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Effect of Sho-saiko-to Extract on HGF and TGF-β Levels of Intraorgans in Liver-injured Rats after Partial Hepatectomy

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Abstract

To examine the effects of Sho-saiko-to extract on liver regeneration, Sho-saiko-to extract (0·75%, 1·5% or 3%) was administered to 70% partial hepatectomized rats with dimethylnitrosamine-induced liver-injury.

S phase cell number, liver retinoid levels, hepatocyte growth factor (HGF) and transforming growth factor-beta (TGF-β) levels in each intraorgan were measured as indicators of liver regeneration. Three to seven days after hepatectomy, HGF and TGF-β levels of the liver and spleen of the Sho-saiko-to extract groups were significantly different from the levels of the ordinary food group ($P < 0{\cdot}05-0{\cdot}1$). HGF levels in the Sho-saiko-to extract groups were approximately 1·3–1·8 times higher in the liver and approximately 1·8–2·1 times higher in the spleen compared with the levels found in the ordinary food group. TGF-β levels in the Sho-saiko-to extract groups were approximately 0·38–0·47 times the level in the liver and 0·58–0·77 times the level in the spleen of the ordinary food group. There was no difference in HGF and TGF-β levels of the kidney and lung between the Sho-saiko-to extract group and the ordinary food group. There was a significant and positive correlation between HGF level and S phase cell number in the liver ($r = 0{\cdot}826$, $P < 0{\cdot}01$). There was a significant and negative correlation between TGF-β level and the retinoid level in the liver ($r = -0{\cdot}696$, $P < 0{\cdot}01$). In addition, the levels of the active constituents of Sho-saiko-to extract (glycyrrhetic acid, baicalin and baicalein) showed high values in the liver and spleen of partial hepatectomized rats, and increased from the third day after partial hepatectomy.

These results show that Sho-saiko-to extract induces liver regeneration by increasing the production of HGF and suppressing the production of TGF-β in the liver and spleen of partial hepatectomized rats. It was considered that the increase in the Sho-saiko-to extract active constituent levels in the liver and spleen greatly influences this action.

Liver regeneration after partial hepatectomy is an important factor, influencing the prognosis in hepatocirrhosis and liver cancer treatment. From the standpoint of cell growth, hepatocirrhosis can be explained as an uncontrolled proliferation of parenchymal and non-parenchymal cells in the liver (Miyazawa 1993). Therefore, various attempts have been made to prevent or treat hepatocirrhosis by controlling cell proliferation. We reported previously that Sho-saiko-to extract was useful for the repair of liver injury, improvement of fibrotic changes of the liver and promoting liver regeneration, and this improvement seems to depend on the suppression of the activation of Ito cells (Miyamura et al 1998). Nakamura et al (1984) reported that hepatocyte growth factor (HGF) is involved in stimulating DNA synthesis in liver regeneration. When an injury was generated in the liver, the expression of HGF mRNA was rapidly increased in non-parenchymal cells such as Ito cells, and played a role in the paracrine mechanism of parenchymal cells in promoting liver regeneration. In the spleen, lung or kidney, expression of HGF was also increased, and liver regeneration was occurring via the endocrine mechanism through the blood (Kinoshita et al 1989, 1991). Transforming growth

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factor-beta (TGF-β) which is the contradictory hepatocyte inhibitor, also appeared when an injury was generated in the liver, and it played a role in control of liver regeneration (Russell et al 1988). Therefore it is necessary to examine the relationship between the production of HGF and TGF-β and liver regeneration.

In this study, we have examined the changes in S phase cell number, liver retinoid level, and HGF and TGF-β levels of each intraorgan after administration of Sho-saiko-to extract in partial hepatectomized rats. We have attempted to elucidate their relationship.

Materials and Methods

Animals

Male Wistar rats, 7-weeks-old, 180–200 g, were purchased from NSC Japan. Animals were acclimatized for seven days at 23 ± 2 °C with free access to pellet food (CE-2, Crea Japan) and water. Healthy rats were then selected and they were assigned to each group.

Materials

Dimethylnitrosamine was from Nacarai Tesque, pentobarbital sodium from Dainippon Pharmaceutical, Sho-saiko-to extract from Tsumura, and bromodeoxyuridine from Wako Pure Chemicals. Glycyrrhizin, glycyrrhetic acid, baicalin and baicalein were from Nacarai Tesque. Other reagents were extra-pure grade from commercial sources.

Preparation of the liver-injured and hepatectomized rats

To induce liver injury the acclimatized rats were administered an intraperitoneal injection of 35 mg kg^{-1} dimethylnitrosamine. Seven days after this treatment the rats, under anaesthesia, underwent resection of approximately 70% of the liver according to the method of Higgins & Anderson (1931).

Administration of Sho-saiko-to extract

Sho-saiko-to extract was administered in pellet food by adding the extract to the food at concentrations of 0·75%, 1·5% or 3%. The groups to be administered Sho-saiko-to extract were fed with this food immediately after hepatectomy. The control group was fed with ordinary food.

Extraction and measurement of intraorgan HGF

After removal, intraorgans (approximately 0·5 g) were weighed, and homogenized with a cell-homogenizer (Eilard) in 2 mL 20 mM Tris–HCl buffer (pH 7·5; containing 0·1% Tween 80, 1 mM phenylethyl sulphonyl fluoride, 1 mM EDTA) at 3000 *g* for 1 min in an ice bath. The homogenate was centrifuged (7000 *g*) for 30 min at 4°C and an intermediate layer of 1 mL was collected. The intermediate layer was applied to the heparin column (Pharmacia Biotech) and it was made to adsorb a 4 mL 20 mM Tris–HCl buffer (pH 7·5) containing 0·5 M NaCl. It dissolved out at 5 mL 20 mM Tris–HCl buffer (pH 7·5) containing 2 M NaCl, and collected in the column outflow solution, 1·5 mL between 0·5–2 mL, which was taken to be the HGF assay measurement sample.

The amount of HGF in the intraorgans was determined following an ELISA method by using a multi-plate-type reader (Corona MTP-32) reported by Tsubouchi et al (1991).

Counting the number of S phase cells

Bromodeoxyuridine (40 mg kg^{-1}) was injected intraperitoneally into rats 1, 3, 5, 7, 10 and 14 days after partial hepatectomy. After 1 h the rat was killed under ether anaesthesia and the liver was removed, quickly fixed with 70% ethanol, dehydrated, embedded in paraffin and sectioned. After removal of the paraffin the section was treated with 4 M HCl for monomerization of DNA. The slices were then washed with phosphate buffer (pH 6–8) and S phase nuclei were stained with 3,3-diaminobenzidine using anti-bromodeoxyuridine monoclonal antibody (Becton Dickinson) by the avidin–biotin peroxide complex method. The number of S phase cells/500 cells was counted under a microscope, and the cell-labelling rate was calculated.

Extraction and measurement of intraorgan TGF-β

Each removed intraorgan (approximately 0·2 g) was weighed, and homogenized with a cell-homogenizer (Eilard) in RD51 (R&D) 1 mL at 3000 *g* for 1 min in an ice bath. The homogenate was centrifuged (7000 *g*) for 30 min at 4°C and 1-mL supernatant was collected. To a 500-μL sample of supernatant was added 100 μL 1 M HCl. This was then incubated for 10 min at room temperature. After incubation, the sample was neutralized (pH 7·2–7·6) by adding 100 μL 1·2 M NaOH/0·5 M HEPES. The sample was then ready for the TGF-β assay.

The amount of TGF-β in the intraorgans was determined following an ELISA method using

recombinant human TGF receptor Type II. Becker et al (1997) has reported the correlation with rats.

Determination of retinoid level in the liver

Retinol palmitate in the liver was measured and expressed as retinoid concentration (Miyamura et al 1998).

Measurement of active constituents of Sho-saiko-to extract in the intraorgans

Although the concentration of glycyrrhizin measured as the active constituent of Sho-saiko-to in the liver was below the detection limit, glycyrrhetic acid (an aglycon) was measured. The liver concentrations of baicalin and baicalein were measured and expressed as combined molarities; Uchida et al (1995) reported their interconversion by in-vivo enzymes.

The amount of each active constituent in the liver was determined by HPLC methods reported by Miyamura et al (1998).

Statistical analysis

Comparison of results from different groups was performed by one-way analysis of variance. Where there was significant variance, the means were compared by the Bonferroni method. The correlation coefficient was determined by a *t*-test.

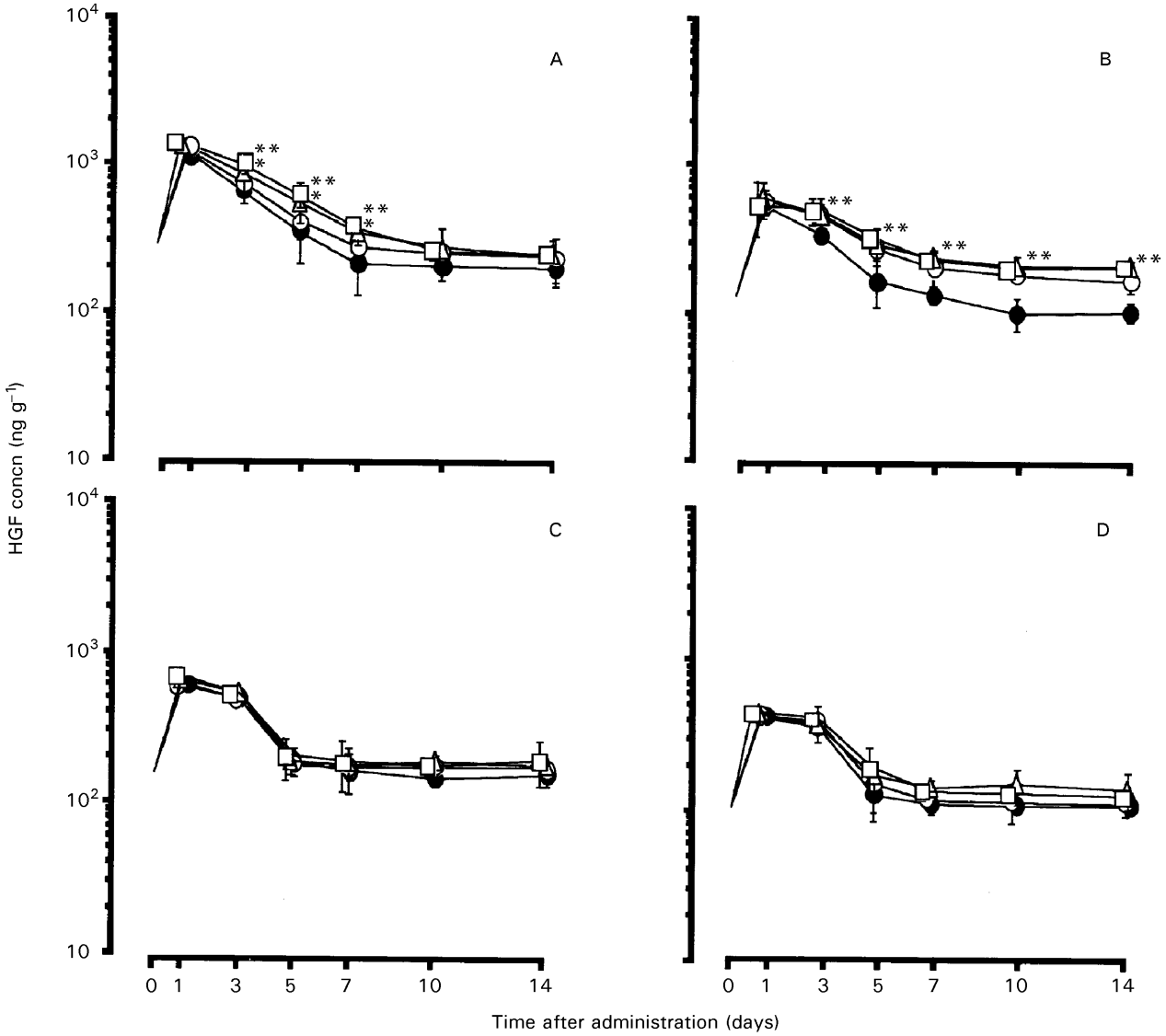

Figure 1. Effect of oral administration of different concentrations of Sho-saiko-to extract on the HGF level in intraorgans of rats with injury after partial hepatectomy. A, liver; B, spleen; C, kidney; D, lung. Sho-saiko-to extract concentrations: 0·75% ○, 1·5% △, 3·0% □, ordinary food ●. * $P < 0{\cdot}1$, ** $P < 0{\cdot}05$, compared with the ordinary food groups. Each point represents the mean ± s.e. of seven experiments.

Results

The effect of the Sho-saiko-to extract on each intraorgan HGF level after partial hepatectomy

In the Sho-saiko-to extract group and the ordinary food group, the HGF levels in the liver reached a peak on the first day after hepatectomy and rapidly decreased thereafter (Figure 1). Three to seven days after partial hepatectomy, the HGF levels of Sho-saiko-to extract groups were higher than in the ordinary food group ($P < 0{\cdot}1-0{\cdot}05$). The values for the Sho-saiko-to extract groups increased approximately 1·3–1·8 times as the dose was increased, compared with the ordinary food group. The HGF levels in the spleen reached a peak on the first day after hepatectomy and decreased thereafter. Three to seven days after partial hepatectomy, the HGF levels in the Sho-saiko-to extract groups were higher than in the ordinary food group ($P < 0{\cdot}05$). The values for the Sho-saiko-to extract groups increased approximately 1·8–2·1 times as the dose was increased compared with the ordinary food group. In the Sho-saiko-to extract group and the ordinary food group, the HGF levels of the kidney and lung rapidly increased on the first day after partial hepatectomy and it rapidly decreased after the third day. The HGF values in the kidney and lung were lower than in the liver.

Changes in the number of S phase cells of the liver after partial hepatectomy

The number of S phase cells was affected by Sho-saiko-to extract, the rate of increase in the Sho-saiko-to extract group being higher than in the ordinary group ($P < 0{\cdot}05$; Table 1). The peak of S phase cells occurred 3–7 days after administration. On the third day of administration the rate of increase in the number of S phase cells was elevated as the dose was increased.

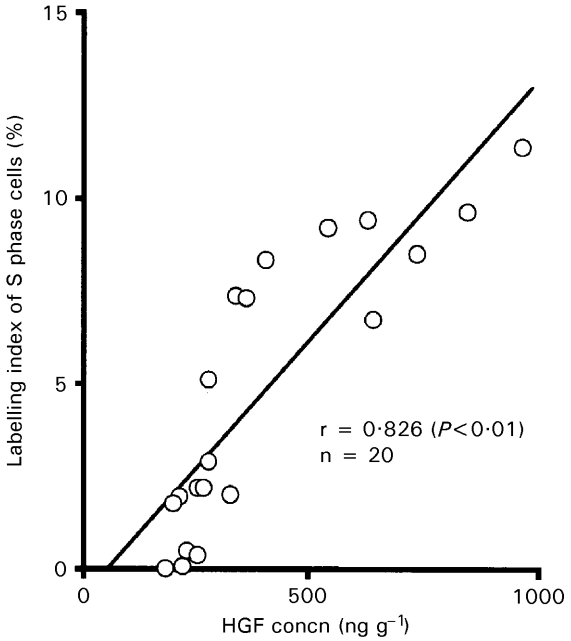

Figure 2. Correlation of HGF level and S phase cell labelling index in the liver of rats with injury after partial hepatectomy.

Correlation of HGF level in the liver and S phase cell numbers of the liver-injured rats after partial hepatectomy

The correlation coefficient was obtained from the values 3–14 days after partial hepatectomy. There was a significant and positive correlation ($r = 0{\cdot}826$, $P < 0{\cdot}01$; Figure 2) between the HGF level and S phase cell numbers in the liver.

The effect of the Sho-saiko-to extract on each intraorgan TGF-β level after partial hepatectomy

In the Sho-saiko-to extract group and the ordinary food group, the TGF-β level in the liver reached a peak on the first day after hepatectomy and rapidly decreased thereafter (Figure 3). Three to seven days

Table 1. Effect of different concentrations of Sho-saiko-to extract on the bromodeoxyuridine labelling index in liver-injured rats after partial hepatectomy.

Dose of Sho-saiko-to extract (%)	Labelling index (%)					
	1 day	3 days	5 days	7 days	10 days	14 days
0·00	5·70 ± 0·10	6·68 ± 0·30	2·31 ± 0·18	2·00 ± 0·10	1·80 ± 0·10	nd
0·75	5·50 ± 0·05	8·50 ± 0·10*	8·40 ± 0·10*	5·10 ± 0·30*	2·20 ± 0·05	nd
1·50	6·00 ± 0·05	9·60 ± 0·50*	9·17 ± 0·31*	7·40 ± 0·53*	2·80 ± 0·10	0·51 ± 0·05
3·00	6·30 ± 0·10	11·30 ± 0·70*	9·10 ± 0·10*	7·30 ± 0·10*	2·20 ± 0·15	0·47 ± 0·10

Each value is the mean ± s.e. of results from seven experiments. *$P < 0{\cdot}05$, compared with the ordinary food group. nd, not detectable.

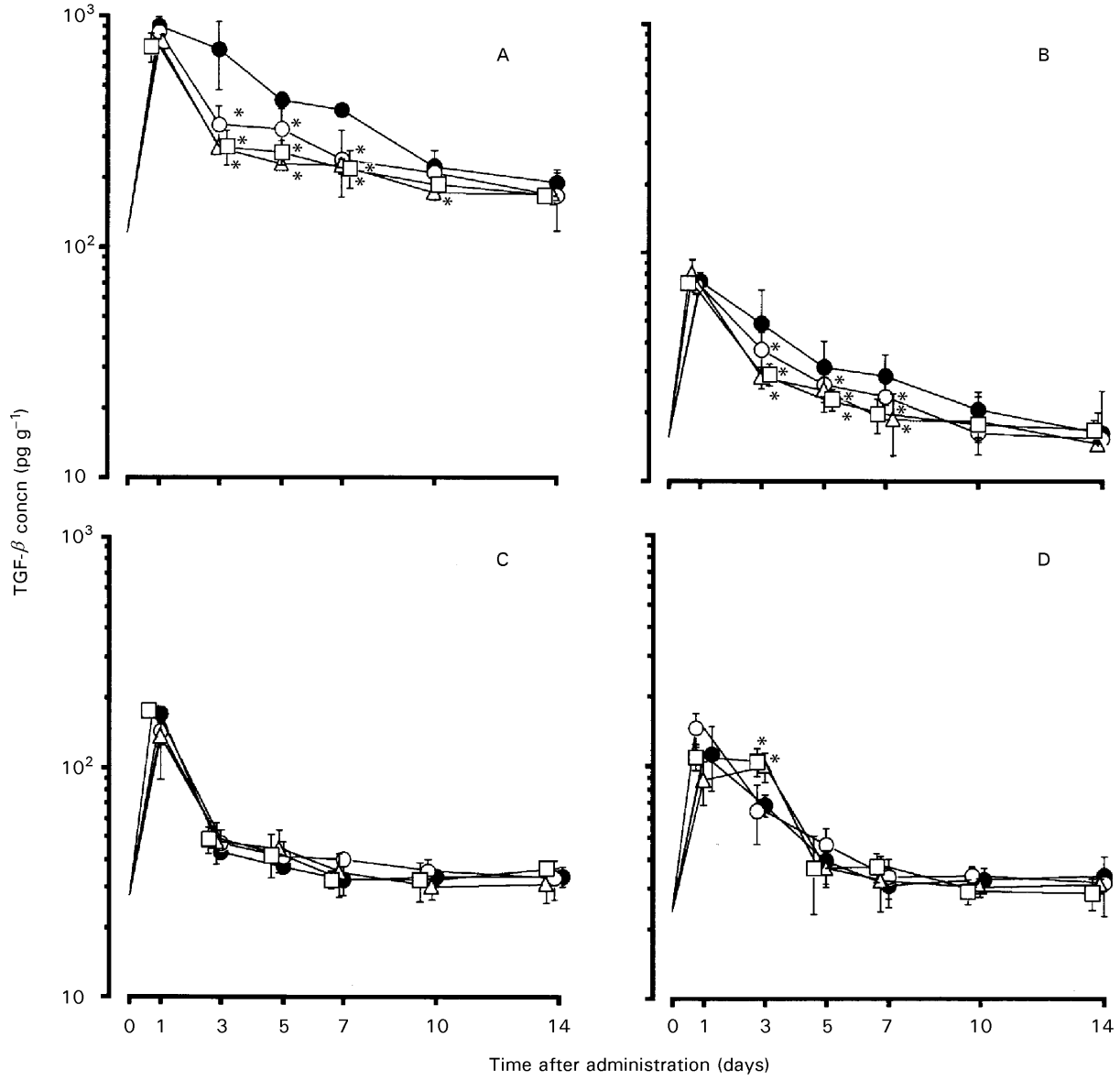

Figure 3. Effect of oral administration of different concentrations of Sho-saiko-to extract on the TGF-β level in intraorgans of rats with injury after partial hepatectomy. A, liver; B, spleen; C, kidney; D, lung. Sho-saiko-to extract concentrations: 0·75% ○, 1·5% △, 3·0% □, ordinary food ●. * $P < 0{\cdot}05$, compared with the ordinary food groups. Each point represents the mean ± s.e. of seven experiments.

after partial hepatectomy, the TGF-β level in the Sho-saiko-to extract groups were lower than in the ordinary food group ($P < 0{\cdot}05$). The levels of TGF-β in the Sho-saiko-to extract groups decreased approximately 0·38–0·47 times as the dose was increased compared with the ordinary food group. The TGF-β levels in the spleen reached a peak on the first day after hepatectomy and decreased thereafter. Three to seven days after partial hepatectomy, the TGF-β levels in the Sho-saiko-to extract groups were lower than in the ordinary food group ($P < 0{\cdot}05$). The TGF-β levels for the Sho-saiko-to extract groups decreased approximately 0·58–0·77 times as the dose was increased compared with the ordinary food group. The kidney and lung TGF-β levels increased after partial hepatectomy and then rapidly decreased. The levels in the kidney and lung were lower than the levels in the liver. There was no difference in the kidney and lung levels of TGF-β between the groups receiving Sho-saiko-to or the ordinary food.

Effect of Sho-saiko-to extract on the liver retinoid levels in rats after partial hepatectomy

Higher liver retinoid levels were found after administration of Sho-saiko-to extract compared with the ordinary food group ($P < 0{\cdot}05$; Table 2).

Table 2. Effect of Sho-saiko-to extract on liver levels of retinol palmitate in liver-injured rats after partial hepatectomy.

Dose of Sho-saiko-to extract (%)	Retinol palmitate (%)						
	Before hepatectomy	1 day	3 days	5 days	7 days	10 days	14 days
0·00	19 ± 3	17 ± 2	9 ± 2	10 ± 3	13 ± 2	17 ± 1	19 ± 1
0·75		17 ± 3	19 ± 2*	27 ± 2*	39 ± 2*	40 ± 3*	43 ± 5*
1·50		20 ± 1	20 ± 2*	38 ± 5*	40 ± 3*	43 ± 2*	50 ± 3*
3·00		22 ± 1	25 ± 2*	40 ± 2*	40 ± 2*	41 ± 2*	47 ± 7*

Each value is the mean ± s.e. of results from seven experiments. *$P < 0.05$, compared with retinoid values of ordinary food group.

Liver retinoid level was higher after administration of higher doses of the Sho-saiko-to extract.

Correlation of TGF-β level and retinoid level in the liver of liver-injured rats after partial hepatectomy
The correlation coefficient was obtained from values 3–14 days after partial hepatectomy. There was a significant and negative correlation ($r = -0.696$, $P < 0.01$; Figure 4) between TGF-β level and the retinoid level in the liver.

Changes in the levels of Sho-saiko-to active constituents in the intraorgans
The glycyrrhetic acid level and baicalin + baicalein level in the liver and the spleen increased from the third day after partial hepatectomy (Table 3). The rate of increase from day 3 to day 7 was greater than at the later times. Although there was an increase in the level of active constituents with increased extract dose, there was no significant difference between the results from the 1·5% dose and the 3% dose ($P > 0.05$). After partial hepatectomy, the active constituents were detected on the third day in the kidney and on the fifth day in the lung. The levels in the kidney and lung were, however, remarkably lower than the level in the liver and the spleen at either administration dose.

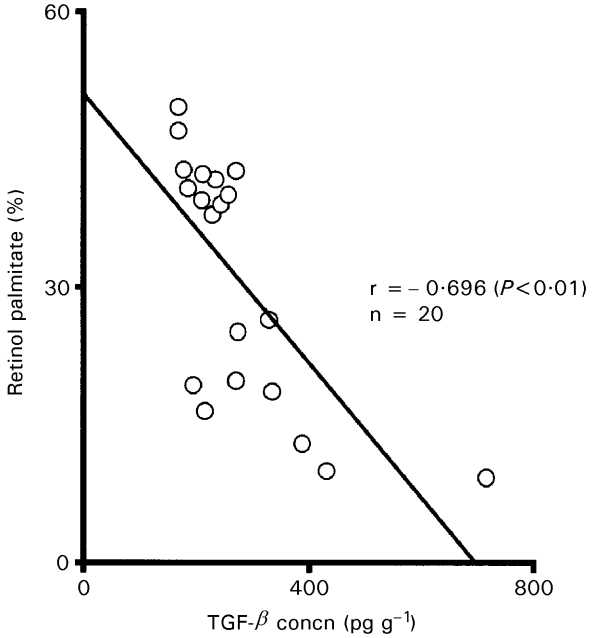

Figure 4. Correlation of TGF-β level and retinol palmitate level (%) in the liver of rats with injury after partial hepatectomy.

Discussion

Peak levels of HGF were detected on day 1 after hepatectomy, with no significant differences between extract administrated and ordinary food groups. It has been reported that remaining hepatocyte cells and non-parenchymal cells greatly increase HGF production via an autocrine mechanism after partial hepatectomy (Ishiki et al. 1992). These results seem to indicate that the Sho-saiko-to extract administration did not influence HGF production via an autocrine mechanism. On days 3–7 after partial hepatectomy of the Sho-saiko-to extract group HGF levels were higher compared with the ordinary food group, and the peak number of S phase cells occurred on days 3–7 after partial hepatectomy. There was a significantly positive correlation ($r = 0.826$). These results show that the Sho-saiko-to extract greatly influenced HGF production via the paracrine mechanism in non-parenchymal cells, and then the increase of liver HGF seemed to induce liver regeneration. Sho-saiko-to extract active constituent (glycyrrhetic acid, baicalin and baicalein) levels in the liver increased from the third day after partial hepatectomy, and the increase in range from day 3 to day 7 was higher with the increase of liver HGF. This suggests that increasing these active constituents greatly stimulated HGF production. However, there was no significant difference between the groups receiving Sho-saiko-to extract at doses of 1·5 and 3%, indicating a necessity for further study of the active constituents and dosage. The HGF production in the spleen was similar to

Table 3. Changes in the levels of Sho-saiko-to active constituents in the organs after administration of Sho-saiko-to extract after partial hepatectomy.

Dose of Sho-saiko-to dose (%)	Level of glycyrrhetic acid (nmol g^{-1})					
	1 day	3 days	5 days	7 days	10 days	14 days
Liver						
0·75	nd	10·1 ± 3·1*	75·2 ± 18·5*	189·3 ± 25·2*	190·5 ± 32·8*	211·1 ± 21·2*
1·50	nd	21·2 ± 3·5*	141·3 ± 20·1	295·3 ± 10·4	318·0 ± 41·2	325·0 ± 23·1
3·00	9·8 ± 3·2	98·0 ± 14·0	164·8 ± 23·5	289·3 ± 15·1	290·0 ± 37·1	295·0 ± 15·6
Spleen						
0·75	nd	7·8 ± 3·2*	27·3 ± 5·2*	43·0 ± 5·6*	69·3 ± 9·7*	75·0 ± 7·0*
1·50	nd	18·2 ± 5·1*	49·1 ± 8·7	89·1 ± 4·5	92·8 ± 6·9	91·8 ± 14·1
3·00	5·7 ± 3·4	32·0 ± 3·5	53·8 ± 10·2	75·0 ± 13·8	82·1 ± 17·6	86·0 ± 15·3
Kidney						
0·75	nd	7·5 ± 1·3*	18·3 ± 4·5	21·3 ± 1·8	23·3 ± 1·6	25·6 ± 3·5
1·50	nd	19·0 ± 1·0*	21·0 ± 3·2	23·5 ± 6·8	25·8 ± 7·4	25·9 ± 6·9
3·00	nd	23·0 ± 1·5	27·0 ± 3·8	29·1 ± 6·5	31·2 ± 1·3	33·3 ± 10·5
Lung						
0·75	nd	nd	2·2 ± 1·5*	4·8 ± 1·2	5·2 ± 1·3*	5·7 ± 1·8*
1·50	nd	nd	4·3 ± 2·1*	6·1 ± 3·2	8·2 ± 1·8	8·9 ± 1·3
3·00	nd	nd	6·1 ± 0·5	6·4 ± 1·5	8·5 ± 0·5	8·8 ± 1·3
Sho-saiko-to dose (%)	Level of baicalin + baicalein (nmol g^{-1})					
	1 day	3 days	5 days	7 days	10 days	14 days
Liver						
0·75	nd	40·9 ± 2·3*	132·3 ± 16·1*	217·7 ± 23·9*	223·9 ± 16·5*	236·6 ± 18·7*
1·50	nd	72·6 ± 6·2*	210·0 ± 16·2	401·0 ± 14·3	450·6 ± 32·3	477·2 ± 15·9
3·00	27·5 ± 6·8	135·0 ± 15·0	285·0 ± 25·0	385·0 ± 33·0	435·0 ± 25·0	447·0 ± 49·1
Spleen						
0·75	nd	20·8 ± 3·1*	37·1 ± 3·8*	63·0 ± 8·9*	70·2 ± 13·8*	75·0 ± 5·8*
1·50	nd	23·5 ± 5·4*	81·2 ± 13·5	135·0 ± 18·2	141·3 ± 21·6	145·0 ± 15·5
3·00	7·6 ± 1·5	43·0 ± 4·0	96·3 ± 11·7	120·5 ± 15·6	137·3 ± 32·6	140·5 ± 20·0
Kidney						
0·75	nd	10·9 ± 3·8*	11·2 ± 4·2*	18·6 ± 5·4	20·1 ± 6·2	31·5 ± 3·2
1·50	nd	21·8 ± 5·6	25·2 ± 6·8	27·3 ± 4·2	27·4 ± 8·5	30·1 ± 6·8
3·00	nd	24·2 ± 3·2	28·2 ± 3·2	31·7 ± 5·2	32·7 ± 6·8	33·7 ± 9·2
Lung						
0·75	nd	nd	4·7 ± 1·2*	5·8 ± 3·7*	6·7 ± 2·8*	7·3 ± 1·5*
1·50	nd	nd	8·5 ± 1·6	9·4 ± 2·5	11·7 ± 1·3	13·5 ± 2·3
3·00	nd	nd	9·1 ± 1·7	10·3 ± 2·2	12·1 ± 1·8	18·1 ± 3·8

Each value is the mean ± s.e. of results from seven experiments. *$P < 0·05$, compared with the result from 3% dose. nd, not detectable.

that in the liver. However, HGF levels in the kidney and lung were not significantly different despite Sho-saiko-to extract administration, and were remarkably lower than liver HGF. These results suggested that Sho-saiko-to extract administration induced an increase in the HGF level in the spleen, and then it influenced liver regeneration via the endocrine mechanism.

We also investigated TGF-β levels in the intraorgans, the action of which is contradictory to HGF. It is known that TGF-β and liver retinoid are related to the activation of Ito cells. TGF-β especially is known to promote the activation of Ito cells (Friedman 1993). In the liver and spleen, the TGF-β levels of Sho-saiko-to extract administered groups were lower than the ordinary food group, and the values for the Sho-saiko-to extract groups decreased as the Sho-saiko-to extract dose was increased. On the other hand, higher liver retinoid levels were found in the groups administered Sho-saiko-to extract rather than ordinary food, showing a significantly negative correlation between TGF-β and the liver retinoid levels ($r = -0·696$, $P < 0·01$). TGF-β production in the kidney and lung is different from that of the liver and spleen, and the effect of Sho-saiko-to extract administration could not be recognized. These results suggest that administration of Sho-saiko-to extract decreased TGF-β production in the liver and spleen, and suppressed the activation of Ito cells. As was shown

before, the increasing active constituent levels in the liver and spleen influenced the production of TGF-β as much as HGF.

In conclusion, administration of Sho-saiko-to extract enhanced liver regeneration. The regeneration mechanism which controls the activation of Ito cells by increasing HGF production and decreasing TGF-β production was promoted by the administration of Sho-saiko-to extract. The level of the active constituents of the extract in the intraorgans seemed to influence the production of HGF and TGF-β.

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