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ASSAY METHOD FOR ANTIHEPATOTOXIC ACTIVITY USING GALACTOSAMINE-INDUCED CYTOTOXICITY IN PRIMARY-CULTURED HEPATOCYTES[1]

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ABSTRACT.—Conditions were investigated to devise an *in vitro* assay method for antihepatotoxic activity using galactosamine-produced injury in primary-cultured mouse and rat liver cells. Employing 1.5-h preincubated hepatocytes prepared from rats, which were much more sensitive to the hepatotoxin, a satisfactory assay procedure was achieved. Some natural products known to exert liver-protective effects *in vivo* were subjected to screening by this *in vitro* assay method to reveal that cynarin, desoxypodophyllotoxin, glycyrrhetinic acid, glycyrrhizin, picroside II, and silybin possessed significant antihepatotoxic activity. The described assay method may be useful for primary screening of antihepatotoxic activity of materials of plant origin. The assay method has a number of advantages including the ability to dispose numerous samples at one time at a low cost, the requirement of small sample sizes, little variation, and good reproducibility of results.

As part of our investigation on clarification of liver-protective principles of crude drugs that have been alleged to be remedies for hepatitis, we have recently devised an assay method for antihepatotoxic activity using carbon tetrachloride (CCl_4)-induced cytotoxicity in primary cultured rat hepatocytes (1). This *in vitro* assay method was shown to be quite suitable for primary screening of antihepatotoxic activity of extracts, fractions, and constituents of plant origin, because (a) many samples can be screened at one time at a low cost, (b) the amounts of samples required are very small, (c) the variation of results is little, and (d) the reproducibility of results is good. Further, in order to examine the utility of this assay method, some natural products known to possess antihepatotoxic activity using *in vivo* assay methods (2), were screened. The results of this *in vitro* assay method are comparable with those *in vivo* assay methods (1).

The model system of liver damage produced by D-galactosamine (GalN) in rats is now recognized to be much like viral hepatitis in humans from both the morphological and functional points of view (3). Further, the mechanisms of GalN-induced liver injury have been extensively examined (4-7). As a result, this model is now accepted as one of the most authentic test systems of liver damage in experimental animals.

However, because this GalN-induced liver lesion can be made only in rats but not in mice, it may not be a practical method to screen materials of plant origin from the standpoint of expense and sample size. Therefore, subsequent to establishment of a model system of liver lesion by CCl_4 for screening of antihepatotoxic activity, development of another test model system of liver lesion by GalN, which can be utilized for pharmacological evaluation, was required.

Recently, Schanne *et al.* (8) showed that adding GalN to primary-cultured rat hepatocytes produced cytotoxicity that was inhibited by uridine and chlorpromazine and, thus, concluded that the *in vitro* cytotoxicity occurs in the same way as the *in vivo* cytotoxicity. In order to devise a suitable test method for screening of antihepatotoxic activity, we have carried out an examination of the conditions for induction and inhibition of cytotoxicity by GalN.

[1]Liver-Protective Drugs, Part 7. This paper is also part 52 in the series on the validity of oriental medicines.

MATERIALS AND METHODS

ANIMALS.—Male Std:ddY mice (20-25 g) and male Std:Wistar rats (200-250 g) were used.

MATERIALS.—The following compounds were purchased: bovine serum albumin Fr. V (Sigma), calf serum (Flow Lab.), collagenase type I (125-250 IU/mg, Sigma), cynarin (Roth), dexamethasone (Sigma), Eagle's minimum essential medium (Eagle's MEM, Nissui), ethyleneglycol-*bis*-(β-aminoethylether) *N,N'*-tetraacetic acid (EGTA, Dotite), D-galactosamine (Sigma), glycyrrhetinic acid (Tokyo Kasei), glycyrrhizin (Minophagen), insulin (Novo Research), methionine (Wako), picroside I, picroside II, potassium penicillin G (Meiji Seika), silybin (Roth), and streptomycin sulfate (Meiji Seika).

Desoxypodophyllotoxin was isolated from podophyllin powder (from *Podophyllum hexandrum*, E. Merck) (9).

ISOLATION AND CULTURE OF HEPATOCYTES.—Liver cells were isolated by a modified procedure of Seglen (10): The abdomen of the mouse or rat was opened under ether anesthesia. A midline incision was made, and the portal vein was cannulated with a needle fitted with a teflon catheter. After the teflon catheter was tied in place and the needle removed, the inferior vena cava was cut below the renal vein. Perfusion of the liver was started with Ca^{2+}-free Hanks' buffer (11), which contained 1% bovine serum albumin Fr. V and 0.5 mM EGTA and was aerated with 95% O_2/5% CO_2 to pH 7.4 at 37°. The flow rate was 30 ml/min. The thoracic portion of the superior vena cava was cannulated, and the inferior vena cava was tied off above the renal vein. After the liver had been perfused for 10 min, recirculation of Ca^{2+}-free Hanks' buffer (100 ml), which contained, additionally, 0.075% collagenase and 4 mM $CaCl_2$, was started. In the case of mice, the same buffer was poured into the inferior vena cava and drained from the hepatic portal vein at the flow rate of 10 ml/min.

After 10-15 min of perfusion, the liver was transferred into a beaker containing Ca^{2+}-free Hanks' buffer (50 ml) and gently dispersed with two forceps. Next, the crude cell suspension was rotated on a rotater under oxygen-carbon dioxide at 37° for 10 min. Then, the cell suspension was cooled on ice and gently filtered through cotton gauze into centrifuge tubes. Finally, the preparation was centrifuged at 50 g for 1 min (for mice, 30 g, 1 min). The supernatant was aspirated off, and the loosely packed pellet of cells was gently resuspended in Ca^{2+}-free Hanks' buffer. This washing procedure was repeated three to five times. Viability of the cells to exclude the dye, trypan blue, was determined by incubating cell suspension (0.1 ml) with 0.4% trypan blue (0.9 ml) and then counting the number of cells that excluded the dye and the number of cells whose nuclei were stained blue.

The culture medium was composed of Eagle's MEM, supplemented with 10% inactivated (56° for 30 min) calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), 10^{-6}M dexamethasone, and 10^{-8}M insulin. Inocula of 5×10^4 cells/0.1 ml/cm^2 were seeded into plastic dishes (Falcon Plastics) and preincubated in a humidified incubator at 36.5° under 5% CO_2 in air for 1.5 or 24 h. When preincubated for 24 h, the medium was replaced after 6 h.

Viability of cultured cells was assayed by trypan blue exclusion. After the direct addition of 0.4% trypan blue, the number of cells, stained and unstained, was counted.

DETERMINATION OF CYTOTOXICITY INDUCED BY GALACTOSAMINE.—In the medium (1.0 ml), to which a sample of dimethylsulfoxide (0.01 ml) was added, 0.1-0.5 mM GalN was dissolved.

After preincubation (1.5 or 24 h), the cells were exposed to the above-prepared medium containing GalN and a sample. At the indicated times after the GalN challenge, glutamic-pyruvic transaminase (GPT) activity in the medium was measured by the method of Karmen (12) using an autoanalyzer (RaBA super, Chugai Pharmaceutical).

As the reference, a similar experiment was conducted (12), except that we did not use GalN in the case of examining the sole action of a sample to hepatocytes.

STATISTICAL ANALYSIS.—The data are shown in mean±S.E. and, statistical significances were evaluated by one-way analysis of variance.

RESULTS

PREPARATION AND CULTURE OF HEPATOCYTES.—Yields of $2\text{-}4\times10^7$ cells/liver with over 90% viability, in the case of mice, and $2\text{-}4\times10^8$ cells/liver with 85-95% viability, in the case of rats, were routinely obtained. We observed practically no contamination with nonparenchymal cells. When placed in the culture medium, isolated hepatocytes attached to the surface of plastic dishes within 60 min and completely within several hours to form a monolayer. Cell attachment was facilitated by prior addition of 10^{-6}M dexamethasone and 10^{-8}M insulin in the medium.

GALACTOSAMINE-INDUCED DAMAGE OF CULTURED HEPATOCYTES.—The time-course and dose-response of GPT activity in the culture medium after GalN challenge to mouse and rat hepatocytes preincubated for 1.5 and 24 h are shown in figures 1 and 2.

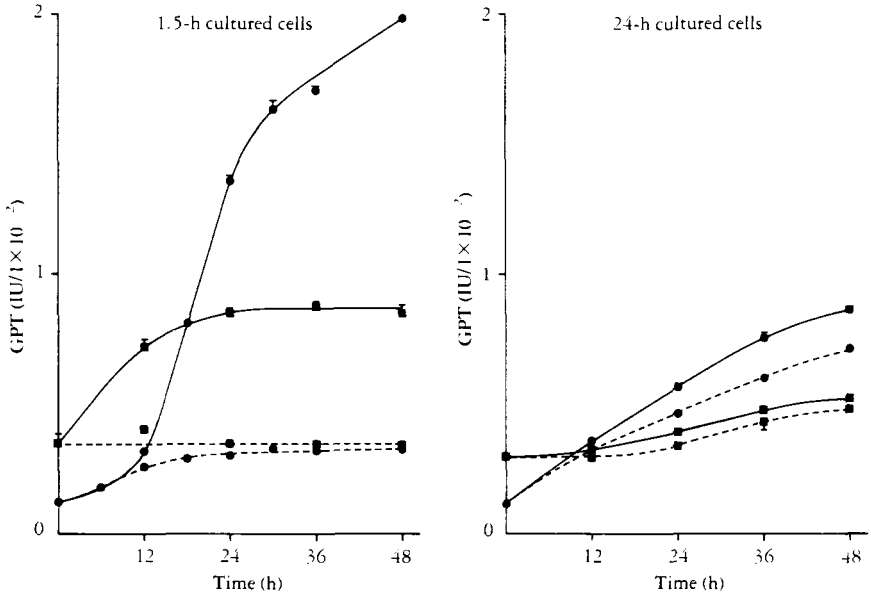

FIGURE 1. Time dependence of galactosamine-induced cytotoxicity in primary cultured hepatocytes; ■=mouse, ●=rat.
After initial 1.5- or 24-h incubation, liver cells were exposed to 0.5 mM GalN in fresh medium, and GPT activity was determined periodically. Solid lines indicate the GPT values in the GalN media, and broken lines indicate those in the control media; n=3 (dishes). One side of the standard error is shown.

Increase of GPT activity in the medium by GalN challenge was significant in rat hepatocytes preincubated for 1.5 h. The GPT value increased markedly after about 12 h and reached its peak, by slow increments, after about 30 h. Dose-dependency was noticed between 0.1 and 0.5 mM of GalN. No remarkable increase of GPT activity in the medium caused by GalN challenge was observed in rat hepatocytes preincubated for 24 h. On the other hand, GPT activity was only very slightly elevated by GalN in mouse hepatocytes preincubated for 1.5 and 24 h.

Change of the viability of rat hepatocytes by addition of GalN was assessed by trypan blue exclusion. As indicated in table 1, significant reduction of the viability by GalN was found in hepatocytes preincubated for 1.5 h, but almost no reduction of the viability caused by GalN was observed in those preincubated for 24 h.

Influence of dexamethasone and insulin on the increase of GPT activity by GalN was measured next. After 1.5 h preincubation in the medium without dexamethasone and insulin, the cells were exposed to 0.5 mM GalN in the medium in the absence or presence of 10^{-6}M dexamethasone or 10^{-8}M insulin. GPT activity in the medium at 30 h thereafter demonstrates that the increase of GPT activity by GalN (100±8 IU/liter) was not affected by the presence of dexamethasone (96±4 IU/liter) or insulin (96±3 IU/liter).

ANTIHEPATOTOXIC ACTIVITY OF KNOWN LIVER-PROTECTIVE NATURAL PRODUCTS.—After preincubation of isolated rat hepatocytes for 1.5 h, cynarin, desoxypodophyllotoxin, glycyrrhetinic acid, glycyrrhizin, methionine, picroside I, pic-

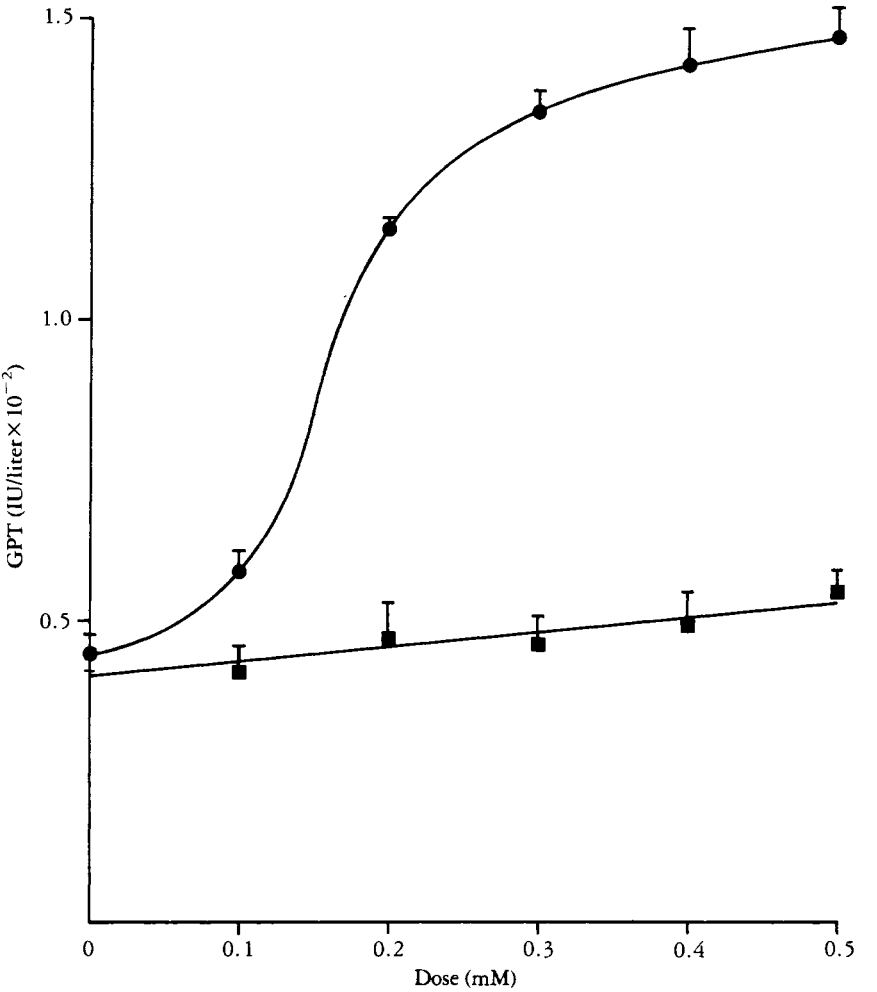

FIGURE 2. Dose dependence of galactosamine-induced cytotoxicity in primary cultured hepatocytes; ■=mouse, ●=rat.
After initial 1.5-h incubation, liver cells were exposed to GalN in fresh medium, and GPT activity was determined 30 h thereafter; n=3 (dishes). One side of the standard error is shown.

roside II, and silybin were added at the doses of 0.01, 0.1, and 1.0 mg, with or without 0.5 mM GalN, to the culture medium (1.0 ml). GPT activity was measured at 30 h after the treatment. The results are shown in table 2.

DISCUSSION

In order to devise a suitable *in vitro* assay method for antihepatotoxic activity employing GalN-produced injury in primary cultured hepatocytes, a number of conditions were examined.

Because the transaminases (GOT and GPT) in the hepatocytes were released into blood concomitant with liver injury (13) and because the activity of both in the serum is in good accord with the extent of liver damage (14), the determination of transaminase activity in the serum is widely adopted in the diagnosis of liver lesion. Hence, the cell injury induced by GalN was evaluated by means of GPT activity, which is easily measured and corresponds well with hepatitis.

Liver lesions produced by GalN in mouse and rat hepatocytes preincubated for 1.5 and 24 h were thus investigated by monitoring GPT activity in the culture medium.

TABLE 1. Viability of rat hepatocytes

Period (h)	Treatment	Viability (%)	
		1.5-h preincubated cells	24-h preincubated cells
0		97	98
30	none	98	95
	GalN 0.5 mM	5	93

As shown in figures 1 and 2, GalN challenge caused a remarkable increase of GPT activity in the case of rat liver cells preincubated for 1.5 h, but produced only slight increases of the enzyme activity in the case of those preincubated for 24 h. This indicates that cultured liver cells preincubated for 1.5 h are much more sensitive to GalN cell damage. Although the reasons for this sensitivity cannot be well explained, a possibility claimed by Ichihara *et al.* (15,16) that membrane injury suffered at the isolation of liver cells has not yet been completely recovered within 1.5 h may be one of the reasons. It is reported that increase of Ca^{2+} in hepatocytes is related to their death induced by

TABLE 2. Effect of natural products on galactosamine-induced cytotoxiciy in primary-cultured rat hepatocytes (n=3 dishes)

Substance	Dose (mg/ml)	GPT		
		GalN		none
		IU/liter	%	IU/liter
Control	—	131±4	100	37±2
Cynarin	0.01	125±3	95	20±2[a]
	0.1	110±5[a]	84	27±2
	1.0	63±2[b]	48	51±2
Desoxypodophyllotoxin	0.01	122±4	93	43±1
	0.1	66±2[b]	50	35±1
	1.0	121±7	92	137±5[d]
Glycyrrhetinic acid	0.01	134±9	102	26±3
	0.1	76±8[b]	58	16±2[a]
	1.0	35±2[b]	27	42±1
Glycyrrhizin	0.01	135±8	103	24±4
	0.1	131±7	100	59±4[c]
	1.0	31±1[b]	24	21±0[a]
Methionine	0.01	140±2	106	24±1[a]
	0.1	148±1[c]	113	28±2
	1.0	160±6[c]	122	25±3
Picroside I	0.01	126±6	96	24±2
	0.1	160±2[d]	122	50±1[c]
	1.0	145±3[c]	111	131±4[d]
Picroside II	0.01	135±6	103	27±2
	0.1	132±4	101	28±1
	1.0	102±1[b]	78	40±3
Silybin	0.01	140±6	106	29±2
	0.1	134±4	103	157±1[d]
	1.0	86±3[b]	66	93±2[d]

[a]Significantly different from the control, effective $p<0.01$
[b]Significantly different from the control, effective $p<0.001$
[c]Significantly different from the control, toxic $p<0.01$
[d]Significantly different from the control, toxic $p<0.001$

GalN (8). Therefore, it is also possible that cultured liver cells preincubated for 24 h are more resistant to Ca^{2+} influx. Further, because the content of cytochrome P-450 in cultured hepatocytes is known to diminish after 24-h culture (17-19), it is possible that inactivation of enzyme systems that link with biochemical sequences participating in the liver lesion caused by GalN may similarly occur, and consequently liver damage may not be readily observable by GalN challenge after 24-h culture.

On the other hand, a lesser increase of GPT activity was found by treatment with GalN in cultured mouse hepatocytes. This is in accord with the previous reports that a model system of liver damage was not produced in mice *in vivo* (4).

Although addition of dexamethasone and insulin to the culture medium is reported to be favorable for maintaining the viability of cultured hepatocytes (15,16), these chemicals may affect GalN-induced cytotoxicity. However, the magnitudes of liver injury determined by GPT activity were found to be essentially identical in both the media with and without 10^{-6}M dexamethasone or 10^{-8}M insulin. Therefore, the described experiments were performed with the culture medium containing dexamethasone and insulin in order to maintain better viability of cells.

The above data led to contrivance of a satisfactory assay procedure consisting of preincubation of hepatocytes in Eagle's MEM containing 10% inactivated calf serum, dexamethasone, and insulin for 1.5 h, addition of 0.5 mM GalN and a sample, and determination of GPT activity in the medium at 30 h thereafter.

After examinations of the mechanism of liver lesion produced by GalN using primary cultured rat hepatocytes, Schanne *et al.* (8) reported that the mechanisms *in vitro* are similar to those *in vivo*. Therefore, it was expected that this *in vitro* assay method would give results similar to those obtained by *in vivo* assay methods. In order to evaluate the utility of this assay method, antihepatotoxic actions of the natural products known to exert liver-protective effects *in vivo* (2) were determined by means of the presently devised procedure utilizing GalN-intoxicated hepatocytes *in vitro*.

As a result, we found that cynarin, desoxypodophyllotoxin, glycyrrhetinic acid, glycyrrhizin, picroside II, and silybin significantly prevented the increases of GPT activity by GalN. Desoxypodophyllotoxin (9), glycyrrhizin (20), and silybin (21,22), which have been reported to exhibit inhibitory activity against GalN-produced liver lesion *in vivo*, showed significant preventive activity against GalN-induced injury in primary cultured liver cells, indicating that this *in vitro* assay may correlate with other *in vivo* measures of antihepatotoxicity.

As evident from table 2, the preventive effect of desoxypodophyllotoxin was not dose-dependent. Thus, the least GPT value was observed at 0.1 mg/ml, and a higher GPT value was found at 1.0 mg/ml. When the cells were preincubated for 1.5 h, desoxypodophyllotoxin alone was added to the medium, and the cells were cultured for a further 30 h. A much higher GPT value was obtained in the group at 1.0 mg/ml than in that at 0.1 mg/ml, indicating that desoxypodophyllotoxin rather disclosed some cytotoxicity at higher concentrations. Since glycyrrhizin, picroside I, and silybin also exhibited some cytotoxicity at certain concentrations, it is concluded that these substances possess both cytotoxic and antihepatotoxic actions and, therefore, GPT activity in the medium represents the balance of both actions, although this reversely-directed action may occur *in vivo*. Hence, the assay method for antihepatotoxic activity using primary-cultured rat hepatocytes *in vitro* is concluded to be useful for primary screening of antihepatotoxic activity of materials of plant origin.

Like the previously reported assay method for antihepatotoxic activity using CCl_4-induced cytotoxicity (1), this assay method employing GalN-induced cytotoxicity has many advantages, for example, a large number of samples can be tested at one time at

low expense, sample sizes can be much reduced, and there is little variation and good reproducibility of results with this method.

Screening of antihepatotoxic principles in plants utilizing this recently devised *in vitro* assay method is now underway.

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