Tumor Res. **18 (Special Issue)**, S 49–S 56 (1983)

Sequential Analysis of Drug Metabolizing Enzymes During Initiation and Promotion in Chemical Hepatocarcinogenesis

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SUMMARY

Changes of drug metabolizing enzymes were investigated in the liver through the process of initiation and promotion by the method of Solt and Farber. The content of cytochrome P-450 decreased on the lst week after the injection of diethylnitrosamine (DEN) (200 mg/kg) but gradually increased to normal level by the 3rd to the 4th week. On the other hand, induction of epoxide hydrase, glutathione S-transferase and glutathione S-epoxide transferase could be seen in cytosol or microsome from the rats killed on the 3rd week in this experiment or on the lst week after the administration of 0.02% 2-fluorenyacetamide (2FAA). It was considered that the induction of epoxide hydrase should be due to the effect of DEN in addition to 2FAA and also that of glutathione S-transferase and glutathione S-epoxide transferase was due to mainly to 2FAA. In the liver killed on the 4th and 5th weeks, hyperplastic nodules were observed sporadically and the immunofluorescence study indicated specific localization of epoxide hydrase or glutathione S-transferase E in these nodules, while hyperplastic nodules were negative islands for cytochrome P-450, as compared with the surroundings.

Key words: Drug metabolizing enzyme, Cytochrome P-450, Epoxide hydrase, Glutathione S-transferase, Glutathione S-epoxide transferase, Glutathione S-transferase E, Hepatocarcinogenesis

INTRODUCTION

In the experimental hepatocarcinogenesis, it has been focussed that the hyperplastic nodules could be ultimate precancerous lesion of the hepatoma (Farber, 1973).

Although preneoplastic antigen (PN antigen) was discovered in the hyper-

plastic liver nodules and hepatoma induced by 2FAA, 3'-methyl-4-dimethylazobenzene (DAB), ethionine, DEN or Diethylnitrosamine (Okita *et al.*, 1975), Levin *et al.* (1978) reported the immunochemical identification of PN antigen with epoxide hydrase which is one of the microsomal drug metabolizing enzymes. However, recently it was concluded that epoxide hydrase was not always identical with PN antigen (Ogine *et al.*, 1982).

Glutathione S-transferase is composed by multiforms of A, B, C, D, E and AA in rat liver (Habig *et al.*, 1974) and these glutathione S-transferase have affinity to epoxide. Moreover, it is known that glutathione S-epoxide transferase is induced by phenobarbital and 3-methylcholanthrene (Mukhtar and Bresnick, 1976) as well as the other microsomal drug metabolizing enzymes.

Therefore, It is the purpose for this communication to observe the changes of these enzymes in the process of initiation and promotion appearing in the liver during hepatocarcinogenesis.

MATERIALS AND METHODS

Animals

Male Wistar rats Weighing 150 g were obtained from Kuroda Animal Farm (Kumamoto, Japan). The animals were starved overnight and killed by decapitation in every week according to the experimental protocol of the initiation and promotion by Solt and Farber (1976) (Fig. 1).

Chemicals

The chemicals purchased for this study were as follows: styrene oxide [7-^{3}H] (specific activity : 9.1 mCi/mmol) from the Radiochemical Centre (Amersham, U. K.); styrene oxide from Katayama Chemical Co. (Tokyo); 1-chloro-2, 4-dinitrobenzene from Nakarai Chemical Co. (Kyoto); glutathione (reduced form) from Sigma Chemical Co. (St. Louis, U. S. A.).

Preparations of microsome and supernatant fraction

The livers were homogenized in TKMS buffer (250 mM sucrose, 24 mM KCI, 5 mM Tris HCI pH 7.6). The homogenate was centrifuged at 10,000×g for 20 min and the supernatant fraction was centrifuged for a further 90 min at 100,000×g. Microsomes were sonicated in TKMS buffer by Sonifer model 200 (Smithkline, U. S. A.) for the following enzyme assay.

Enzyme assay

Cytochrome P-450 content was determined in microsome by the procedure of Omura and Sato (1964). Epoxide hydrase activity was measured in microsome using the method of Oesch *et al.* (1971) with [7-^{3}H] styrene oxide as a substrate. Glutathione S-transferase activity was measured in cytosol fraction with 1-chloro-2, 4-dinitrobenzene as a substrate by the procedure of Habig *et al.* (1974).

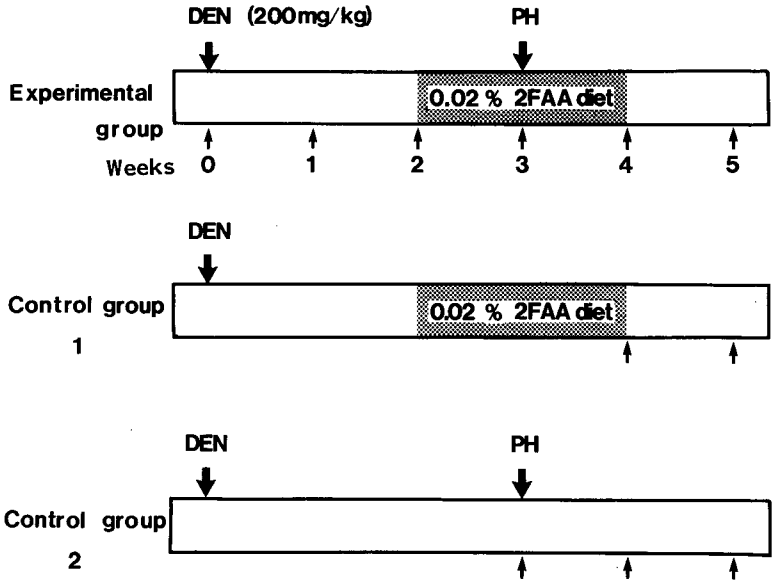

Fig. 1 Experimental design according to Solt and Farber and its controls.

Glutathione S-epoxide transferase was quantitated in cytosol using the method of Pacifici *et al.* (1981) with [7-^{3}H] styrene oxide. Protein concentration was determined by the method of Lowry *et al.* (1951). The preparation of cytochrome P-450, epoxide hydrase and glutathione S-transferase E

Microsomal cytochrome P-450 was purified from phenobarbital treated rat by the method of West *et al.* (1979). Purification of microsomal epoxide hydrase was described in elswhere (Ogino *et al.*, 1982). Glutathione S-transferase E was purified with apparent homogenity from cytosol of phenobarbital treated rat liver using the 3 steps of the chromatography of DEAE-cellulose, CM-cellulose and glutathione affinity.

Antiserum

Antisera to purified cytochrome P-450, epoxide hydrase and glutathione S-transferase E were produced in the rabbits after immunization of each enzyme with Freund's complete adjuvant.

Immunofluorescence

The livers from the rats killed in every week owing to experimental protocol were fixed in precooled 95% ethanol containing 1% glacial acetic acid for paraffin embedding method as described by Sainte-Marie (1962).

RESULTS

The changes of cytochrome S-450, epoxide hydrase, glutathione S-transferase and glutathione S-epoxide transferase on the several critical points were shown in Fig. 2. On the lst week after a single i. p. injection of DEN (200 mg/kg), epoxide hydrase and glutathione S-transferase slightly increased, but glutathione S-epoxide transferase remained at a normal level, while cytochrome P-450 dramatically decreased. On the 3rd week from the beginning of this experiment (the lst week after administration of 0.02% 2FAA mixed in basal diet), epoxide hydrase, glutathione S-transferase and glutathione S-epoxide transferase declined rapidly; at this stage, sporadically the early focal preneoplastic nodules were seen in the liver. On the 5th week, epoxide hydrase and glutathione S-transferase increased remarkably, but on the contrary, cytochrome P-450 decreased to the value of the lst week.

Control study was performed in two groups (1. DEN+2FAA, 2. DEN+PH). Regarding the control study 1, the levels of epoxide hydrase and cytochrome P-450 reached to the maximal level by the 4th week and glutathione S-epoxide transferase

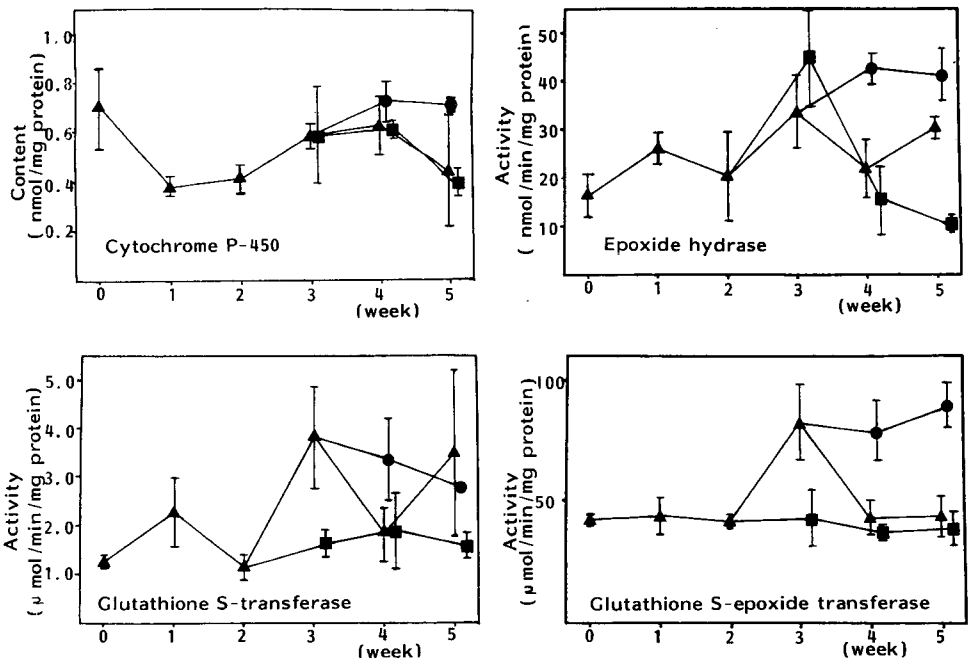

Fig. 2 Changes of hepatic drug metabolizing enzymes.
experimental group: (▲); control group 1: (●); control group 2: (■)

reached the maximal level by the 5th week. While glutathione S-transferase declined gradually from the maximal level of the 3rd week. In the control study 2, glutathione S-transferase and glutathione S-epoxide transferase remained at normal levels through the experiment, but epoxide hydrase and cytochrome P-450 increasingly reached to the maximal level equal to the level of experimental group by the 4th week ; the enzymatic level rapidly decreased to the normal values on the 5th week.

Immunofluorescence analysis of cytochrome P-450, epoxide hydrase and glutathione S-transferase E was shown in Fig. 3. Immunofluorescence study using

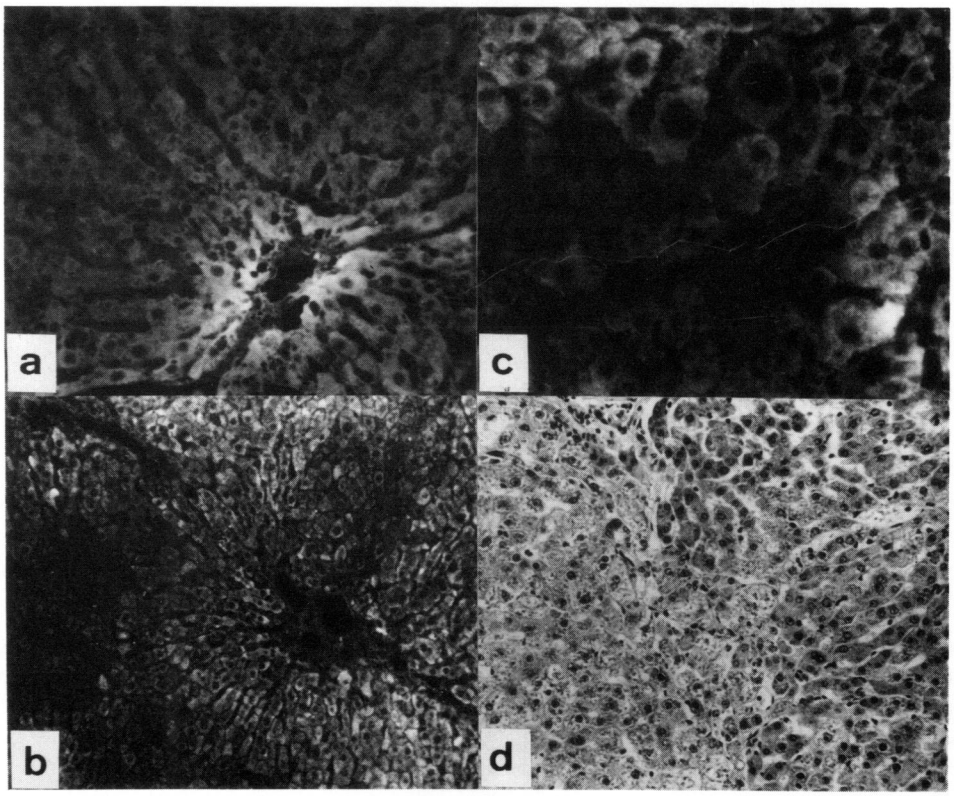

Fig. 3 **A** Immunofluorescence study of cytochrome P-450, epoxide hydrase and glutathione S-transferase E on the lst week of the experiment.' (a) Immunofluorescence of cytochrome P-450 (×32). Specific fluorescence is seen in the hepatocyte around the central vein. (b) Immunofluorescence of epoxide hydrase (×32). Specific fluorescence is seen in the hepatocyte around the portal tract. (c) Immunofluorescence of glutathione S-transferase E (×175). A specific fluorescence is seen in the hepatocyte around the portal tract. The large cell has also specific fluorescence in the cytoplasm. (d) Hematoxylin-eosin stain (×32). Hyperbasophilic hepatocytes are seen around the portal tract.

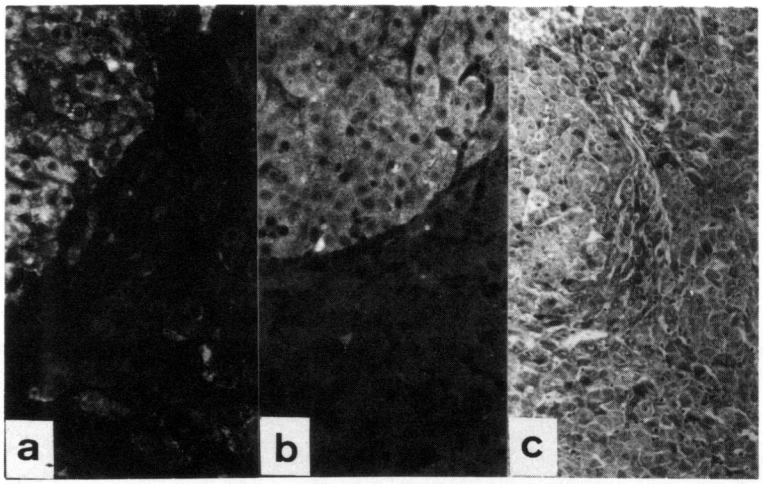

Fig. 3 B Immunofluorescence study of epoxide hydrase and glutathione S-transferase E on the 4th week of the experiment. (a) Immunofluorescence of epoxide hydrase (×72). Specific fluorescence is observed in early focal preneoplastic nodule. (b) Immunofluorescence of glutathione S-transferase E (×72). Specific fluorescence is seen in early focal preneoplastic nodule. (c) Hematoxylin-eosin stain (×32). Notes early focal preneoplastic nodule.

anti cytochrome P-450 serum revealed negative fluorescence in early focal preneoplastic nodule and feeble fluorescence around the lesion on the 5th week of the experiment, but on the lst week, there was slight fluorescence in the hepatocyte around the portal tract. The localization of epoxide hydrase was observed in the hepatocyte around the portal tract on the lst to 3rd week and specific fluorescence was observed predominantly in the early focal preneoplastic nodules in the liver killed on the 4th week. A specific fluorescence of glutathione S-transferase E was observed in the hepatocyte around the portal tract in the liver on the lst week after treatment of DEN and also in the early focal preneoplastic nodules seen on th 4th weeek, in the same manner of the epoxide hydrase.

DISCUSSION

Using the experimental design by Solt and Farber (1976), it was demonstrated that cytochrome P-450 of mixed function oxydase system as phase 1 reaction decreased on the lst week, while the successive drug metabolizing enzymes of epoxide hydrase, glutathione S-transferase and glutathione S-epoxide transferase increased on the 3rd week of the experiment and that the induction of these enzymes on the 3rd week was suppressed transiently by a procedure of partial hepatectomy. However, these enzymes except cytochrome P-450 had a tendency to increase by

the 5th week.

It is well known that in hyperplastic nodule and hepatoma induced by continuous feeding of 2FAA, induction of PN antigen and epoxide hydrase can be seen. On the other hand, Åström and Depierre (1981) clarified the induction of epoxide hydrase and glutathione S-transferase in the rat liver after short term treatment with 2FAA. Therefore, it may be considered that the high levels of epoxide hydrase, glutathione S-transferase and glutathione S-epoxide transferase come from the direct interaction of 2FAA.

According to Solt and Farber (1976), the purpose to treat the rats with 2FAA for one week before partial hepatectomy is to create a mitoinhibitory effect to normal hepatocyte, and partial hepatectomy stimulates the proliferation of putative preneoplastic hepatocytes. Soon after partial hepatectomy, the levels of enzymes decreased temporally, but raised again. We do not know the mechanism of inhibited enzyme induction after partial hepatectomy.

The livers killed on the 4th and 5th week showed the presence of hyperplastic nodules. Immunofluorescence study of epoxide hydrase and glutathione S-transferase E indicated the significant localizations in the hyperplastic nodules. It is documented that the hyperplastic foci appears arround the portal tract. Therefore, immunohistochemical findings of epoxide hydrase and glutathione S-transferase E may support that the initiated hepatocytes, which from hyperplastic nodules, originate from the arround portal tract.

The drug metabolizing enzymes was determined biochemically through the experiment. However, on the contrary with immunohistochemistry, these changes show biochemically too complex to analyze these in association with initiation and promotion of the hepatocarcinogenesis.

REFERENCES

ÅSTRÖM, A. and DEPIERRE, J.: Characterization of the induction of drug metabolizing enzymes by 2-acetylaminofluorene. Biochim. Biophys. Acta **673,** 225-233 (1981).

FARBER, E.: Hyperplastic liver nodule. Methods Cancer Res. **7,** 345-375 (1973).

HABIG, W. H., PABST, M. J., and JAKOBY, W. B.: Glutathione S-transferases. J. Biol. Chem. **249,** 7130-7139 (1974).

LEVIN, W., LU, A. Y. H., THOMAS, P. E., RYAN, D., KIZER, D. E. and GRIFFIN, M. J.: Identification of epoxide hydrase as the preneoplastic antigen in rat liver hyperplastic nodules. Proc. Natl. Acad. Sci. USA **75,** 3240-3243 (1978).

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. **193,** 265-275 (1951).

MUKHTAR, H. and PRESNICK, E.: Effects of phenobarbital and 3-methycholanthrene administration on glutathione-S-epoxide transferase activity in rat liver. Biochem. Pharmacol. **25,** 1081-1084 (1976).

OESCH, F., JERINA, D. M. and DALY, J.: A radiometric assay for hepatic epoxide hydrase activity with [7-^{3}H] styrene oxide. Biochim. Biophys. Acta **227,** 685-691 (1971).

OGINO, M., OKITA, K., TSUBOTA, W., NUMA, Y., KODAMA, T. and TAKEMOTO, T.: Some biochemical properties of the preneoplastic antigen in rat liver hyperplastic nodules. Gann **73,** 349-353 (1982).

OKITA, K., KLIGMAN, L. and FARBER, E.: A new common marker for premalignant and malignant hepatocytes induced in the rat by chemical carcinogenesis. J. Natl. Cancer Inst. **54,** 199-202 (1975).

OMURA, T. and SATO, R.: The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem. **230,** 237-2378 (1964).

SOLT, D. and FARBER, E.: New principle for the analysis of chemical carcinogenesis. Nature **263,** 701-703 (1976).

SAINT-MARIE, G.: A Paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem. **10,** 250-256 (1962).

WEST, S. D., HUANG, M. T., MIWA, G. T. and and LU, A. Y. H.: A simple and rapid procedure for the purification of phenobarbital inducible cytochrome P-450 from rat microsomes. Arch. Biochem. Biophys. **193,** 42-50 (1979).