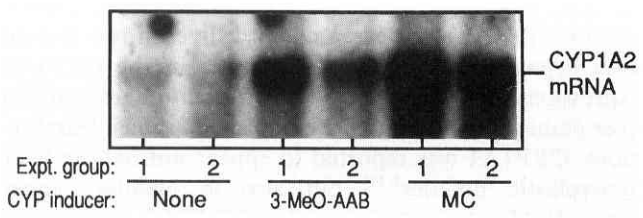

Fig. 2. Analysis of CYP1A2 mRNA in the liver by Northern blotting. Total RNA preparations were obtained from the pooled livers of three control rats (group 1) and three DEN-treated rats (group 2). An aliquot of the RNA preparation (20, 10 and 5 μg/lane for CYP inducer-untreated, 3-MeO-AAB-treated and MC-treated rats, respectively) in each group of rats was subjected to Northern blotting using a synthetic oligonucleotide probe for CYP1A2 mRNA.

controls (group 1 rats). Without CYP inducer treatment, microsomal activities for Trp-P-2 and Glu-P-1 activation in the group 2 rats were 29% and 22% of those of the corresponding group 1 rats, respectively.

In both groups of rats, treatment with 3-MeO-AAB, a selective inducer of CYP1A2,[22] resulted in a significant increase of the activities of hepatic microsomes for the activation of both Trp-P-2 and Glu-P-1, although the total amount of CYP enzymes was not affected by the treatment. Likewise, treatment with MC, a CYP1A (1A1 and 1A2) inducer,[12, 22] increased the microsomal activity in both groups of rats. However, the induction level of the enzyme activity towards both amines, especially Glu-P-1, by MC was lower in the group 2 rats than in the group 1 rats.

Regardless of CYP inducer treatment, the microsomes of group 2 rats gave a higher Trp-P-2/Glu-P-1 activation ratio than did the corresponding group 1 rats, indicating

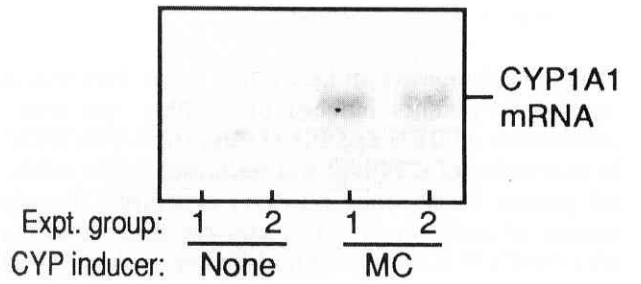

Fig. 3. Analysis of CYP1A1 mRNA in the liver by Northern blotting. Total RNA preparations were obtained from the pooled livers of three control rats (group 1) and three DEN-treated rats (group 2). An aliquot of each RNA preparation (25 μg/lane) was subjected to Northern blotting using a synthetic oligonucleotide probe for CYP1A1 mRNA.

that the substrate-specificities of the microsomes were different among the two groups of rats.

Expression of CYP1A enzyme proteins Levels of hepatic CYP1A1 and CYP1A2 proteins in the rats of groups 1 and 2 were comparatively examined by Western blotting using a monoclonal antibody, APL-2. The results are depicted in Fig. 1. A component with a molecular mass of 54,000 corresponding to CYP1A2 was detected from the group 1 rats (control), but not the group 2 rats. On the other hand, a component with a molecular mass of 56,000 corresponding to CYP1A1, was detected from the group 2 rats but not the group 1 rats.

Treatment of the rats with either 3-MeO-AAB or MC resulted in an increase of the components corresponding to CYP1A1 and CYP1A2 in both group 1 and group 2 rats. However, the increased levels of the two components, especially CYP1A2, induced by 3-MeO-AAB and MC were lower in the group 2 rats than in the corresponding group 1 rats.

Expression of CYP1A mRNAs Total RNA was prepared from the livers of each group of rats and the levels of CYP1A mRNAs were determined by Northern blot analysis using oligonucleotides as probes. As shown in Figs. 2 and 3, CYP1A2 mRNA, but not CYP1A1 mRNA, was detected in rats of both groups 1 and 2. The level of CYP1A2 mRNA, however, was lower in the group 2 rats than in the group 1 rats.

Treatment with either 3-MeO-AAB or MC increased the level of CYP1A2 mRNA in both groups of rats (Fig. 2). Inducibility of CYP1A2 mRNA was lower in the group 2 rats than in the group 1 rats. Likewise, MC treatment resulted in an increase of the level of CYP1A1 mRNA in both groups of rats, whereas the inducibility of CYP1A1 mRNA was slightly lower in the group 2 rats than in the group 1 rats (Fig. 3).

DISCUSSION

We have demonstrated herein that in the liver bearing hyperplastic nodules induced by treating rats with a combination of DEN and PH (DEN/DEN/PH/DEN), the expression of CYP1A2 was decreased at the mRNA and protein levels, while CYP1A1 appeared. The total amount of microsomal CYPs and the microsomal activity for CYP1A2-mediated mutageneses of heterocyclic aromatic amines were significantly decreased in the liver bearing hyperplastic nodules.

Buchmann *et al.*[7,8] showed by immunohistochemical methods that the expression of some CYP enzymes, especially CYP1A2, was suppressed in DEN-induced (pre)neoplastic lesions in rat liver, and this has been confirmed in the present experiment by demonstrating the decrease of CYP1A2 at the level of mRNA as well as protein. Inducibility of CYP1A2 in the liver bearing hyperplastic nodules was also demonstrated to decrease in terms of the mRNA and protein levels. On the other hand, CYP1A1 was expressed in the liver bearing hyperplastic nodules, although the induction of CYP1A1 by MC was decreased at the mRNA and protein levels.

These changes in the expression of CYP1A1 and CYP1A2 were similar to those observed previously in the liver bearing hyperplastic nodules produced by the method of Solt and Farber.[5,11] For the liver hyperplastic nodules developed by either the present method (DEN/DEN/PH/DEN) or the method of Solt and Farber (DEN/AAF/PH/AAF),[5,11] decrease in the expression of CYP1A2 might be favorable for escape from contact with cytotoxic metabolites of DEN and/or AAF, because CYP1A2 efficiently mediates the bioactivations of AAF[23] and DEN.[24] However, the expression of CYP2E1, which is one of the CYP enzymes responsible for bioactivation of DEN,[25] was not suppressed in the liver bearing hyperplastic nodules developed by the present method with a combination of DEN and PH (data not shown). These results suggest that development of liver lesions leading to carcinogenesis affects certain forms of CYP but not all forms of CYP responsible for the bioactivation of carcinogens.

CYP1A1, which is undetectable in normal rat liver, appeared in the liver bearing hyperplastic nodules developed by either the method of Solt and Farber (DEN/AAF/PH/AAF)[5,11] or the present method (DEN/DEN/PH/DEN). Appearance of CYP1A1 in the DEN/AAF/PH/AAF system might be attributable to the ability of AAF[25] to induce CYP1A enzymes. On the other hand, since DEN has no capacity for inducing CYP1A1 in normal rat liver, the mechanism of appearance of CYP1A1 in the DEN/DEN/PH/DEN system remains unclear.

Nevertheless, the present findings suggested that the expressions of CYP1A1 and CYP1A2 would be controlled by different host factor(s) and confirmed that the expression of CYP1A2 was preferentially suppressed in the liver bearing hyperplastic nodules. Since the expression of CYP1A2 also decreased in the liver of rats treated with hepatotoxic agents,[12–15] decrease in the CYP1A2 expression might be a phenotypical event characterizing liver damage, including (pre)neoplastic lesions. Furthermore, CYP1A1 was reported to appear not only in liver hyperplastic nodules[5,11] but also in bladder cancer tissues[26] of rats, suggesting that CYP1A1 might be a marker enzyme for (pre)neoplastic alteration of several tissues, such as liver and bladder in the rat.

Further studies on the expression of CYP enzymes in the liver should provide important information for the diagnosis and drug-therapy of liver diseases, including cancer.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received January 13, 1995/Accepted March 20, 1995)

REFERENCES

1) Hashimoto, Y., Degawa, M., Kojima, M. and Hishinuma, T. Induction of carcinogen activation enzyme(s) by feeding of a carcinogenic tryptophan pyrolysate correlates to sex difference in the carcinogenesis of the mouse. *Gann*, **73**, 508–510 (1982).
2) Degawa, M., Kojima, M., Hishinuma, T. and Hashimoto, Y. Sex-dependent induction of hepatic enzymes for mutagenic activation of a tryptophan pyrolysate component, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, by feeding in mice. *Cancer Res.*, **45**, 96–102 (1985).
3) Degawa, M., Hishinuma, T., Yoshida, H. and Hashimoto, Y. Species, sex and organ differences in induction of a cytochrome P-450 isozyme responsible for carcinogen activation: effects of dietary hepatocarcinogenic tryptophan pyrolysate components in mice and rats. *Carcinogenesis*, **8**, 1913–1918 (1987).
4) Degawa, M., Kojima, M. and Hashimoto, Y. Species difference between rats and mice in activities of enzymes activating aromatic amines: effect of dietary 3-methoxy-4-aminoazobenzene. *Gann*, **75**, 966–975 (1984).
5) Degawa, M., Miura, S. and Hashimoto, Y. Expression and induction of cytochrome P450 isozymes in hyperplastic nodules of rat liver. *Carcinogenesis*, **12**, 2151–2156 (1991).

6) Cameron, R., Sweeney, G. D., Jones, K., Lee, G. and Farber, E. A relative deficiency of cytochrome P-450 and arylhydrocarbon [benzo(*a*)pyrene] hydroxylase in hyperplastic nodules induced by 2-acetylaminofluorene in rat liver. *Cancer Res.*, **36**, 3888–3893 (1976).
7) Buchmann, A., Kuhlmann, W., Schwarz, M., Kunz, W., Wolf, C. R., Moll, E., Friedberg, T. and Oesch, F. Regulation and expression of four cytochrome P-450 isoenzymes, NADH-cytochrome P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase in preneoplastic and neoplastic lesions in rat liver. *Carcinogenesis*, **6**, 513–521 (1985).
8) Buchmann, A., Schwarz, M., Schmitt, R., Wolf, C. R., Oesch, F. and Kunz, W. Development of cytochrome P-450-altered preneoplastic and neoplastic lesions during nitrosamine-induced hepatocarcinogenesis in the rat. *Cancer Res.*, **47**, 2911–2918 (1987).
9) Conney, A. H. Induction of microsomal cytochrome P-450 enzymes: the first Berardie lecture at Pennsylvania State University. *Life Sci.*, **39**, 2493–2518 (1986).
10) Solt, D. and Farber, E. A new principle for the analysis of chemical carcinogenesis. *Nature*, **263**, 701–703 (1976).
11) Hashimoto, Y., Miura, S. and Degawa, M. Altered expression of cytochrome P-450 isozymes by aromatic amine carcinogens in experimental animals: correlation with hepatocarcinogenesis. *In* "Xenobiotics and Cancer," ed. L. Ernster, H. Esumi, Y. Fujii, H. V. Gelboin, R. Kato and T. Sugimura, pp. 129–136 (1991). Japan Scientific Societies Press, Tokyo/Taylor & Francis Ltd., London.
12) Degawa, M., Ueno, H., Miura, S., Ohta, A. and Namiki, M. A simple method for assessment of rat cytochrome P-448 isozymes responsible for the mutagenic activation of carcinogenic chemicals. *Mutat. Res.*, **203**, 333–338 (1988).
13) Degawa, M., Mikami, K., Namiki, M. and Hashimoto, Y. Inhibition of the induction and activity of hepatic P450IA isozymes by *in vivo* administration of carbon tetrachloride to rats. *Biol. Pharm. Bull.*, **16**, 1248–1250 (1993).
14) Degawa, M., Arai, H., Miura, S. and Hashimoto, Y. Preferential inhibitions of hepatic P450IA2 expression and induction by lead nitrate in the rat. *Carcinogenesis*, **14**, 1091–1094 (1993).
15) Degawa, M., Arai, H., Kubota, M. and Hashimoto, Y. Ionic lead, a unique metal ion as an inhibitor for cytochrome P450IA2 (CYP1A2) expression in rat liver. *Biochem. Biophys. Res. Commun.*, **200**, 1086–1092 (1994).
16) Miller, J. A., Miller, E. C. and Finger, G. C. Further studies on the carcinogenicity of dyes related to 4-aminoazobenzene. The requirement of an unsubstituted 2-position. *Cancer Res.*, **17**, 387–398 (1957).
17) Degawa, M., Tanimura, S., Agatsuma, T. and Hashimoto, Y. Hepatocarcinogenic heterocyclic aromatic amines that induce cytochrome P-448 isozymes, mainly cytochrome P-448H (P450IA2), responsible for mutagenic activation of the carcinogens. *Carcinogenesis*, **10**, 1119–1122 (1989).
18) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
19) Omura, T. and Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.*, **239**, 2370–2378 (1964).
20) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
21) Reval, P., Iversen, P. L. and Bresnick, E. Induction of cytochromes P450IA1 and P450IA2 as determined by solution hybridization. *Biochem. Pharmacol.*, **41**, 1719–1723 (1991).
22) Degawa, M., Kojima, M., Sato, Y. and Hashimoto, Y. Induction of a high spin form of cytochrome P-448 in the liver microsomes by 4-aminoazobenzene derivatives. *Biochem. Pharmacol.*, **35**, 3565–3570 (1986).
23) Goldstein, J. A., Weaver, R. and Sundheimer, D. W. Metabolism of 2-acetylaminofluorene by two 3-methylcholanthrene-inducible forms of rat liver cytochrome P-450. *Cancer Res.*, **44**, 3768–3771 (1984).
24) Yamazaki, H., Oda, Y., Funae, Y., Imaoka, S., Inui, Y., Guengerich, F. P. and Shimada, T. Participation of rat liver cytochrome P450 2E1 in the activation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine to products genotoxic in an acetyltransferase-overexpressing *Salmonella typhimurium* strain (NM2009). *Carcinogenesis*, **13**, 979–985 (1992).
25) Åström, A., Birberg, W., Pilotti, A. and DePierre, J. W. Induction of different isozymes of cytochrome P-450 and microsomal epoxide hydrase in rat liver by 2-acetylaminofluorene and structurally related compounds. *Eur. J. Biochem.*, **154**, 125–134 (1986).
26) Masuko, T., Sugahara, K., Nakajima, S. and Hashimoto, Y. Altered expression of immunohistochemically detected cytochrome P-450 component(s) in nitrosamine-induced rat urinary bladder lesion. *Jpn. J. Cancer Res.*, **78**, 840–845 (1987).