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Demonstration of Initiation Potential of Carcinogens by Induction of Preneoplastic Glutathione *S*-Transferase P-Form-positive Liver Cell Foci: Possible *in vivo* Assay System for Environmental Carcinogens

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In a development trial for an initiation bioassay system, 7 known carcinogens and 1 suspected carcinogen were examined. In experiment 1, group 1 animals were initially subjected to partial hepatectomy (PH) 12 h before administration of diethylnitrosamine, 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ), captafol, α-hexachlorocyclohexane or diethylstilbestrol (DES), then 2 weeks later underwent a promotion procedure comprising administration of phenobarbital (0.05% in diet) for 8 weeks and D-galactosamine (300 mg/kg, i.g.) at week 3. Group 2 received the promotion protocol alone as in group 1. Initiating potential was assayed on the basis of significant increase in values of preneoplastic placental form glutathione *S*-transferase-positive (GST-P$^+$) foci of more than 3 cells in cross section at week 10. Numbers and areas of GST-P$^+$ foci in group 1 given IQ, captafol and DES were significantly increased as compared to group 2, confirming the validity of the protocol as an initiation assay. In Experiment 2, group 1 rats were subjected to PH and 12 h later received a suspected carcinogenic mixture of opium pyrolysate (OP) or carcinogenic pesticide *p,p′*-dichloro-diphenyltrichloroethane or hexachlorobenzene. Application of a modified promotion procedure comprising cholic acid (0.15%) and carbon tetrachloride (1 ml/kg, i.g.) revealed significant initiation potential for OP. Overall the results indicate that the current protocols may be useful for detection of the initiation potential of carcinogens irrespective of their mutagenicity.

Key words: Initiation potential — Environmental carcinogen — GST-P-positive focus — Rat liver

Since conventional detection methods for assaying carcinogenicity of chemicals using long-term *in vivo* rodent experiments are costly, various *in vitro* tests based on genotoxicity have been introduced as practical systems for the assay of initiation potential of chemicals.[1–4] However, recent increase in the numbers of agents tested has revealed that prediction of initiation potential on the basis of *in vitro* genotoxicity alone is not satisfactory.[5, 6] Therefore the advantages of using *in vivo* medium-term bioassay systems in various organs, and especially that based on the two-step concept of liver carcinogenesis, have been stressed.[7–9]

Several bioassay systems primarily for determining promotion potential of carcinogens have recently been developed,[7, 8] taking advantage of the availability of good marker enzymes such as γ-glutamyl transpeptidase[10] or the placental form of glutathione *S*-transferase(7-7) (GST-P)[11, 12] for preneoplastic liver populations that can serve as end-point marker lesions.[13] Using this concept, a reliable screening system has indicated that the majority of liver carcinogens,[14] irrespective of their mutagenicity, give positive results in good agreement with long-term carcinogenicity results.[15, 16]

Since carcinogens are considered to possess both initiation and promotion potential, it should also be possible to predict their carcinogenicity by simply assessing the initiation stage. It has been shown that several non-liver as well as liver carcinogens may initiate the induction of enzyme-altered hepatocellular focal lesions or tumors when administered during a phase of replicative DNA synthesis.[9, 17] It is generally accepted that cellular proliferation is critical for initiation of hepatocarcinogenesis[18, 19] and two-thirds partial hepatectomy (PH) has been extensively utilized as one of the most effective methods to enhance initiation.[17, 19]

In the present investigation, we studied the validity of our protocol for the assay of initiation potential by testing known carcinogens such as diethylnitrosamine

Abbreviations: GST-P, placental-form glutathione *S*-transferase; PH, partial hepatectomy; DEN, diethylnitrosamine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PB, phenobarbital; DGA, D-galactosamine; GST-P$^+$, GST-P-positive; CCl_4, carbon tetrachloride; OP, opium pyrolysate; DDT, *p,p′*-dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; CA, cholic acid; α-HCH, α-hexachlorocyclohexane; DES, diethylstilbestrol.

(DEN), the pyrolysis product 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ),[20] captafol,[21,22] α-hexachlorocyclohexane (α-HCH)[23,24] or diethylstilbestrol (DES),[25] followed by a promotion procedure utilizing the hepatopromoter phenobarbital (PB).[26,27] The suspected carcinogen, opium pyrolysate (OP),[28,29] a pyrolysis product of opium, was tested along with known carcinogenic pesticides, *p,p'*-dichlorodiphenyltrichloroethane (DDT)[30] and hexachlorobenzene (HCB)[31,32] as positive controls, using a modified protocol in which PB was replaced by a new hepatopromoter, cholic acid (CA).[33] Secondary stimuli to enhance the promotion effect were given by administration of a necrogenic dose of D-galactosamine (DGA) in Experiment 1[34] and carbon tetrachloride (CCl_4)[17–19] in Experiment 2. A potent mutagen, OP, was chosen for testing in Experiment 2 because of its suspected role in human neoplasia and because it has not been subjected to long-term assay in the rat.[28,29]

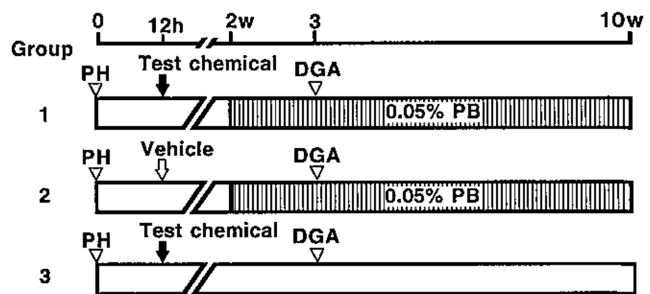

Fig. 1. Experimental protocol for Experiment 1. See "Materials and Methods" for details. [ ], basal diet; [hatched], basal diet containing 0.05% PB; PH, two-thirds partial hepatectomy; DGA, D-galactosamine, 300 mg/kg, i.p. Initiating potentials of test compounds were assayed on the basis of significant increase in quantitative values of GST-P+ foci larger than 3 cells in cross section in group 1 as compared to group 2.

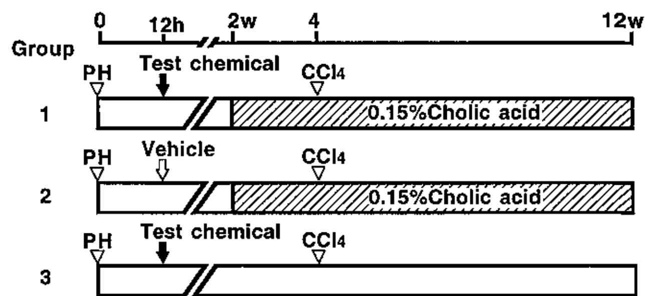

Fig. 2. Experimental protocol for Experiment 2. [hatched], basal diet containing 0.15% cholic acid; CCl_4, carbon tetrachloride, 1 ml/kg, i.g. See the legend to Fig. 1 for other symbols.

MATERIALS AND METHODS

A total of 260 male 6-week-old F344 rats (Charles River Japan, Inc., Atsugi) housed five per plastic cage on wood chips for bedding, were maintained under constant conditions (12 h light/dark cycle, 60% humidity at 22±2°C) on Oriental MF basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. After 1 week of acclimatization, the animals were divided into two experimental series (Experiments 1 and 2), which were further divided into three groups each.

In Experiment 1, group 1 rats were initially subjected to PH 12 h before administration of DEN, IQ, captafol, α-HCH or DES, and then 2 weeks later placed on basal diet containing PB (0.05%) for 8 weeks. The timing of test compound administration, 12 h after PH, was based on our previous experiments.[17,35,36] One week from the start of PB feeding, all animals were given an i.p. injection of DGA at a dose of 300 mg/kg/body weight, dissolved in saline (5 ml/kg). Doses were chosen after preliminary experiments using 5 animals for each compound given 12 h after PH, allowing 4/5 survivals. Doses and routes of administration of all compounds are shown in Table I. Group 2 received saline or corn oil 12 h after PH and was given DGA and PB as in group 1. Group 3 was treated with test compounds after PH and DGA as in group 1 but not given PB. All surviving rats were killed at week 10 (Fig. 1).

In Experiment 2, as in Experiment 1, group 1 rats were initially subjected to PH and 12 h later were given OP, DDT or HCB. Then from week 3 they were subjected to feeding of CA (0.15%) mixed into the basal diet for 10 weeks. Two weeks from the start of CA feeding, rats were given CCl_4 (1 ml/kg dissolved in corn oil at 1:1 dilution) by gastric intubation. Survivors were killed at the end of week 12. Group 2 rats received dimethyl sulfoxide (DMSO, 2 ml/kg) or corn oil (5 ml/kg) as vehicle controls and were also subjected to the promotion procedure of CA and CCl_4. Group 3 rats were administered test compounds as in group 1 then placed on basal diet. CCl_4 was also given as in group 1 (Fig. 2). Dose and routes of administration of all compounds are shown in Table II.

Immediately after killing, the livers were excised and sections 2–3 mm thick were cut with a razor blade. Three slices, one each from the right posterior, caudate and anterior lobes, were fixed in ice-cold acetone for immunostaining with antibody to placental-form GST-P,[37] a sensitive marker for preneoplastic hepatocyte lesions in the rat.[11,12,38] The numbers and areas of GST-P+ foci of more than 3 cells in cross section (approximately 30 μm in diameter) were measured with the aid of a color video

image processor (VIP-21C, Olympus-Ikegami Tsushin Co., Ltd., Tokyo) (Fig. 3). The results were assessed in terms of quantitative data for GST-P$^+$ foci in groups 1 and 2. Group 3 served to assay potential for induction of GST-P$^+$ foci without the promotion treatment. Statistical analysis was carried out by using Student's t test. Differences were considered to be significant if $P<0.05$.

RESULTS

Quantitative data from Experiment 1 are summarized in Table I. Numbers and areas of GST-P$^+$ foci in the rats given DEN were significantly increased compared to saline control values in group 2 ($P<0.001$) in a dose-dependent manner. Similarly, levels in animals given IQ, captafol and DES were significantly increased compared to respective control values in group 2 ($P<0.001$ to 0.05). Rats given α-HCH showed a significant increase in number ($P<0.05$) as compared to group 2. However, the values for captafol, α-HCH, and DES were far lower than for DEN and IQ. In group 3, values for GST-P$^+$ foci induced with all the test compounds were significantly lower than in group 1 (Table I).

In Experiment 2, numbers of GST-P$^+$ foci in rats given OP and DDT in group 1 were significantly increased ($P<0.05$) compared to the respective DMSO and corn oil controls group 2. The number of foci in rats given HCB also showed a tendency for increase, although this was not significant (Table II).

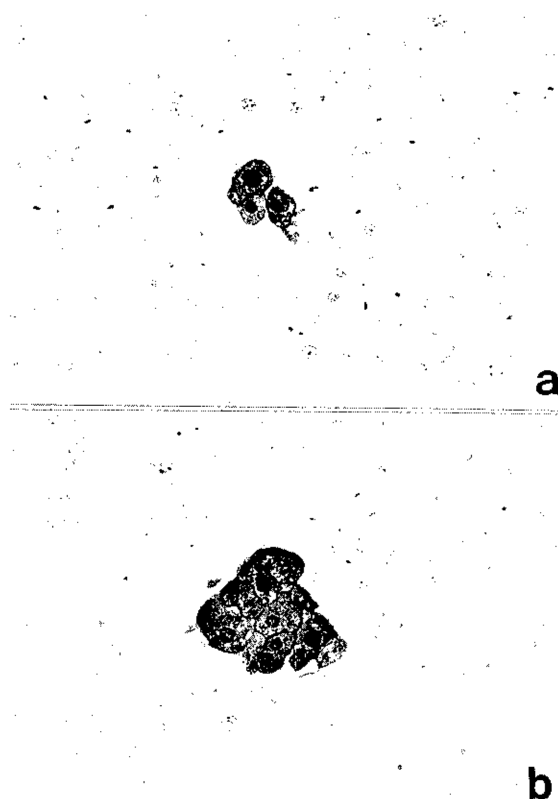

Fig. 3. Smallest (a) and average-sized (d) GST-P$^+$ foci counted for quantitative analysis.

Table I. Quantitative Values for GST-P$^+$ Foci in Experiment 1

Group	Treatment	Dose (mg/kg)	Promotion procedure	Vehicle[a]	Route	No. of rats	GST-P$^+$ foci[b] Number/cm^2	GST-P$^+$ foci[b] Area (μm^2)/cm^2
1	DEN	40	+	S	i.p.	14	59.02±10.02***	795387±251103***
	DEN	20	+	S	i.p.	13	51.86±13.75***	573570±211181***
	DEN	10	+	S	i.p.	15	46.88±10.41***	406449±120657***
	IQ	100	+	O	i.g.	15	14.95±5.10***	158599±67022***
	Captafol	300	+	O	i.g.	9	0.75±0.57	6435±5100*
	α-HCH	500	+	O	i.g.	14	0.35±0.25*	3443±3783
	DES	10	+	S	i.g.	15	0.57±0.25***	7844±7404**
2	Saline	5 ml/kg	+	S	i.p.	12	0.03±0.08	621±1473
	Corn oil	5 ml/kg	+	O	i.g.	14	0.13±0.13	2033±2156
3	DEN	40	−	S	i.p.	4	13.21±2.44	220366±50556
	DEN	20	−	S	i.p.	5	15.83±4.66	98221±58809
	DEN	10	−	S	i.p.	4	9.86±2.85	90603±29011
	IQ	100	−	O	i.g.	4	2.75±0.86	15499±7075
	Captafol	300	−	O	i.g.	4	0.22±0.12	2151±1497
	α-HCH	500	−	O	i.g.	4	0	0
	DES	10	−	S	i.g.	4	0	0

a) S, saline; O, corn oil.
b) Larger than 3 cells in cross section.
$P<0.05$ (*), 0.01 (**) or 0.001 (***) as compared to the respective control value in group 2.

Table II. Quantitative Values for GST-P+ Foci in Experiment 2

Group	Treatment	Dose (mg/kg)	Promotion procedure	Vehicle[a]	Route	No. of rats	GST-P+ foci[b] Number/cm²	Area (μm²)/cm²
1	OP	60	+	D	i.p.	4	3.84±1.84**	7174±1370
	DDT	50	+	O	i.g.	14	1.20±0.70**	3727±2708
	HCB	5000	+	O	i.g.	10	0.91±0.65	3836±3526
2	DMSO	2.5 ml/kg	+	D	i.p.	10	0.52±0.49	7978±3353
	Corn oil	5 ml/kg	+	O	i.g.	15	0.72±0.41	7969±8748
3	OP	60	−	D	i.p.	5	0.61±0.36	9248±12377
	DDT	50	−	O	i.g.	5	0.74±0.60	5844±4746
	HCB	5000	−	O	i.g.	4	0.79±0.41	5478±7087

a) D, DMSO (dimethyl sulfoxide); O, corn oil.
b) Larger than 3 cells in cross section.
$P<0.01$(**) as compared to the respective control value in group 2.

DISCUSSION

In the present study, in order to facilitate growth of initiated hepatocytes for their reliable detection and quantitation, we used the well-known promoting agent PB in Experiment 1 and CA[33, 39] in Experiment 2. Although their potencies as promoting agents are far less than that previously reported for 2-acetylaminofluorene,[17, 18, 40, 41] they were chosen because of the environmental hazard concomitant with dietary application of a strong carcinogen. The disadvantage in the present case could be compensated for by increasing the sensitivity of measurement by way of reducing the size of effective GST-P+ foci. In Experiment 1, the validity of the method for quantitative analysis, measuring GST-P+ foci larger than 3 cells in cross section, was confirmed by the observation of significant and dose-dependent induction of GST-P+ foci in response to low doses of the hepatocarcinogen DEN. A variety of other agents including the potent carcinogen IQ and captafol, α-HCH, and DES induced significant numbers of GST-P+ foci of a size smaller than reported for previous assay models.[14–16, 35)

Although it could be argued that such small lesions might not be directly relevant to hepatocellular carcinoma development, use of putative preneoplastic foci has become well established for assay purposes. Since even single GST-P+ hepatocytes positively correlate with exposure to various hepatocarcinogens,[42] it can be considered that larger GST-P+ foci are reliable indicators of carcinogenesis.[15, 16] In this context, it should be noted that the non-mutagenic compounds, α-HCH,[24] DES[25] and DDT[30] also induced significantly increased values for GST-P+ foci. This is in agreement with our previous report that ethyl carbamate (urethane), vinyl carbamate, safrole and dieldrin could induce preneoplastic γ-glutamyl transpeptidase-positive hepatocytes in a similar model in which test compounds were given after PH, followed by a potent promotion procedure comprising 2-acetylaminofluorene and CCl_4.[17]

With regard to positive testing of non-mutagens, the present results are in line with the increasing evidence of dissociation between mutagenicity and carcinogenicity. It has also been shown that mutagenicity may not always indicate initiation potential for *in vivo* carcinogenesis, although the number of compounds tested in these initiation assay protocols is still limited.[17, 35, 36, 43, 44]

Use of CA in the current model merits some comment. In a previous experiment aimed at the development of a screening assay system for promoting activity of environmental carcinogens, two species of bile acids, CA and deoxycholic acid, exerted positive effects as assayed by quantitation of γ-glutamyl transpeptidase or GST-P+ foci.[14, 33] CA also promoted carcinogenesis in the liver as well as other organs after initiation with N-nitrosomethylurea.[39]

Hepatotoxins cause necrosis followed by compensatory regeneration, which in turn stimulates proliferation of initiated as well as non-initiated cells. DGA and CCl_4 were chosen for this purpose on the basis of our previous observation that both agents result in significant levels of cell division.[34, 35, 45]

Previous studies have indicated an epidemiological association between ingestion of OP and an elevated incidence of esophageal cancer development among populations in north-east Iran.[28, 29, 46, 47] Laboratory studies showed that OP, a complex mixture containing compounds with a hydroxyphenanthrene moiety exhibiting strong mutagenicity in bacteria, can induce sister-chromatid exchanges in mammalian cells and transformed Syrian hamster embryo cells.[47, 48] OP was found to induce neoplastic lesions in the respiratory tract when applied topically to the trachea but proved negative in an initiation study of skin carcinogenesis.[47] The results of Experiment 2 are thus the first to show unequivocal

initiation activity for OP in rat liver carcinogenesis. Accordingly, since the initiation step may be less organ-specific than promotion in terms of determining the organ in which a tumor develops, it is possible that OP might also act as an initiator in sites other than the liver. Actually, epidemiological studies have implicated OP in esophageal cancer.[29, 46] Further studies by application of an appropriate promoter for esophageal carcinogenesis, or use of multi-organ carcinogenesis bioassay protocols in which OP could be given during the promotion stage, should clarify this point.[49, 50]

Since HCB has been shown to be carcinogenic to the mouse and hamster,[31, 32] our result does not allow a clear conclusion as to the validity of the protocol in Experiment 2.

The earlier observation that several conventional non-liver carcinogens such as benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene and 1,2-dimethylhydrazine could also initiate hepatocarcinogenesis under certain conditions[17] suggests that the current system might be applicable for the detection of initiating activity of carcinogens possessing organotropic sites other than the liver. However, since the reliable detection of initiation activity of weak initiating agents in the liver requires a strong promoter so that foci can become visible, further investigation to find non-hazardous promoting agents is required for optimization of the present initiation assay model. While the results are not conclusive as to which model is superior with regard to practical application, that used in experiment 1 appears to be advantageous because of its shorter duration.

Finally, since only a single administration of initiating agent is sufficient, the current system has a particular advantage when the quantity of available test compound is limited. For example, the total amount of OP required for this experiment was less than 200 mg. Furthermore, the model is economical because of its relatively short duration. Although only a few compounds have been tested so far, the preliminary evidence shown in the present report suggests that the system may find practical application, as with other models.

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