Self-regulating Factor Present in Culture Supernatants of Regenerating Hepatocytes in Adult Rats

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Abstract : The auto-regulation mechanism of liver regeneration after partial hepatectomy was investigated in rats. Hepatocytes isolated from the individual remnant livers at various times after 70% removal of the liver were cultured, and determination of DNA synthesis of the cultured hepatocytes and collection of culture supernatants were performed. The maximum value of DNA synthesis was observed at 1 day after the operation and then gradually decreased, and the liver weights recovered rapidly during 2-4 days. The culture supernatants harvested from these primary cultured hepatocytes isolated from the rats at 2, 3 and 4 days after the operation suppressed dose-dependently DNA synthesis of hepatocytes which were isolated from normal rats and stimulated by epidermal growth factor (EGF) and insulin *in vitro*. The supernatants were non-cytotoxic against hepatocytes, and the activity of them was fairly stable for acid and heat treatments but not for proteolytic enzyme. No inhibition of the binding of EGF to the receptors on hepatocytes was observed. Furthermore, the factor showed an inhibitory activity of mitogen-induced DNA synthesis of human peripheral blood lymphocytes in culture. These results suggested that hepatocytes themselves might modulate the process of liver regeneration by secreting a growth inhibitory factor.

Key words : hepatocyte proliferation inhibitor, liver regeneration, partial hepatectomy, self-regulation, primary culture of hepatocytes.

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Introduction

It is well known that the liver has a potent ability to regenerate after injury, and the normal liver mass is fully restored within about 7 to 10 days when 70% of the liver is removed (Grisham, 1962). Both mechanisms of promotion and termination of liver regeneration are considered to be attractive for the precisely regulated process and in relation to hepatocyte carcinogenesis. Recently, several hepatocyte growth factors have been reported (Nakamura *et al.*, 1986 a; Miyazawa *et al.*, 1989; Zarnegar & Michalopoulos, 1989) and a part of the promoting mechanism appears to have been elucidated, whereas the terminating mechanism is still obscure.

Although several hepatocyte proliferation inhibitors have been detected from sera or liver homogenates during the past few decades (Iype & McMahon, 1984), the inhibitors in sera were not determined by the tissues by which they were produced or whether those in

liver homogenates were really excreted from the liver cells and not the products accompanied by cell destruction. Nakamura *et al.* (1986a, b) recently discovered a hepatocyte growth factor and two kinds of inhibitors from rat platelets. One of the inhibitors was identical to transforming growth factor β (TGF- β) which had been already reported to inhibit hepatocyte proliferation (Nakamura *et al.*, 1985). Nakamura *et al.* (1983a) also detected the presence of a cell surface protein which inhibited hepatocyte proliferation through cell-cell contact. Fausto & Mead (1989) revealed that during liver regeneration both mRNA of TGF- β and transforming growth factor α (TGF- α ; a growth promoter) were increased in sinusoidal cells and in hepatocytes, respectively, and suggested that liver regeneration was controlled by events locally occurring in the liver. Additionally, the process of liver regeneration was also modulated by the immune system, e. g. interleukin-1 β (Nakamura *et al.*, 1988), interferons (Nishiguchi *et al.*, 1988) and humoral factors derived from activated macrophages (Takahashi *et al.*, 1988).

We attempted to investigate whether hepatocytes themselves participated in regulating liver regeneration by using primary culture of adult rat hepatocytes and *in vitro* induction of hepatocyte DNA synthesis by epidermal growth factor (EGF) and insulin (Richman *et al.*, 1976). Consequently, the inhibitory activity on hepatocyte proliferation was found in the culture supernatants of regenerating hepatocytes, and the properties of the inhibitory factor were partially characterized.

Materials and Methods

Materials

William's medium E, penicillin-streptomycin solution and mono-poly resolving medium were purchased from Flow Laboratories, North Ryde, New South Wales, Australia, and fetal calf serum (FCS) and RPMI 1640 medium were from Grand Island Biological Co., Grand Island, NY. Insulin, aprotinin, trypsin and trypsin inhibitor (soybean) were obtained from Sigma Chemical Co., St. Louis, MO. EGF purified from mouse submaxillary glands was from Toyobo Co. Ltd., Osaka, and collagenase was from Nitta Gelatin Co., Yao. Concanavalin A (con A) was from E-Y Laboratories, San Mateo, CA, and rabbit anti-mouse EGF (Rb α mEGF) was from Biomedical Technologies Inc., Stoughton, MA. [6- 3 H]thymidine ([3 H]TdR; 15 Ci/mM) was from New England Nuclear, Boston, MA, and [125 I]EGF (143 μ Ci/ μ g) and ACS- II were from Amersham International Plc., Amersham, UK. Whatmann GF/C glass microfibre filters were purchased from Whatmann Ltd., Maidstone, UK, and an ultrafiltration unit was from Amicon Co., Danvers, MA. Tissue culture plastic plates (Corning) were from Iwaki Glass Co., Tokyo.

Partial hepatectomy

Male Wistar rats weighing 250–300 g were partially hepatectomized (removal of 70% of the liver) under ether anesthesia by the method of Higgins & Anderson (1931).

Remnant livers of individual rats operated on were excised at 3, 6, 12 h and every 24 h after the operation. A sham operation was performed by dissection free from fibrous attachments around the liver and gentle manipulation of it.

Primary culture of hepatocytes

Hepatocytes were isolated from rats by perfusing the liver *in situ* with 0.05% collagenase by the method of Seglen (1976). After perfusion, the liver was excised and weighed. Viability by trypan blue exclusion test and purity of hepatocytes isolated were more than 80% and 96%, respectively.

Hepatocytes were inoculated usually at 0.5×10^5 cells/cm²/0.2 ml into type I collagen-coated plastic plates, and were cultured in William's medium E supplemented with 10% FCS, 10^{-7} M insulin, 50 U/ml penicillin and 50 μ g/ml streptomycin at 37°C for 3 h in 5% CO₂ and 95% air. Then unattached cells were removed and the culture medium was changed to serum- and hormone-free medium containing 0.5 μ g/ml aprotinin unless otherwise stated.

Collection of culture supernatants

1) Hepatocytes isolated from hepatectomized rats: Hepatocytes were inoculated into collagen-coated dishes (10 cm in diameter) and cultured for 3 h. Then the medium was removed and the dishes were rinsed twice with warm PBS, and 12 ml of William's medium E alone was added. After culture for 24 h, the medium was collected and centrifuged at $700 \times g$ for 10 min, and the supernatant was stored at -20°C . Just before using it following experiments, the supernatant was defrosted and dialyzed against William's medium E for 24 h to 48 h at 4°C using cellulose tubing (20/32; Union Carbide Co.), and finally filter-sterilized.

For concentrating culture supernatants, an ultrafiltration unit with a DM5 diaflo membrane (MW 5,000 cut-off) was used.

2) Normal hepatocytes stimulated by EGF and insulin *in vitro*: Hepatocytes isolated from non-treated rats (normal hepatocytes) were inoculated into dishes and cultured for 20 h as described above. Then the medium was changed to 12 ml of William's medium E containing 0.5 μ g/ml aprotinin and the various concentrations of EGF and 10^{-7} M insulin were added. The supernatants from the culture from 20 to 50 h after plating were harvested, and the culture was continued for another 30 h without growth factors. Both supernatants of the 20–50 h culture (Sup: 20–50) and the 50–80 h culture (Sup: 50–80) were stored and treated as described above.

Assay of DNA synthesis

1) Regenerating hepatocytes: Hepatocytes from hepatectomized rats were inoculated into 1.6 cm-diameter wells of collagen-coated multi-well plastic plates. The cells were pulsed with [³H]TdR (1 μ Ci/well) from 4 to 24 h after plating in the absence and presence

of 10 mM hydroxyurea which was added 1 h before addition of [3 H]TdR (Nakayama *et al.*, 1985; McGowan, 1986). Then the cells were washed *in situ* three times with cold PBS followed by two washes each with 5% trichloroacetic acid and 95% ethanol. The cells were air-dried and then solubilized with 1 ml of 2% sodium dodecyl sulfate (SDS) containing 2 mM EDTA and 20 mM NaHCO₃ (Nakayama *et al.*, 1985). An aliquot of this solution (0.5 ml) was used for determining cellular protein by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard, and 0.2 ml of the solution was for measuring the radioactivity which was counted in a Beckman LS 7000 after mixing with 5 ml of ACS-II. The activity of replicating DNA synthesis (dpm/ μ g protein) was calculated as the difference between values with and without hydroxyurea, which were the means of triplicate wells, respectively.

2) Effect of culture supernatants on DNA synthesis of normal hepatocytes stimulated by EGF and insulin: As the cell density used (0.5×10^5 cells/cm²) was a half of the confluent cell density, hepatocytes could grow without the effect of contact inhibition (Nakamura *et al.*, 1983b). Normal hepatocytes could not acquire competence to respond to EGF and insulin until 20 h after plating at this density (Nakamura *et al.*, 1983b; Sand & Christoffersen, 1987). Normal hepatocytes were cultured for 20 h as described above. Then the medium was changed to the supernatants above mentioned, supplemented with 0.5 μ g/ml aprotinin, and 10 ng/ml EGF and 10^{-7} M insulin were added 3 h later. EGF induced maximum DNA synthesis of hepatocytes at the concentration of 10 ng/ml in the presence of the constant concentration of insulin (10^{-7} M) in this assay system. [3 H]TdR (1 μ Ci/well) was added 32 h after seeding and the culture was continued for the following 20 h. Then incorporation of [3 H]TdR and cellular protein were measured. Values are expressed as per cent of control (William's medium E with 10 ng/ml EGF and 10^{-7} M insulin). When the effect of supernatants on DNA synthesis of regenerating hepatocytes was examined, the medium was changed to supernatants after a 3 h-attachment period and [3 H]TdR incorporation into the cells during 4 to 24 h after plating was measured.

Physical and chemical treatments of culture supernatants

Culture supernatants at 2 days after the operation were concentrated 5-fold by using a DM5 diaflo membrane for the treatments. The supernatants were incubated in 1 N acetic acid at 4°C for 12 h, or were incubated at 60°C for 2 h in a water bath. To check susceptibility to proteolytic enzyme, the supernatants were treated with trypsin (10 μ g/ml) at 37°C for 2 h and then the reaction was stopped by adding trypsin inhibitor (20 μ g/ml). After the treatments, samples were thoroughly dialyzed against William's medium E and then filter-sterilized.

Effect of culture supernatants on the binding of [125 I] EGF to EGF receptors on hepatocytes in primary culture

Normal hepatocytes were inoculated at 2×10^5 cells/well and cultured in the same way as described above until 20 h after plating. Then the medium was changed to 0.25 ml of

culture supernatants. [^{125}I] EGF (1.4 ng/ml) was added to each well 3 h later and incubated at 4°C or at 37°C under shaking. The specific activity of [^{125}I] EGF was approximately 3×10^5 cpm/ng. The cells were washed three times with cold PBS and solubilized with 1 ml of 2% SDS containing 2 mM EDTA and 20 mM NaHCO_3 . Each sample (0.5 ml) was transferred to a test tube and the radioactivity was counted using an Aloka Auto Well Gamma System ARC 500. Cellular protein was subsequently measured by the method of Lowry *et al.* (1951). The non-specific binding to plastic culture plates without cells was less than 0.2% of the total [^{125}I] EGF added. The antisera to EGF (Rb α mEGF; 5 μl /well) was added as a positive inhibitory control of the binding.

Effect of culture supernatants on DNA synthesis of human peripheral blood lymphocytes (PBL)

Human PBL were isolated from heparinized blood from a normal volunteer by using mono-poly resolving medium, and were washed three times with RPMI 1640 medium. PBL (5×10^5 cells) were cultured in 0.2 ml of supernatants, which had been dialyzed against RPMI 1640 medium, supplemented with 5% FCS and 10 $\mu\text{g}/\text{ml}$ con A in a flat-bottomed microtiter culture plate at 37°C for 72 h in 5% CO_2 and 95% air. The cells were pulse-labeled with 0.5 μCi of [^3H]TdR for the last 18 h and were harvested on glass microfibre filters. The radioactivity of the incorporated [^3H]TdR was counted by using a liquid scintillation counter as described above. Each sample was tested in triplicate wells.

Statistical analysis

Statistical analysis was made with the Student t-test, and differences with a $P < 0.05$ were considered significant.

Results

Time course of liver regeneration

The weights of the remnant livers after 70% partial hepatectomy increased abruptly and rapidly during 2 to 4 days after the operation, and thereafter increased slowly (Fig. 1). The liver weights were restored to approximately 80% of the average liver weight of non-treated rats at 7 days after the operation. DNA synthesis of regenerating hepatocytes started 12 h after partial hepatectomy, and reached a notable peak 24 h after the operation. Then DNA synthesis was more highly maintained until 3 days after the operation than it was from 4 days after the operation. There was little DNA synthesis of hepatocytes isolated from the rats at 2 days after the sham operation.

Effect of culture supernatants on hepatocyte DNA synthesis

Whether the culture supernatants of hepatocytes isolated from hepatectomized rats affected hepatocyte proliferation was studied. DNA synthesis of normal hepatocytes in

Fig. 1. Liver weights and hepatocyte DNA synthesis after 70% partial hepatectomy in rats. Liver weights (●) are expressed as Mean $\pm$ SD, and the number of rats operated on at each time is in parenthesis. For DNA synthesis of cultured hepatocytes (○), the mean value from 5–9 rats at each time is expressed. DNA synthesis of hepatocytes at 2 days after the sham operation (□) is also the mean value from 6 rats.

Fig. 2. Effect of culture supernatants of hepatocytes from hepatectomized rats on hepatocyte DNA synthesis. The results are expressed as Mean $\pm$ SD from 6 individual supernatants at each time indicated. DNA synthesis was significantly suppressed by the supernatants at 2, 3 and 4 days after partial hepatectomy compared with the supernatants from non-treated rats (* P < 0.01, ** P < 0.001).

Table 1. Effect of culture supernatants on DNA synthesis of hepatocytes in the different cell cycles

Culture supernatants prepared from rats	Normal hepatocytes + EGF + insulin	Regenerating hepatocytes
Non-treated	91.5 ± 4.0 (6)	91.7 ± 9.8 (6)
2 days after sham operation	101.1 ± 13.4 (6)	95.4 ± 5.4 (6)
2 days after partial hepatectomy	55.1 ± 10.2 (6)*	83.7 ± 8.4 (8)

Normal hepatocytes were in G₁ phase when the supernatants were added (at 20 h after plating), and regenerating hepatocytes from the rat at 24 h after partial hepatectomy were in S phase. In the culture of regenerating hepatocytes, the supernatants were added at 3 h after plating. The results are expressed as Mean ± SD. The number of supernatants tested is in parenthesis. **P* < 0.001; compared with the supernatants from non-treated rats.

culture was induced with 10 ng/ml EGF and 10^{-7} M insulin (EGF-stimulating normal hepatocytes). Culture supernatants at 2, 3 and 4 days after partial hepatectomy (Sup-2, -3 and -4) suppressed DNA synthesis of normal hepatocytes to approximately 60% of control (Fig. 2). It coincided with the rapid growth of remnant livers (during 2 to 4 days after the operation), but did not coincide at the peak of DNA synthesis (at 1 day after the operation).

Inhibition on DNA synthesis of hepatocytes in the different cell cycles is shown in Table 1. DNA synthesis of normal hepatocytes stimulated after the addition of supernatants was significantly suppressed by Sup-2, while DNA synthesis of regenerating hepatocytes, which were isolated at 1 day after the operation, was only slightly suppressed by Sup-2.

Effect of concentrated culture supernatants on hepatocyte DNA synthesis

Sup-2 was concentrated by using a DM5 diaflo membrane. Sup-2 concentrated 5-fold and 10-fold suppressed DNA synthesis of the EGF-stimulating normal hepatocytes to 46.7% and 15.9% of control, respectively (Fig. 3). DNA synthesis of unstimulated normal hepatocytes cultured in William's medium E was 21.7% of control. However, the level of cellular protein from cultured hepatocytes was not significantly decreased even by the addition of 20-fold concentrated Sup-2 compared with the filtrate which had passed through a diaflo membrane. When hepatocytes died, they were detached from culture plates; the number of viable cells did not alter when the level of cellular protein was not changed. Therefore this inhibitory activity on DNA synthesis was not derived, if any, from the cytotoxic activity in the culture supernatants. The 10-fold concentrated Sup-2 contained approximately 0.25 mg/ml of protein (determined by the method of Lowry *et al.*, 1951). Since rat serum albumin (fraction V, Sigma) at the concentration of 0.1 to 4 mg/ml had no effect on DNA synthesis of the EGF-stimulating normal hepatocytes (data not shown), a possibility that the inhibitory effect of the supernatants resulted from the interaction between growth factors and a high concentration of protein used should be excluded.

Fig. 3. Effect of concentrated culture supernatants on hepatocyte DNA synthesis and cellular protein. Culture supernatants from 6 or 7 rats at 2 days after partial hepatectomy were pooled and concentrated 20-fold, and then serially diluted. Cellular protein (■) was determined from triplicate cultures without hydroxyurea. Closed circles (●) indicate DNA synthesis in the supernatants with 10 ng/ml EGF and 10^{-7} M insulin, and an open circle (○) indicates DNA synthesis in William's medium E without growth factors. The results are expressed as Mean $\pm$ SD of four experiments using separate pooled supernatants.

Table 2. DNA synthesis of normal hepatocytes stimulated by EGF and insulin in the two serial culture periods

Concentration of EGF (ng/ml)	Relative DNA synthesis (%)	
	20–50 h culture	50–80 h culture
0	8.8 $\pm$ 5.0	14.2 $\pm$ 9.3
1	48.2 $\pm$ 35.8	34.2 $\pm$ 14.1
5	88.1 $\pm$ 14.1	65.1 $\pm$ 22.7
10	82.3 $\pm$ 18.4	84.8 $\pm$ 23.9
20	100	100

EGF and insulin were present during 20–50 h. Hepatocytes were pulsed with [3 H]TdR for the last 20 h in each culture period. The mean values of [3 H]TdR incorporation at 20 ng/ml of EGF were 1007 dpm/ μ g protein in the 20–50 h culture and 1311 dpm/ μ g protein in the 50–80 h culture. The results are expressed as Mean $\pm$ SD from 4 rats.

Effect of culture supernatants from normal hepatocytes stimulated by EGF and insulin *in vitro* on hepatocyte DNA synthesis

When EGF and insulin were added to the culture of normal hepatocytes at 20 h after

plating, DNA synthesis was dose-dependently induced at the concentration of added EGF for both 30 h-periods (20–50 h and 50–80 h), as shown in Table 2. In spite of no supplement with growth factors for the latter 30 h, there was no significant difference between the values of [^3H]TdR incorporation observed in these two periods (at 10 or 20 ng/ml of EGF). The inhibitory activities of these culture supernatants (Sup: 20–50 and Sup: 50–80) are indicated in Fig. 4. Sup: 20–50 was difficult to inhibit DNA synthesis of the EGF-stimulating normal hepatocytes, but Sup: 50–80 suppressed DNA synthesis in response to the added dose of EGF.

Effect of physical and chemical treatments of culture supernatants on their inhibitory activity

The inhibitory activity of culture supernatants was fully conserved after treatments under heat or acid conditions, but disappeared by proteolytic enzyme digestion as shown in Table 3. These results indicated that a protein-like substance was an essential part of the inhibitory factor.

Effect of culture supernatants on the binding of [^{125}I] EGF to EGF receptors on hepatocytes

As shown in Fig. 5, the binding of [^{125}I]EGF to hepatocytes in fresh medium reached

Fig. 4. Effect of culture supernatants of normal hepatocytes stimulated by various concentrations of EGF on hepatocyte DNA synthesis. The concentrations of EGF which was added to collect the supernatants of the EGF-stimulating normal hepatocytes (as shown in Table 2) were on the ordinate. The results are expressed as Mean $\pm$ SD from 4 supernatants. Open bars: the supernatants of the 20–50 h culture, hatched bars: the supernatants of the 50–80 h culture.

Table 3. Effect of physical and chemical treatments of culture supernatants on their inhibitory activity

Treatment	DNA synthesis (% of control)
Medium	11.5 ± 9.0
Medium+EGF (E)+insulin (I)	100
Culture supernatants	
No treatment+E+I	29.1 ± 20.2
Heat 60°C 2 h+E+I	8.0 ± 3.7
1 N acetic acid 4°C 12 h+E+I	7.7 ± 1.6
Trypsin (10 µg/ml) 37°C 2 h+E+I	93.8 ± 7.8

Culture supernatants from 7 rats at 2 days after partial hepatectomy were pooled and concentrated 5-fold, and then treated as indicated. The results are expressed as Mean ± SD of three repeated experiments. William's medium E treated similarly had no effect on hepatocyte DNA synthesis.

Fig. 5. Time course of the binding of [125 I] EGF to EGF receptors on hepatocytes at 4°C or 37°C. Normal hepatocytes were incubated with [125 I] EGF in William's medium E at 4°C (○) or 37°C (●). The results are expressed as Mean ± SD of triplicate wells.

maximum value for 2 h at 4°C or for 1 h at 37°C, respectively, under the conditions used here. Although the peak value was not changed by further incubation at 4°C, the binding was gradually decreased by longer incubation after 1 h at 37°C. The binding of EGF to hepatocytes was neither inhibited by Sup: 50–80 (EGF 20 ng/ml) nor by Sup-2 (Table 4). That RbāMEGF completely inhibited the binding reinforced the evidence.

Table 4. Effect of culture supernatants on the binding of [125 I] EGF to EGF receptors on hepatocytes

Incubation	Sample	Radioactivity bound (cpm/ μ g protein)	%
4°C 2 h	Medium	91.1 $\pm$ 2.0	100
	Culture supernatants (2 days after partial hepatectomy)		
	Filtrate	83.4 $\pm$ 4.2	91.5
	Concentrated	84.0 $\pm$ 6.2	92.2
	0.1 $\times$	85.4 $\pm$ 5.1	93.7
	1 $\times$	83.9 $\pm$ 1.9	92.1
	5 $\times$	84.3 $\pm$ 1.4	92.5
	10 $\times$	85.0 $\pm$ 0.6	93.3
	Culture supernatants (stimulated <i>in vitro</i>)		
	I	86.7 $\pm$ 2.5	95.2
	II	90.9 $\pm$ 1.8	99.8
	III	83.9 $\pm$ 2.8	92.1
	IV	84.5 $\pm$ 3.8	92.8
37°C 1 h	Rb α mEGF	0.6 $\pm$ 0.2	0.7
	NRbS	88.1 $\pm$ 1.5	96.7
	Medium	114.2 $\pm$ 4.5	100
	Culture supernatants 10 $\times$	123.0 $\pm$ 5.4	107.7

Culture supernatants of hepatocytes from 6 rats at 2 days after partial hepatectomy were pooled and concentrated 10-fold, and then serially diluted. I, II, III and IV were supernatants of normal hepatocytes from 4 individual rats stimulated by 20 ng/ml EGF and 10^{-7} M insulin *in vitro* (as shown in Fig. 4). The results are expressed as Mean $\pm$ SD of triplicate wells. NRbS; normal rabbit serum.

Table 5. Effect of culture supernatants on DNA synthesis of human PBL stimulated by con A

Sample	[3 H] TdR incorporated (cpm)	%
Medium con A (–)	3,584 $\pm$ 221	14.3
con A (+)	24,990 $\pm$ 3,564	100
Culture supernatants (2 days after partial hepatectomy)		
Filtrate	25,453 $\pm$ 1,467	101.9
Concentrated	23,330 $\pm$ 178	93.4
0.1 $\times$	21,993 $\pm$ 1,450	88.0
1 $\times$	17,340 $\pm$ 1,525	69.4
5 $\times$	11,210 $\pm$ 908	44.9
10 $\times$	6,677 $\pm$ 618	26.7
Culture supernatants (stimulated <i>in vitro</i>)		
I	16,580 $\pm$ 2,101	66.3
II	10,233 $\pm$ 1,318	40.9
III	10,303 $\pm$ 252	41.2
IV	11,236 $\pm$ 1,624	45.0

Culture supernatants indicated were the same as described in Table 4. The results are expressed as Mean $\pm$ SD of triplicate wells.

Effect of culture supernatants on DNA synthesis of human PBL

To investigate tissue- and species-specificity of the culture supernatants for DNA synthesis, human PBL were cultured in the presence of the supernatants and stimulated with con A. [^3H]TdR incorporation into human PBL was suppressed dose-dependently by Sup-2 (Table 5). Sup: 50–80 also inhibited [^3H]TdR incorporation. Thus, the inhibitory activity on DNA synthesis was not restricted to rat hepatocytes.

Discussion

The mechanism of termination of liver regeneration might be a complex process participated in by several elements such as serum factors (Nadal *et al.*, 1981), platelet derived factors (Nakamura *et al.*, 1986b), interaction with nonparenchymal cells (Braun *et al.*, 1988) and contact inhibition of hepatocytes (Nakamura *et al.*, 1983a). Besides those, we showed the presence of an inhibitory activity on DNA synthesis in the supernatant from the culture of regenerating hepatocytes. The evidence implies that hepatocytes modulate the process of liver regeneration by a self-regulating mechanism.

The results are summarized as follows. 1) The inhibitory activity on DNA synthesis was observed in the supernatants at 2, 3 and 4 days after the operation while the weights of the remnant livers increased rapidly, but not in the supernatants at 1 day after the operation when the maximum DNA synthesis of regenerating hepatocytes occurred (Figs. 1, 2). Therefore, this factor seemed to have been secreted from hepatocytes behind the time of the peak DNA synthesis. This was confirmed by the result that normal hepatocytes stimulated with EGF and insulin secreted the inhibitory activity into culture supernatants at the later period of stimulation (Fig. 4). Vintermyr & Døskeland (1987) presented that when EGF and insulin were added to the culture of normal hepatocytes 20 h after plating, DNA synthesis of them started at 17 h and reached the peak at 35 h after the addition of growth factors under almost the same conditions used here. Therefore, in spite of comparably induced DNA synthesis in the former (20–50 h) and the latter (50–80 h) periods, it was considered that only the supernatants at the latter period suppressed DNA synthesis of the EGF-stimulating normal hepatocytes. In addition, the inhibitory activity increased in proportion to the degree of DNA synthesis during the 50–80 h culture. Probably, it appeared to reflect the number of hepatocytes shifted to S phase in response to each concentration of EGF added. The consideration that this inhibitory factor might be derived from the dead hepatocytes or hepatocytes cultured under inappropriate conditions was not always deniable.

2) One possibility that this inhibitory factor inhibited the binding of EGF to the receptors was conceivable. According to the reason for the binding difference between at 4°C and at 37°C, as shown in Fig. 5, the inhibitory activity was examined at the both temperatures. The result showed that the factor did not act as a competitive inhibitor with EGF (Table 4). The decrease of the binding after 60 min at 37°C has been ex-

plained by down regulation of the receptors (Moriarty & Savage, 1980). Another possibility of thymidine degradation was also conceivable. However, thymidine degrading enzymes extracted from the rat liver were heat-labile (Miyamoto & Terayama, 1971), but the factor was heat-resistant. 3) Because the factor affected normal hepatocytes (at 20 h after plating) in G₁ phase (Nakamura *et al.*, 1983b) but not regenerating hepatocytes in S phase as shown in Table 1, it seemed to prevent the hepatocyte transition from G₁ to S phase. The prevention of G₁-S transition was seen in the action of several growth inhibitors (Nadal *et al.*, 1981; Russell *et al.*, 1988). 4) As the factor suppressed DNA synthesis of hepatocytes without any change in the levels of cellular protein, as shown in Fig. 3, it was considered to act without cytotoxic effect. 5) The inhibitory activity of the factor was stable against treatments of heat and acid, and unstable for trypsin digestion (Table 3). Since the inhibitory activity was fully retained in the cellulose tubing (20/32) after a long term dialysis, the factor seemed likely to be a protein-like substance with high-molecular weight. In order to characterize the factor, the purification was carried out. However, the activity was unexpectedly found in a wide range of the effluent from a gel filtration column chromatography (unpublished data). These results may indicate that the factor is composed of a complex form with other substances or molecular elements of heterogeneous forms itself. Further application of other isolation procedures is under investigation. 6) The inhibitory activity appeared to be less tissue- and species-specific since con A-induced proliferation of human PBL was suppressed by this factor, as shown in Table 5.

After 70% partial hepatectomy in adult rats, most of the remnant hepatocytes proliferate within 36 h (Grisham, 1962; McGowan, 1986). In fact, the most synchronized proliferative response of hepatocytes was observed in DNA synthesis at 24 h after partial hepatectomy, as shown in Fig. 1. Then the synchronization tended to be lost and a lowered level of DNA synthesis continued (Fausto *et al.*, 1987). Assuming simply that all of the remnant hepatocytes would proliferate with the initial DNA synthesis, it is not necessary for all of them to divide twice in order that the number of cells be completely restored to the preoperative level. In addition, the hepatocyte growth factor was still detected in the sera from hepatectomized rats at least 48 h after the operation (Nakamura *et al.*, 1984). Thus secretion of the inhibitory factor during 2 to 4 days after partial hepatectomy might serve the purpose of preventing unregulated excessive proliferation of the liver. Since hepatocytes essentially cannot continue to proliferate without growth factors, the inhibitory action of this factor *in vivo* is to prevent the hepatocytes in G₁ phase from transition to S phase in the presence of growth factors. Mead & Fausto (1989) revealed that hepatocytes stimulated by EGF secreted TGF- α which promoted proliferation of hepatocytes and shared EGF receptor. However, as the preincubated mixture of EGF and insulin with Sup-2 similarly suppressed DNA synthesis of hepatocytes (data not shown), the factor is considered to inhibit hepatocyte proliferation independently even if growth promoters such as TGF- α are simultaneously present *in vivo*.

Concerning differences between this growth inhibitory factor and other inhibitors re-

ported, similarity to the properties of TGF- β which is a heat- and acid-stable protein must be discussed. TGF- β is a 25 kD homodimeric protein found in both normal and transformed cells, and is less species-specific; 99% homology between the human and mouse TGF- β amino acid sequences (Massag, 1987). TGF- β was reported to be a potent inhibitor of hepatocyte DNA synthesis both *in vivo* (Russell *et al.*, 1988) and *in vitro* (Carr *et al.*, 1986, Russell, 1988). Additionally, Kehrl *et al.* (1986) suggested a self-regulating role of TGF- β in human T lymphocyte proliferation. Although the activity of TGF- β was observed in several normal tissues including the liver of an adult mouse (Roberts *et al.*, 1981), Derynck *et al.* (1985) detected TGF- β mRNA in many human tumor cell lines but not in a normal adult human liver tissue. Recently, Braun *et al.* (1988) showed that an increase of TGF- β mRNA was observed in nonparenchymal cells but not in hepatocytes in the rat regenerating liver. According to the study of insulin-like growth factor I (IGF-I) in the fetal liver, although IGF-I was immunostained in fetal hepatocytes, its mRNA was detected only in nonparenchymal cells (Han *et al.*, 1987a, b). These phenomena might be possibly accounted for by accumulation of exogenous IGF-I into the fetal hepatocytes. Thus, further studies are necessary to elucidate exactly the mechanism for producing (or secreting) the inhibitory factor by hepatocytes in the hepatectomy.

Nevertheless the characteristics of this inhibitory factor are similar to those of TGF- β , the factor might be distinguishable from TGF- β because the secretion of the factor is associated with DNA synthesis and proliferation of hepatocytes, and its production seems likely to be scheduled in a series of the gene expression concerned with hepatocyte proliferation.

Alternatively, it is widely known that regenerating hepatocytes in adult rats reproduce alpha fetoprotein which had been synthesized in the fetal liver (Sell *et al.*, 1974). As an example of fetal products, Mllerian inhibiting substance (MIS) which was considered to be a naturally occurring inhibitor of EGF receptor tyrosine kinase was found in the fetal and neonatal genital organs (Cigarroa *et al.*, 1989; Coughlin *et al.*, 1987). In addition, MIS was reported to be a member of the TGF- β gene family (Massag, 1987) and to inhibit growth of a certain human ovarian carcinoma cell line (Fuller *et al.*, 1982). The possibility of producing such a fetal growth inhibitor by hepatocytes during liver regeneration might still remain.

In conclusion, we demonstrated that a self-regulating mechanism of hepatocyte proliferation was possibly present by using the *in vitro* culture system. This mechanism might play an important role in prevention of unregulated proliferation of hepatocytes and in appropriate termination of liver regeneration in concert with other systems. It is of interest whether proliferation of hepatoma cells or of hepatocytes induced by other growth factors, especially hepatocyte growth factor (Nakamura *et al.*, 1986a; Gohda *et al.*, 1986), is suppressed by this factor or not. To clarify these problems including the differences of this factor with TGF- β or other growth inhibitors, further purification of the factor is necessary.

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ラット再生肝細胞の培養上清中に存在する増殖抑制因子

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要 旨： ラット肝切除後の肝再生停止機構に対する肝細胞自身の関与の有無について検討した。70%切除後の残存肝では、1日後に急峻なDNA合成のピークが、2-4日間に急激な重量の増加がみられた。経時的に肝細胞を分離培養して得られた培養上清のうち、術後2-4日のものが、初代培養系で上皮成長因子とインスリンにより誘発される正常肝細胞のDNA合成を抑制した。この抑制活性は、濃度依存的に作用し、細胞毒性はなく、S期の肝細胞には効果がなかった。その性状は、熱および酸処理に安定な蛋白性物質で、種および臓器特異性に乏しかった。また、上皮成長因子の肝細胞膜上の受容体への結合を阻害しなかった。その生理的意義は、初回分裂後の細胞を含むG₁期にある肝細胞が、増殖因子に反応してS期に進行するのを阻止することにあると推測される。以上の結果から、肝細胞自身が増殖抑制因子を分泌して、肝再生過程を自ら制御している可能性が示唆された。

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