Modifying Effects of Clofibrate on the Phenotype of Glutathione S-transferase Isoenzymes (YaYa, YbYb, YcYc, YkYk and YpYp) during Chemical Hepatocarcinogenesis in Rat Livers

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SUMMARY

The modifying effects of clofibrate (ethyl-a-p-chlorophenoxyisobutylate) on the phenotype of glutathione S-transferase (GST) isoenzymes (YaYa, YbYb, YcYc, YkYk and YpYp) were investigated by using immunohistochemical techniques. Among a family of GST isoenzymes, the YcYc alone remarkably increased in epithelial cells of bile ducts of rat livers treated with clofibrate. In altered foci and neoplastic nodules under chemical hepatocarcinogenesis, the YpYp isoenzyme which is highly inducible, is surely effective for determining the localization of these lesions, but the other YaYa, YbYb, YcYc and YkYk-positive foci could also be clearly distinguished from the surrounding hepatocytes. And some of neoplastic lesions showed positive staining not for YpYp, but for YaYa, YbYb, YcYc and YkYk. Studies on the modifying effects of clofibrate under chemical hepatocarcinogenesis indicated that not only the expression of YpYp but alst the other GST isoenzymes were suppressed in neoplastic nodules.

The possible roles of GST isoenzymes in the liver cells and the possible mechanisms for the suppression of these isoenzymes by hypolipidemic agents treatment during chemical hepatocarcinogenesis are discussed.

Abbreviations:
Clofibrate (ethyl-a-p-chlorophenoxyisobutylate), GSH (glutathione), GSH-Px (glutathione peroxidase), CAT (catalase), SOD (superoxide dismutase), GSTs (glutathione S-transferases), BD (basal diet), DEN (diethylnitrosamine), 2-AAF (2-acetylaminofluorene), DAB (3, 3'-diaminobezine tetrahydro-chloride), gamma-GT (gamma-glutamyl transpeptidase).

Key words: Glutathione S-transferase isoenzymes, Clofibrate, Chemical hepatocarcinogenesis, Bile ducts, Lipid peroxidation

INTRODUCTION

Clofibrate (ethyl-a-p-chlorophenoxyisobutylate), widely known hypolipidemic agent and peroxisome prolifearator(1, 8, 9, 20), induces altered foci, neoplastic nodules and hepatocellular carcinomas in rat livers during chemical hepatocarcinogenesis(26, 27). The mechanism(s) by which these drugs such as clofibrate induce neoplastic lesions in rat liver, have been assumed to involve an excessive generation of oxygen radicals and hydrogen peroxides by β-oxidation of lipids in relation to peroxisome proliferation(5, 27), because none of these agents cause detectable genotoxicity in various assays including the Salmonella mutagenesis assay(29, 33, 34).

While superoxide dismutase (SOD)(EC 1. 15. 1. 1), catalase (CAT) (EC 1. 11. 1. 6) and glutathione peroxidase (GSH-Px) (EC 1. 11. 1. 9) which consist of Se-dependent and Se-independent enzymes, in the enzymes involved in lipid metabolism are known to have highly significant functions of destroying and removing active oxygen, hydrogen peroxides and fatty acid hydroperoxides that are produced in livers during chemical hepatocarcinogenesis(18).

Ther are glutathione S-transferases (GSTs)(EC 2. 5. 1. 18) in another enzyme system which deal with lipid hydroperoxides. GSTs are a family of enzymes that have a capacity to bind a large number of lipophilic substances and xenobiotics including carcinogens(6, 22), and that have an intracellular transport function(11, 12). Multiple forms of the rat enzymes show dimeric combinations of subunit types designated as YaYa, YbYb, YcYc, YkYk, YnYn and YpYp (3, 14, 18), and it has been suggested that each enzyme may have specific functions according to separate genetic regulation.

Recently, evidences have been accumulated to show that clofibrate noncompetitively inhibits the GST activity in rat livers(1, 9), and YpYp (GST-P) is suppressed in neoplastic lesions of rat livers by hypolipidemic agents treatments (25), otherwise is highly inducible during chemical hepatocarcinogenesis (21, 31, 32). Therefore, it is important to analyze the changes on localization and expression of GST isoenzymes under clofibrate treatment in order to understand its roles, because some forms of GST isoenzymes possess Se-independent GST-Px activity, and use a variety of organic hydroperoxidases such as cumene hydroperoxidases as substrates(5, 7). However, very little information is available as to the changes of expression and localization of each GST isoenzyme

which exist in normal rat livers by administration of clofibrate.

In this study, immunohistochemical analyses were carried out to elucidate change of the phenotypic expression and localization of GST isoenzymes in rat livers treated with clofibrate alone and together with clofibrate during the chemical hepatocarcinogenesis.

MATERIALS AND METHODS

Purification of GST isoenzymes from rat kidney

Glutathione S-transferases (GSTs) were prepared as described previously (11, 12, 13). In brief, cytosol (105,000×g supernatant) prepared from kidneys of male F344 Du/Crj rats (Charles River Co., Atsugi) in 10 mM Tris HCl/1.0 mM EDTA pH 8.0 was applied to a Sephadex G100 column. The fractions containing GST activity were pooled, and applied to a lysyl-GSH affinity column(19). The multiple forms of GSTs eluted with 10 mM S-methyl glutathione (GSH) were resolved by a chromatofocusing column using PBE118 and 94(13). Isoenzymes were identified by their subunit structure on SDS-polyacrylamide gel electrophoresis and catalytic properties with selected substrates(13, 18).

Preparation of antibodies

Anti-rat kidney GST (YaYa, YcYc, Yb_1Yb_1, Yb_2Yb_2, YkYk and YpYp) antisera were raised in rabbit as described previously(11, 13). Only anti-Yb_1Yb_1 antibody was cross reactive with a subunit Yb_2Yb_2, and other antibodies were all specific for each corresponding isoenzyme. IgG fractiones of the antisera were prepared by using MAPS II Kit.

Treatment of animals with carcinogens

Three types of hepatocarcinogens, dietylnitrosamine (DEN, Tokyo Kasei Indust., Tokyo), 2-acetylaminofluorene (2-AAF, Tokyo Kasei Indust., Tokyo) and clofibrate (Shizuoka Caffeine Co., Shizuoka), were used in the present study.

Male F344 rats aged 6 weeks were maintained on basal diet (BD) (MF, Oriental Yeast Coyo) and tap water ad Iibitum. These rats were divided into four groups. Rats except group 1 and 2 were intraperitonealy injected with dietylnitrosamine DEN in 0.9% saline at a dose of 200 mg/kg body weight, and fed on BD for 2 weeks. Then, they were given a diet containing 0.02% 2-AAF for next two weeks, and two-third partial hepatectomy was performed as 21st day of this experiment. The rats in group 2 and 3 were given a diet containing 0.25% clofibrate for 14 weeks from the beginning of 8 th week of the experiment. Group 1 are untreated rats.

Immunohistochemistry

All animals were killed by anesthetizing with Nembutal. The livers were excised and cut into 2~3 mm slices which were postfixed in ice-cold acetone, and embedded in paraffin. Serial sections were cut with a thickness of 5 μm and placed on glass slides. Sample preparation was followed in this sequence.: Benzen solution (Katayama Chemical) wash (2×15 min), 0.05 M phosphate buffer pH 7.4 containing 0.15 M NaCl (PBS) wash (3×5 min), 95% methanol (Katayama Cemical) containing 0.06% H_2O_2 (Mitsubisi Chemical Co.) for 30 min to suppress endogenous peroxidase activity, PBS wash (3×5 min), Block Ace (Dainihon Co.) to supress non-specific reaction for 20 min, PBS wash (3×5 min), and they were incubated with each antibody (diluted with PBS supplemented with 0.35 M NaCl in the range of 1:1000 to 1:2000) in a chamber at 4℃ for 12 hours. After washing with PBS (3×5 min), incubation with goat anti-rabbit IgG antibodies labelled with horseradish peroxidase (Seikagaku kogyo Co., LTD) was performed at room temperature for 30 min. After PBS washing again (3×5 min), peroxidase binding sites were detected by 3,3'-diaminobenzine tetrahydrochloride (DAB, Katayama Chemical) in 0.05 M Tris HCl pH 7.6 buffer (10).

RESULT AND DISCUSSION

The phenotypic expression of hepatic enzymes such as gammaglutamyl transpeptidase (gamma-GT) and glucose-6-phosphatase quite often change in that they have altered foci and neoplastic nodules of rat livers induced by various chemical carcinogens(4, 15, 23, 31, 32), and in additions recent investigations have indicated that peroxisome proliferators induce neoplastic lesions that have no gamma-GT activity(24) and show negative stains for YpYp (GST-P form)(25). In contrast to these reports, very little information is available on the expression and behavior of the other GST isoenzymes except for YpYp in rat livers by treatment of hypolipidemic agents. Therefore, we analysed the expression of GST isoenzymes using immunohistochemical techniques in order to examine the alterations of these isoenzymes in rat livers by clofibrate treatment during chemical carcinogenesis.

In hepatocytes, YaYa, YbYb, YcYc and YkYk in group 1 rat (fed with BD) showed almost the same distribution as that of group 2 (fed with BD+clofibrate) (Fig. 1). That is, parenchymal cells within the centrilobular venous region exhibited a greater intensity of staining than that within periportal and midzonal regions, except for the fact that a part of periportal region was moderately stained in group 2 (Fig. 2) while this part of group 1 was almost non-negative.

These observations are consistent with the previously reported findings that

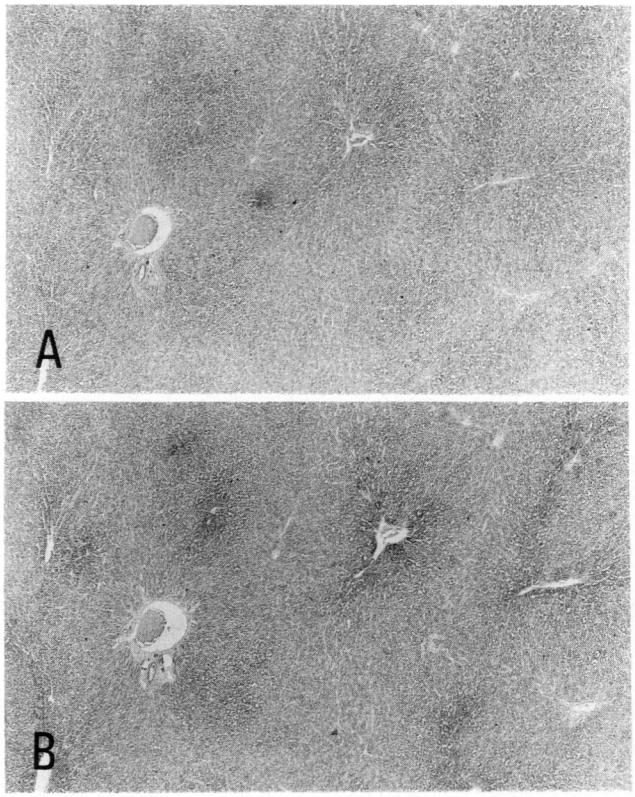

Fig. 1 Immunohistochemical localization of glutathione S-transferase isoenzymes within the liver of an untreated male rat in group 1 (×30). 5 μm serial sections of the same focus were staine for immunoperoxidase activity using 3, 3'-DAB as described in Materials and Methods. A: YaYa, B: YbYb.

centrilobular cells have a significant capacity for detoxification of toxic endogenous and exogenous substances(2, 28). The expression of YpYp, however, was not evident in parenchymal cells in both group 1 and 2.

The antibodies against YaYa, YbYb, YcYc and YpYp in intralobular bile ducts lined by cuboidal epithelium of group 1 rat stained with the slightly~moderate strong intensity as compared with those in the surrounding hepatocytes immunohistochemically, but the staining intensity of YkYk was not apparent in (Fig. 3). Bile duct epithelium in rats of group 2 treated with clofibrate displayed an apparent increase in the YcYc isoenzyme, however concentrations of the other GST isoenzymes were decreased (Fig. 4). In rats of group 3 (Fig. 5), the concentrations of YbYb and YcYc in the epithelium of bile ducts were remarkably increased as compared with those in surrounding hepatocytes. In rats of group 4 (Fig. 6), the moderate increase of YaYa and intense decrease of YcYc was obser-

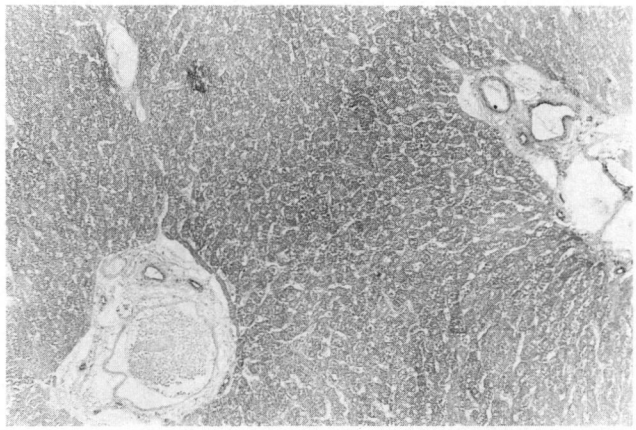

Fig. 2 Immunohistochemical localization of glutathione S-transferase isoenzyme (YbYb) within the liver of an clofibrate-treated male rat in group 2 (×120). Parenchymal cells within the periportal regions as well as the centrilobular regions were more intensively stained for YbYb than those within midzonal regions of the lobule.

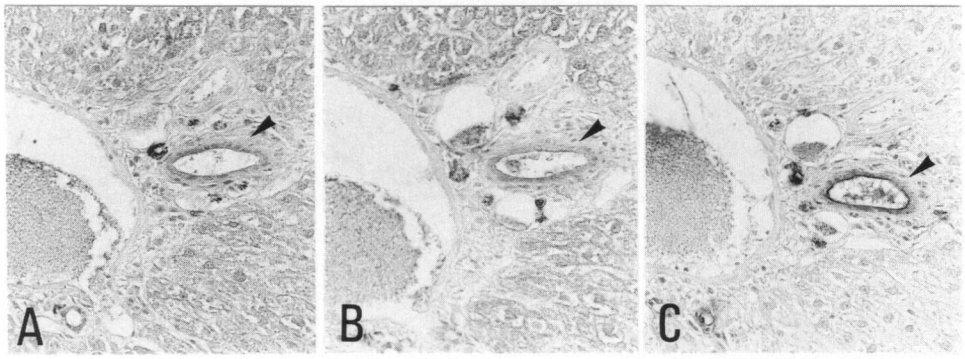

Fig. 3 Immunohistochemical demonstration of glutathione S-transferase isoenzymes within the bile ductual epithelium of an untreated male rat in group 1 (×240). YpYp(C) stained more intensively than the other GST isoenzymes in epithelial cells of bile ducts as shown by an arrow. A: YaYa, B: YbYb, C: YpYp.

ved.

Table 1 summarizes the changes of GST isoenzymes in the bile duct epithelium of group 1, 2, 3 and 4 compared with those in surrounding hepatocytes. It is noteworthy that YcYc isoenzyme in rat bile duct epithelium of group 2 and 3 treated with clofibrate remarkably increased, because this isoenzyme possesses the most highest hydroperoxidase activity among all GST isoenzymes. There fore, this may indicate that YcYc isoenzyme in bile ducts of epithelial cells are distributed for the roles in detoxification of such potentially toxic metabolites of clofibrate, and plays a significant role of detoxifing these hydroperoxides instead

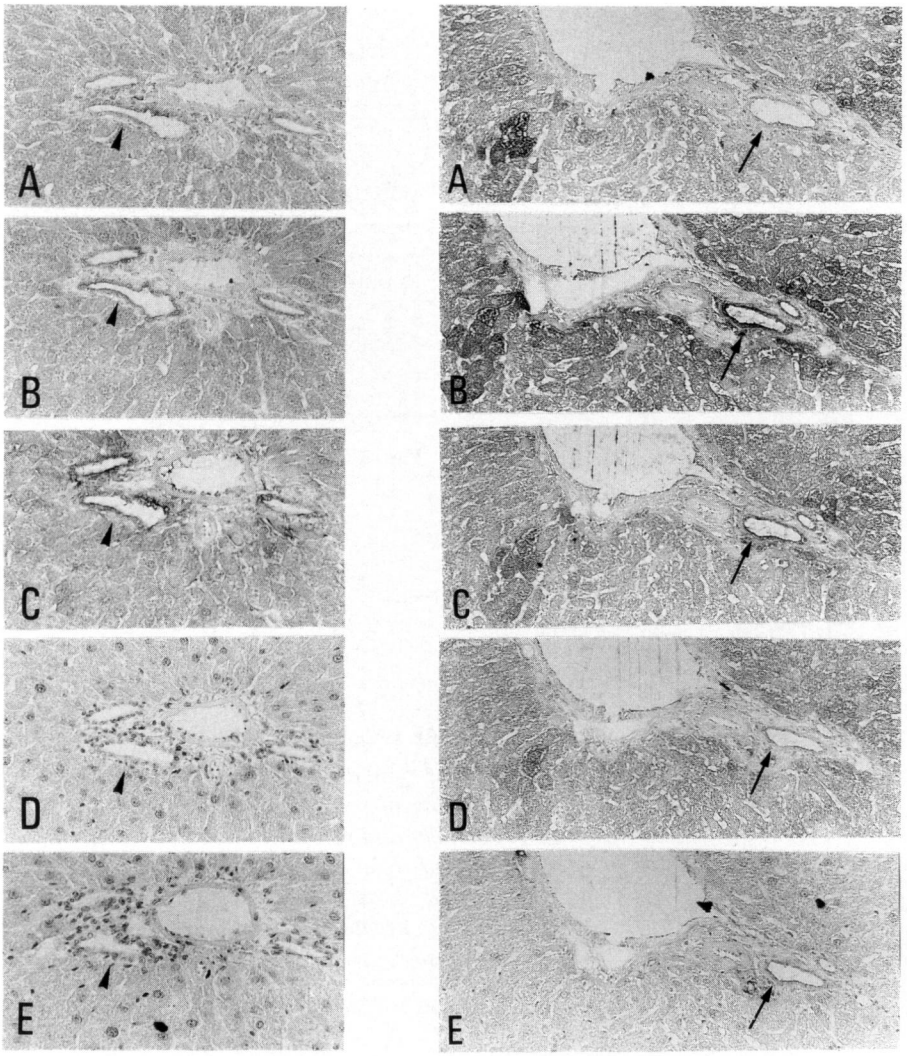

Fig. 4 Immunohistochemical demonstration of five glutathione S-transferase isoenzymes within epithelial cells in serial sections of the same bile ducts in group 2 (×240).
A: YaYa, B: YbYb, C: YcYc,
D: YkYk, E: YpYp.

Fig. 5 Immunohistochemical demonstration of five glutathione S-transferase isoenzymes within epithelial cells in serial sections of the same bile ducts in group 3 (×240).
A: YaYa, B: YbYb, C: YcYc,
D: YkYk, E: YpYp.

of enzymes that is inadequate to cope with excessive oxigen radicals and H_2O_2 generated by lipid peroxidation. While, the increment of YbYb concentration in bile duct epithelium of group 3 may indicate that this isoenzyme participates in

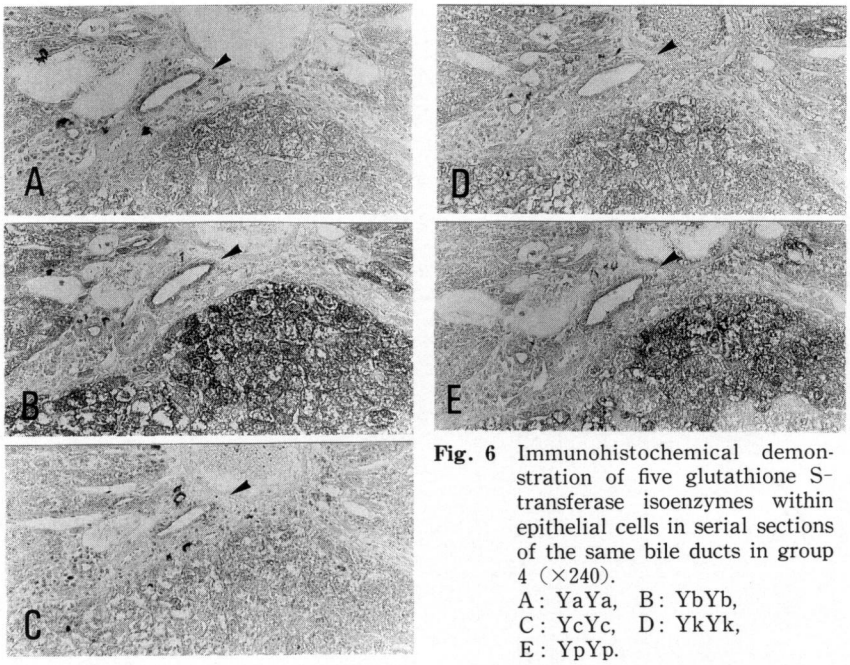

Fig. 6 Immunohistochemical demonstration of five glutathione S-transferase isoenzymes within epithelial cells in serial sections of the same bile ducts in group 4 (×240).
A: YaYa, B: YbYb,
C: YcYc, D: YkYk,
E: YpYp.

Table 1 *Changes on glutathione S-transferase isoenzymes (YaYa, YbYb, YcYc, YkYk andYpYp) in epithelial cells of bile ducts of group 1, 2, 3 and 4 as compared with surrounding hepatocytes immunohistochemically.*

	Group 1	Group 2	Group 3	Group 4
YaYa	↗	→	→	↗
YbYb	↗	↗	↑	↗
YcYc	↗	↑	↑	→
YkYk	→	→	→	→
YpYp	↑	→	↗	↗

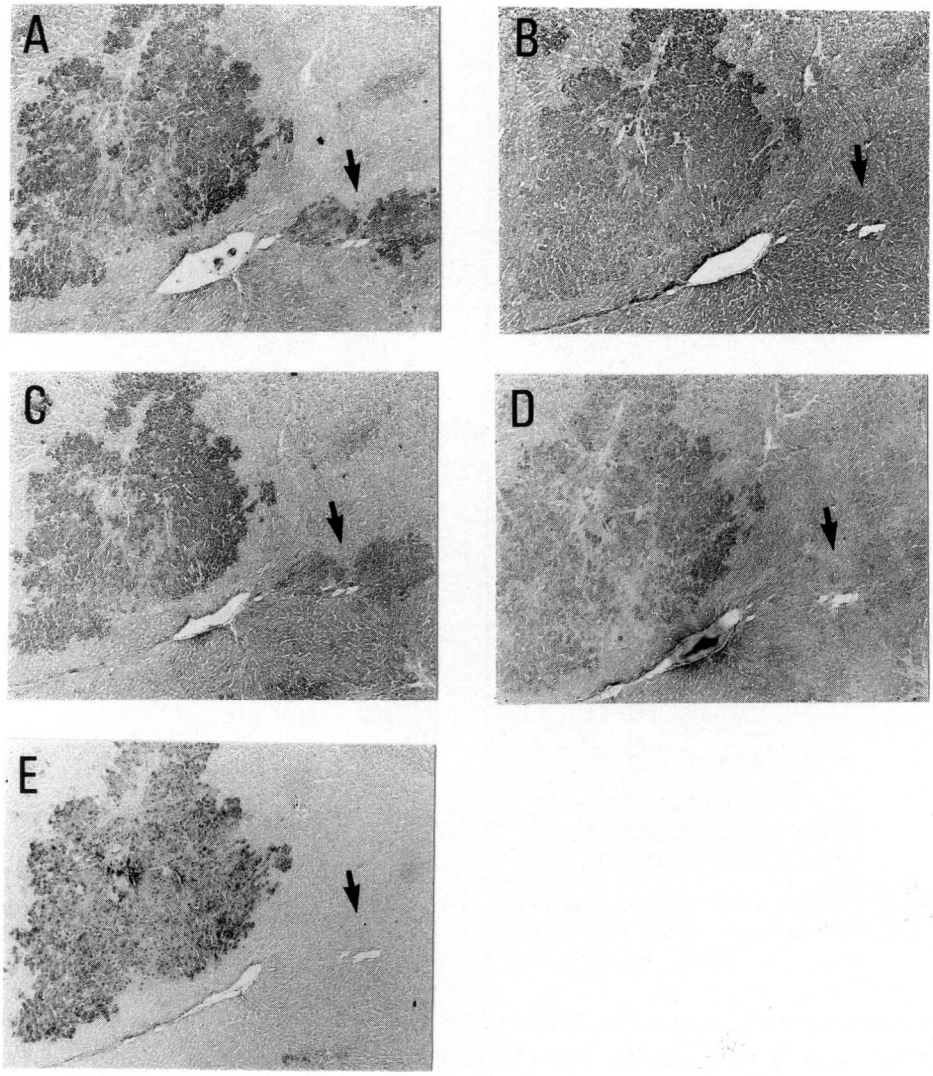

Fig. 7 Immunohistochemical demonstration of glutathione S-transferase isoenzymes in an altered area of rat liver in group 4 (×30). Serial sections of the same focus indicate that YaYa, YbYb, YcYc and YkYk are detectable in YpYp (GST-P)-positive foci. An arrow show YpYp-negative foci. A: YaYa, B: YbYb, C: YcYc, D: YkYk, E: YpYp.

detoxification of DEN and 2-AAF because it showed no increase of YbYb in group 2, and the induction of YaYa in group 4, likewise. In Kupffer and sinusoidal cells of all groups, very little GST isoenzymes were found.

These findings suggest that each isoenzyme of GSTs may have an different

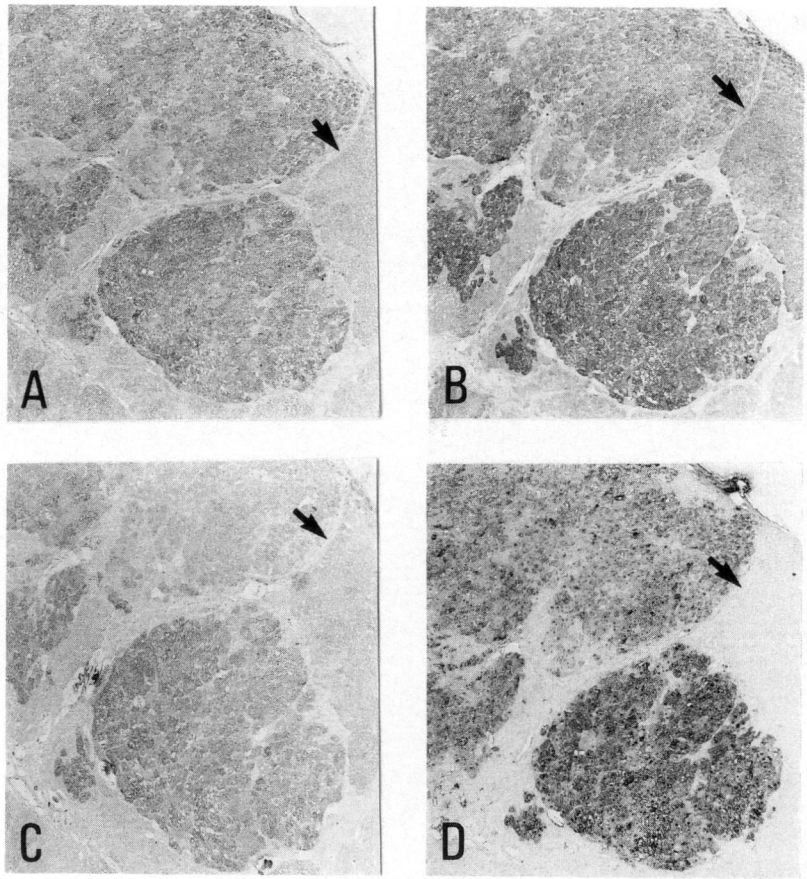

Fig. 8 Immunohistochemical demonstration of glutathione S-transferase isoenzymes in neoplastic nodules of rat liver in group 4 (×30). An arrow shows YpYp-negative nodules. A: YaYa, B: YbYb, C: YkYk, D: YpYp.

function for detoxification in their specific cellular localization (especially in parenchymal cells and epithelial cells of bile ducts).

Increased activities of various cytosolic GST isoenzymes have been reported in the altered foci and hyperplastic nodules induced by various carcinogens(4, 15, 23, 31, 32). In this experiments, YaYa, YbYb, YcYc, YkYk and YpYp concentration in a small altered foci at an earlier stage of group 4 are already increasing (Fig. 7). YpYp (GST-P), which is virtually nonexistant in normal rat liver, is surely effective for determining the localization of the altered foci and hyperplastic nodules as described previouly(21, 31, 32), but the other YaYa, YbYb, YcYc and YkYk-positive foci and nodules can be clearly distinguished from surrounding hepatocytes, likewise (Fig. 7 and 8). Especially some of YaYa, YbYb,

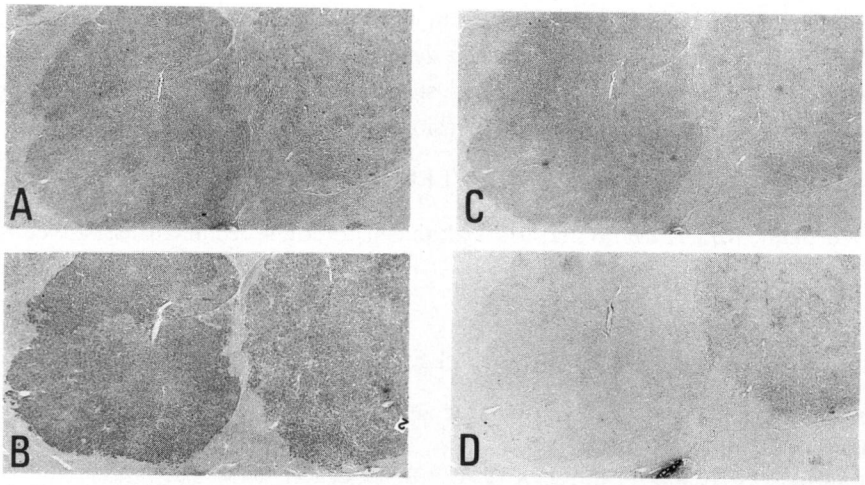

Fig. 9 Immunohistochemical demonstration of glutathione S-transferase isoenzymes in neoplastic nodules of rat liver in group 3 (×15). The staining intensity of YpYp(D) in neoplastic nodules was suppressed the strongest among GST isoenzymes.

YcYc and YkYk positive foci and nodules showed a negative stain for YpYp as indicated with an arrow in Fig. 7 and 8.

These immunohistochemical studies indicate that all GST isoenzymes were induced to restrict to altered foci and nodules under carcinogens such as 2-AAF and DEN, and that YpYp (GST-P) did not always show a positive staining for the other GST isoenzymes positive foci and nodules.

On the other hand, the concentration of YaYa, YcYc, YkYk and YpYp-positive nodules in rats of group 3 treated with clofibrate decreased as compared with the levels in untreated livers of group 4 immunohistochemically (Fig. 9), that of YpYp-positive nodules especially were suppressed the strongest among each GST isoenzyme positive nodules (Fig. 9-D).

These immunohistochemical observations indicate that not only the expression of YpYp but also that of the other GST isoenzymes were suppressed in altered foci and neoplastic lesions on the modifying effects of clofibrate under chemical hepatocarcinogenesis, and coincide with the previously reported findings that peroxisome proliferators such as ciprofibrate and nafenopin inhibit GSTs activity(1, 9). In addition, these hypolipidemic agents do not interact with GSTs as the results of analyzing for binding to GST isoenzymes by circular dichroic displacement assay(17), and do not express such genotoxicity as the other chemical hepatocarcinogenesis(27, 31, 32). But, these drugs produce an exessive generation of oxygen radicals and H_2O_2 and bring about the decrease of superoxide dismutase and GSH-Px in rat livers(7), and that the excessive increase of H_2O_2

and lipid peroxide are much higher than that of catalase(16). As a result, the produced OH^- radicals may damage the gene expressions of GST isoenzymes and the other detoxifing enzymes, and may show an increased potential of evolving ultimately into a malignant neoplastic lesions.

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