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Strain Differences in Susceptibility to 2-Acetylaminofluorene and Phenobarbital Promotion of Hepatocarcinogenesis: Immunohistochemical Analysis of Cytochrome P-450 Isozyme Induction by 2-Acetylaminofluorene and Phenobarbital

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Strain differences in the expression of cytochrome P-450 isoenzymes (P-450s) during enhancement of hepatocarcinogenesis by 2-acetylaminofluorene (2-AAF) and phenobarbital (PB) were investigated immunohistochemically using monoclonal antibodies against phenobarbital (PB) (APF3) or 3-methylcholanthrene (3-MC) (APH8) inducible P-450s. LEW, SD, WBN, F344, SHR, NAR, Wistar and ODS rats were studied, the first five strains proving to be less susceptible to 2-AAF induction of APH8 while responding strongly to the promoting influence of this chemical, as reported previously. The other three strains, NAR, Wistar and ODS, demonstrated greater inducibility, this correlating with an observed resistance to promotion by 2-AAF. PB administration was not associated with any strain difference in APF3 cytochrome P-450 inducibility except in the ODS rat, in which its effects were minimal. The results provide direct evidence that differential expression of cytochrome P-450 species plays a major role in determining responsiveness to hepatocarcinogenesis-promoting agents such as 2-AAF.

Key words: Strain differences — Rat hepatocarcinogenesis — Cytochrome P-450 — Immunohistochemistry

Previously, we investigated strain differences in susceptibility to promotion of carcinogenesis by 2-AAF[5] or PB using preneoplastic GST-P-positive foci as the end-point lesion, and demonstrated that LEW, SD, WBN and F344 rats were most susceptible to hepatopromotion by both compounds, whereas NAR and SHR were intermediate, and Wistar and in particular its mutant ODS strain were far less susceptible.[1)]

The cytochrome P-450 monooxygenase system is responsible for the metabolism of various xenobiotics, including drugs and environmental pollutants. Although most cytochrome P-450-mediated metabolism leads to detoxification, certain reactions can lead to activation through the generation of reactive intermediates that may be carcinogenic, mutagenic or cytotoxic.[2-4)] It is known that 2-AAF is metabolized by P-450 to various active and inactive forms,[2, 3)] while both PB and 2-AAF are inducers of microsomal enzymes such as P-450s.[5)] Inducible P-450 isoenzymes can be divided into two general classes: one is the phenobarbital-inducible type, including P-450b and P-450e which possess mixed function oxygenase activities towards several substrates. The other is the polycyclic aromatic hydrocarbon-inducible type, sometimes termed P-448, such as P-450c or P-450d, which is known to be elevated after administration of 3-methylcholanthrene or benzo[*a*]pyrene.[5, 6)] In this study, two monoclonal antibodies which bind respectively to PB type P-450 (APF3) or MC type P-450 (APH8),[7, 8)] were used to investigate strain differences in liver P-450 expression phenotype and to assess whether any variation might underly the observed differences in susceptibility to the potent hepatocarcinogen (2-AAF) and the hepatopromoting agent (PB) in our medium-term bioassay system.[1)]

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[5] Abbreviations: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; PB, phenobarbital; P-450, cytochrome P-450; GST-P, glutathione *S*-transferase placental form; PH, partial hepatectomy.

MATERIALS AND METHODS

Male rats of the following strains were used in the experiment: F344 (Charles River Japan, Inc., Kanagawa), Wistar (Aburabi Lab., Shionogi Pharmaceutical Co., Ltd., Shiga), osteogenic disorder rat (ODS) (Aburabi Lab., Shionogi Pharmaceutical Co., Ltd.), spontaneous hypertensive rat (SHR) (Charles River Japan, Inc.), Lewis (LEW) (Charles River Japan, Inc.), WBN (WBN) (Shizuoka Laboratory Animal

Center, Hamamatsu), Sprague-Dawley (SD) (Shizuoka Laboratory Animal Center) and Nagase analbuninemic (NAR) (Sasaki Institute, Tokyo). All rats were 6 weeks of age at the start of the experiment and were housed in plastic cages in an air-conditioned room at 24±2°C. Animals of each strain were divided into three groups: Group 1 was given a single intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg) dissolved in 0.9% NaCl to initiate hepatocarcinogenesis, and then after 2 weeks on basal diet, received 0.02% 2-AAF or 0.05% PB in powder diet. All rats were subjected to partial hepatectomy (PH) at week 3. Each 2-AAF or PB group comprised 15 rats. Group 2 animals were given DEN and PH alone in the same manner, and whereas group 3 rats were injected with 0.9% NaCl instead of DEN solution and then subjected to administration of 2-AAF or PB as with group 1, and PH. Immediately after killing of the rats under ether anesthesia at week 8, the livers were removed, and three 2–3 mm thick slices, one each from the right lateral cranial, the right lateral caudal, and the caudate lobes, were fixed in cold acetone for subsequent immunohistochemical demonstration of P-450 binding. As control groups, untreated rats of each group were killed in the same manner as for P-450 immunohistochemistry.

Mouse monoclonal antibodies against rat cytochrome P-450 isoenzymes were raised as described previously[7, 8]: APH8 monoclonal antibody (IgG1) against the high-spin form of cytochrome P-448 (P-448H; Levin's P-450d) which cross-reacts slightly with a low-spin form (P-448L; Levin's P-450c); APF3 monoclonal antibody (IgG1) against PB-inducible rat cytochrome P-450s (Levin's P-450b or P-450e) which is specific for these forms and does not cross-react with cytochrome P-448 isoenzymes.

The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[9] was used to determine the localization of cytochrome P-450 binding. Affinity-purified biotin-labeled goat anti-mouse immunoglobulin IgG and avidin-biotin-peroxidase complex (ABC) (Vectastain ABC kit, PK 4002) were obtained from Vector Laboratories Inc. (Burlingame, CA). Paraffin sections were routinely passed through petroleum benzin and a graded alcohol series and then treated sequentially with 1% BSA, mouse anti P-450 monoclonal antibodies, biotin-labeled horse anti-mouse IgG and ABC. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counter-stained with hematoxylin to facilitate microscopic examination. As a negative control for the specificity of anti-P-450 monoclonal antibody binding, preimmune mouse serum was used instead of the antibodies.

Expression of P-450 isoenzyme in GST-P-positive preneoplastic foci and in background parenchyma was compared with normal levels of binding in each of the rat strains using serial sections.

RESULTS

The results are summarized in Tables I and II. After initiation by DEN followed by PH without 2-AAF or PB treatment, expression of APF3 or APH8 in both foci and their surrounding hapatocytes did not differ among the various strains except in the ODS case, where both GST-P-positive foci and background parenchyma had elevated expression of P-450 isoenzymes. Only APH8 binding was positive in the centrilobular liver areas of LEW, SD, WBN, F344, SHR, NAR and Wistar strains, whereas APF3 and APH8 were also strongly stained in all GST-P-positive foci in the ODS strain. 2-AAF treatment without prior DEN initiation (with PH) was associated with increased APH8 binding in all strains of rats examined. However, the localization was variable with the enzyme being induced throughout the background parenchyma of LEW, WBN, F344, SHR, Wistar and ODS strains, in the periportal area of SD, and in the centrilobular zone of NAR. Strong binding was observed within foci in the Wistar and ODS cases and a slight reaction in SHR. 2-AAF with DEN initiation induced strong APH8 or APF3 binding in the background parenchyma of NAR, Wistar and ODS animals and also to a variable extent in the foci in these strains (Fig. 1). In LEN, SD, WBN, F344 and NAR, APH8-positive hepatocyte foci expressed as (++) in Table I were negative for GST-P and compressed by surrounding GST-P-positive foci. In those cases, larger lobular structure expressing "P" or "C" could not be distinguished because of dense development of large nodules or foci. PB treatment (with or without DEN initiation) was associated with increased APF3 binding in both focal lesions and surrounding parenchyma of all strains (Fig. 2), with the exception of ODS animals where strong expression was evident, particularly in the foci, even without treatment. Interstrain differences in the staining intensity and localization of P-450 in the hepatocytes of untreated rats were not observed except in ODS rats. Their staining patterns of P-450s were closely similar to that observed in surrounding hepatocytes of the respective group given DEN alone.

DISCUSSION

Positive binding of APH8 to surrounding hepatocytes and lack of APH8 binding to GST-P-positive foci after treatment with 2-AAF were observed in the relatively more susceptible strain groups. On the other hand, strong binding of APH8 in the cells within GST-P-positive foci as well as surrounding hepatocytes was observed in rat groups resistant to 2-AAF treatment. Strong binding of

Table I. Induction of Cytochrome P-450 in Different Strains of Rats Treated with 2-AAF

Strain	Treatment											
	DEN				DEN+2-AAF				2-AAF			
	APH8		APF3		APH8		APF3		APH8		APF3	
	sur.	foci	sur.	foci	sur.	foci	sur.	foci	sur.	foci	sur.	foci
LEW	±c	−	−	−	±(++)	−	−	−	++	−	−	−
SD	±c	−	−	−	±(++)	−	−	−	+p	−	−	−
WBN	±c	−	−	−	±(++)	−	−	−	+	−	−	−
F344	±c	−	−	−	±(++)	−	−	−	++	−	−	−
SHR	±c	−	−	−	±(++)	−	−	−	++	±	−	−
NAR	±c	−	−	−	++c	+∼++	++c	−∼+	++c	−	±c	−
Wistar	±c	−	−	−	++c	+∼++	++c	−∼++	++w	++	++w	+
ODS	++c	+ ++	+c	++	++w	++	+c	++	++w	++	++c	++

c, centrilobular area; p, periportal area; w, whole lobules; (++), APH8-positive hepatocytes; foci, GST-P-positive foci; sur., hepatocytes surrounding GST-P-positive foci; APH8, monoclonal antibody recognizing PB-type P-450; APF3, monoclonal antibody recognizing MC-type P-450.

Table II. Induction of Cytochrome P-450 in Different Strains of Rats Treated with PB

Strain	Treatment					
	DEN+PB				PB	
	APH8		APF3		APH8	APF3
	sur.	foci	sur.	foci		
LEW	±c	−	++c	+∼++	±c	++c
SD	−	−	++c	+∼++	±c	++c
WBN	−	−	++c	+∼++	±c	++c
F344	−	−	++c	+∼++	±c	++c
SHR	−	−	++c	+∼++	±c	++c
NAR	−	−	++c	+∼++	±c	++c
Wistar	−	−	++c	+∼++	±c	++c
ODS	+c	±∼+	+c	++	++c	++c

Abbreviations are the same as in Table I.

APF3 in surrounding hepatocytes and at variable binding intensity within the foci of GST-P positive hepatocytes were also found in NAR, Wistar and ODS resistant rats. It is known that the cytochrome P-450 content of early hyperplastic nodules is lower than in untreated or surrounding liver, as shown both biochemically and immunohistochemically.[10-14] A relationship between the expression of drug-metabolizing enzymes and the proliferative potential of preneoplastic foci has been indicated by Farber and his colleagues.[15] They suggested that a decrease in monooxygenase activity with increasing levels of detoxifying enzymes allows preneoplastic cells to escape from toxic environmental effects, leading to a selective increase in the proliferative potential of these cells.

2-AAF induced P-450s as recognized by APH8 positivity in normal hepatocytes (unpublished data) in F344 rats, in agreement with the report that 2-AAF selectively induced microsomal 2-AAF N-hydroxylase.[3] Accordingly it is concluded that P-450s recognizable with APH8 are selectively capable of metabolizing 2-AAF. In the present study, the preneoplastic foci of more susceptible strains of rats, LEW, SD, WBN, F344 and SHR, did not show induction of cytochrome P-450 by 2-AAF treatment. However, the less susceptible strains, NAR, Wistar and ODS, demonstrated a clear response. Therefore, it is suggested that in less susceptible strains, 2-AAF might cause more toxic effects in the preneoplastic foci, leading to a lower growth rate than in the lesions of more susceptible strains.

PB is a well-known hepatopromoter and inducer of P-450s, although the mechanisms underlying the promotion remain unclear. It was earlier demonstrated that individual P-450 isozymes can still be induced in cells of focal neoplastic cell populations by inducers of drug-metabolizing enzymes such as 3-methylcholanthrene or

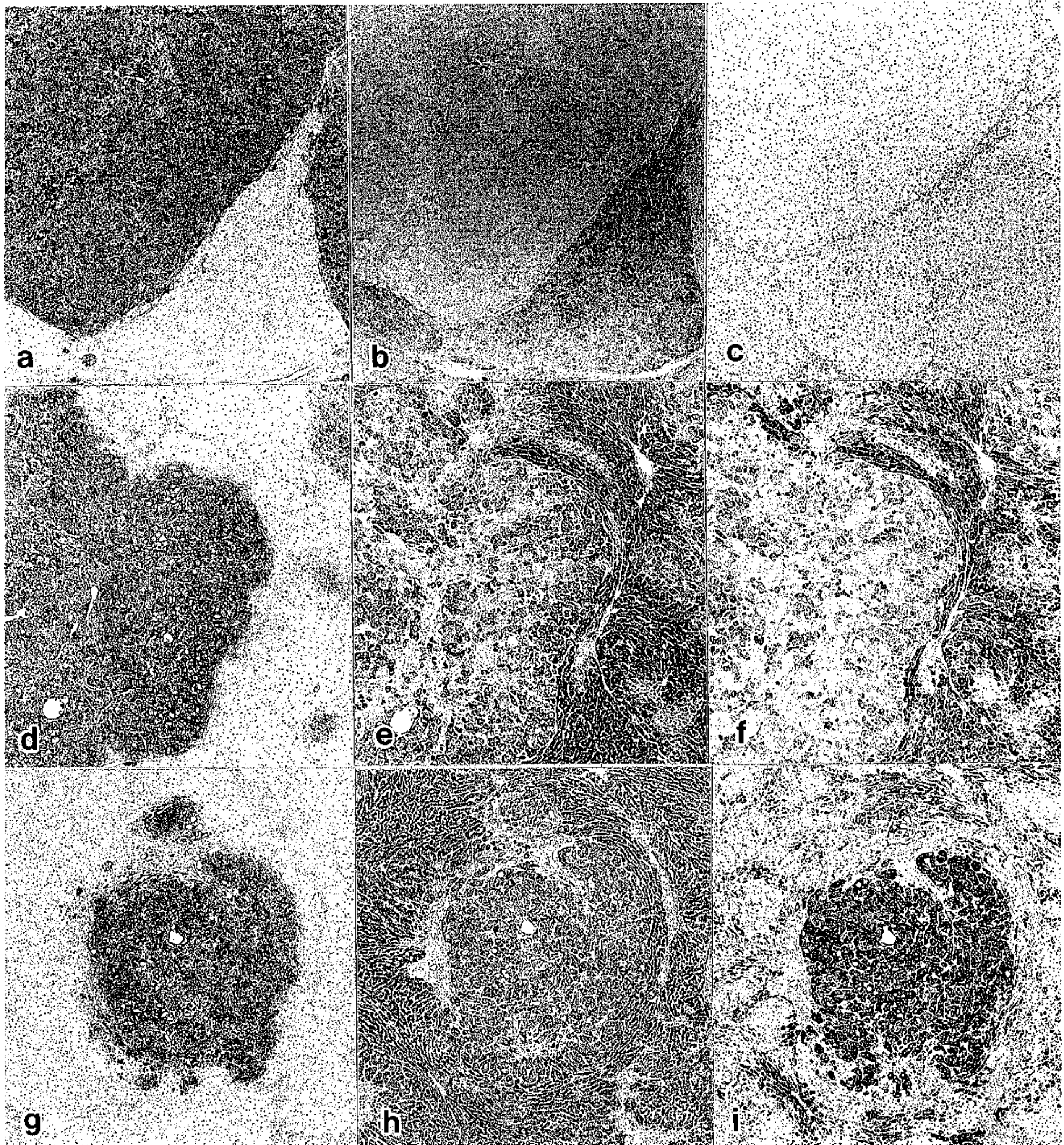

Fig. 1. Serial sections through hepatic altered foci induced by 2-AAF with DEN initiation. a), b) and c) LEW rat. d), e) and f) Wistar rat. g), h) and i) ODS rat. a), d) and g) GST-P. b), e) and h) MC type P-450 recognized by APH8. c), f) and i) PB type P-450 recognized by APF3. In LEW, slight binding of APH8 is apparent in hepatocytes surrounding GST-P-positive foci. Note strong APH8 or APF3 binding in the background parenchyma and variable levels in foci cells in a Wistar rat. In ODS, strong APH8 binding is seen in both the foci and the background parenchyma diffusely, and strong APF3 binding is evident in the foci and centrilobular area of the background parenchyma.

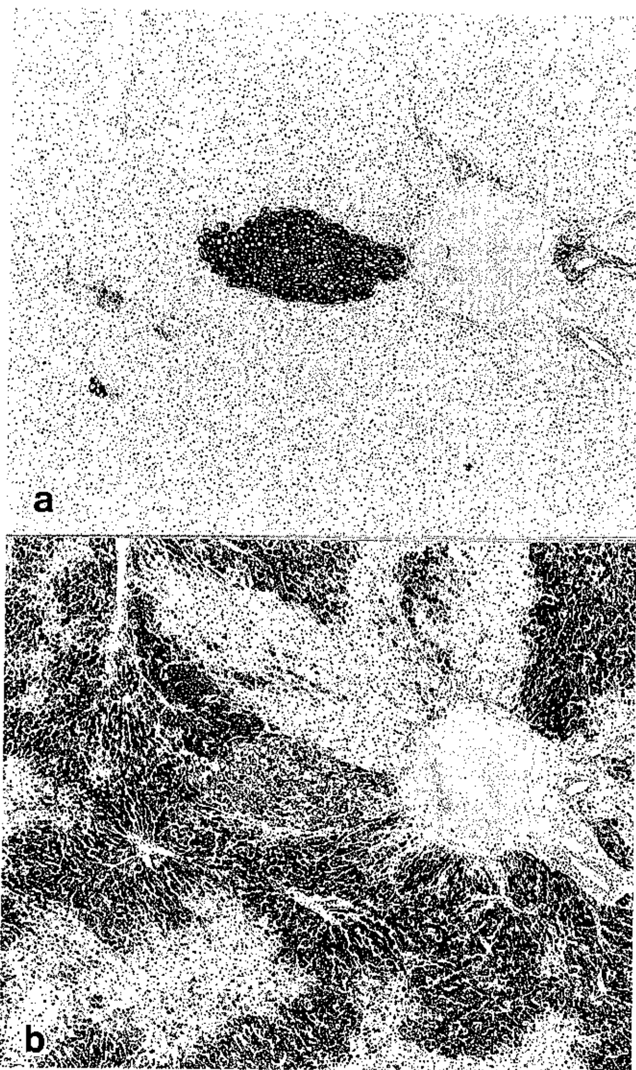

Fig. 2. Serial sections through a hepatic altered focus of an LEW rat induced by PB with DEN initiation. a) GST-P, b) PB type P-450 recognized by APF3. Increased APF3 binding is evident in both the focus and centrilobular area in the background parenchyma, although the level is appreciably lower in the focus.

phenobarbital.[16, 17] In this study, after treatment with PB, P-450 recognized by APF3 was strongly induced in preneoplastic foci in all strains except ODS, although at intensities slightly lower than in the pericentral hepatocytes. While the results with APF3 binding did not differ between the strains, its relatively lower binding within foci cells relative to surrounding hepatocytes might explain the promotion activity of PB in terms of P-450-dependent toxicity. It is conceivable that PB is too strong an inducer to allow variation in P-450 response to be detectable among the different strains of rats investigated.

The ODS strain of rats, derived from Wistar/Shi,[18] has a hereditary defect in L-ascorbic acid-synthesizing ability.[19] Since this deficiency interferes with long-term maintenance of ODS rats, they received a 0.05% L-ascorbic acid supplement to the basal diet in this study. It is known that L-ascorbic acid is required for the induction of P-450 in ODS rats,[20] and therefore, its addition to the diet might have influenced the phenotype of the cytochrome P-450 induction. Whether the genetic defect in ability to synthesize ascorbic acid is directly involved in resistance to promotion by 2-AAF or PB, or whether such a defect is relevant to some other gene expression causing resistance requires further study. However, it is interesting that man is also genetically incapable of synthesizing ascorbic acid.

In summary, obvious strain differences in the phenotype of induction of P-450 were observed in rats treated with 2-AAF, the level of induction being inversely correlated with susceptibility to the cancer-promoting effect. The results clearly suggest that cytochrome P-450 may play a major role in determining the response to the hepatopromoting agent, 2-AAF, and that strain differences exist in the regulating system of P-450 encoding structural genes. In this respect, further investigations regarding activities of detoxifying enzymes, including glutathione *S*-transferases, between different species and strains appear necessary.

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