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SEQUESTRATION OF A HEPATOCYTE GROWTH FACTOR IN EXTRACELLULAR MATRIX IN NORMAL ADULT RAT LIVER [1]

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Perfusion of normal adult rat livers with Hanks' solution containing 1 M NaCl in situ led to the releasing of a large amount of hepatocyte growth factor (HGF). During the first 5 min of perfusion, the HGF content of the perfusate reached a maximum level, while the LDH activity due to release from the cells was negligible. The liver HGF content did not decrease with age. The liver HGF content in rats injured by CCl_4 injection decreased temporarily and then recovered rapidly to a normal level. These results indicate that HGF is sequestered in the extracellular matrix in the subendothelial space in normal adult rat liver and its effect will be either neutralized or potentiated by other local factors.

Many studies of stimulatory factors in hepatocyte proliferation in primary cultures have been reported (1-5).

We have demonstrated that a stimulatory factor for hepatocyte DNA synthesis (hepatocyte growth factor, HGF) was secreted from nonparenchymal liver cells (NPC) in a coculture of hepatocytes with NPC (6). Recently we found that the NPC-derived HGF was secreted mainly from Kupffer cells, and had an affinity for heparin.

Basic fibroblast growth factor is characterized by its high affinity towards heparin and has been shown to bind to heparan sulfate in extracellular matrix (ECM) both in vitro and in vivo (7-8). The presence of heparan sulfate as the major glycosaminoglycan in the extracellular matrix (ECM) between the

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layer of hepatocytes and endothelial cells forming sinusoids (Disse's space) (9) raises the possibility that the ECM contains a heparin-binding HGF.

In this report, we show that a large amount of HGF is deposited and sequestered in the ECM in Disse's space of normal adult rat liver.

MATERIALS AND METHODS

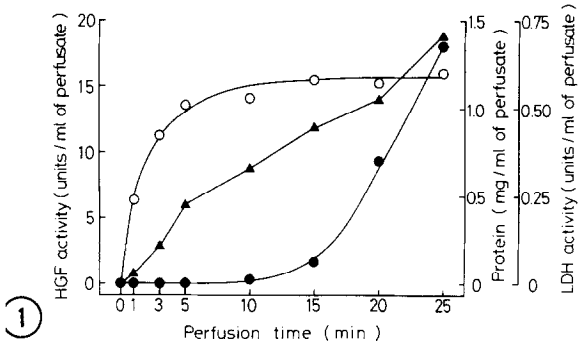
Cell culture and medium. Adult rat hepatocytes were isolated from 6-week-old male Wistar rats, as previously reported (6) and cultured in a mixture of 3 volumes of Dulbecco's modified Eagle's essential minimum medium (DEM) and 1 volume of Ham's F-12 supplemented with 5 ng/ml of insulin, 0.4 μg/ml of dexamethazone, 100 units of penicillin and 100 μg of streptomycin per ml, and 2.5% fetal calf serum (FCS).
Extraction of HGF from liver. Rats were anesthetized by intraperitoneal injection of Nembutal (1.5 ml/kg), the abdomen was opened, and polyethylene cannulae were rapidly inserted into both the portal vein and the hepatic vein to form a circulation system in the liver. The liver was first perfused at 4 °C from portal vein with a small volume (< 30 ml) of Ca^{2+}-free Hanks' solution containing 0.5 mM ethyleneglycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid at a flow rate of 20 ml/min. After the liver and perfusate appeared to be free of blood, the liver was perfused at a flow rate of 15 ml/min with 100 ml of Hanks' solution containing 1 M NaCl, and 10 ml samples of perfusate were harvested at appropriate intervals as shown in Fig. 1. Five ml of the perfusate solution was diluted 10 times with cold distilled water and applied independently to a Heparin-Sepharose CL-6B column (1.5 cm x 1 cm, preequilibrated with 10 mM phosphate buffer,pH 7.0, containing 0.15 M NaCl). The protein absorbed on the column was washed with 20 ml of the preequilibrating buffer and eluted with 2.5 ml of 10 mM phosphate buffer containing 1 M NaCl. The eluate was dialyzed against serum free DEM overnight at 4 °C, and sterilized by filtration through a 0.22-μm filter (Millex GV, Millipore). To determine the HGF activity a portion of each fraction was added to the basal hepatocyte culture medium 24 h after plating. DNA synthesis was measured by the method described previously (6) by scintillation counting of [methyl-^{3}H]-thymidine (20 Ci/mmol, obtained from Du Pont/NEN Research Products) incorporated into DNA. One unit of HGF activity was defined as the amount required for increasing in hepatocyte DNA synthesis two-fold as compared to that in the absence of HGF. The lactate dehydrogenase (LDH) activity and the protein content of each perfusate was determined by the methods described by Kornberg (10) and Lowry et al. (11), respectively.
Treatment of rats with CCl_4. Male Wistar rats (6 weeks old, weighing 135-170 g) were used. Carbon tetrachloride was dissolved in olive oil (1:1, v/v) and injected intraperitoneally at a dose of 1 ml/kg body weight. Rats were anesthetized at various times after CCl_4 administration, the abdomen was opened, and blood was harvested from the abdominal aorta and centrifuged at 1500 x g for 20 min. Then,

the livers were perfused at 4°C for 5 min with cold Hanks' solution containing 1 M NaCl as described above. To determine the HGF activity a portion of each serum was dialyzed overnight against serum-free DEM at 4°C, passed through a 0.22-μm Millipore filter and added to the FCS-free basal hepatocyte culture medium to give a final concentration of 10%.

RESULTS AND DISCUSSION

Presence of HGF in normal adult rat liver. An affinity of the NPC-derived HGF towards heparin led us to the idea that the HGF may be sequestered in the ECM in Disse's space in normal adult rat liver. This idea was confirmed by the results shown in Fig. 1. A large amount of HGF appeared in the perfusate of the liver. During the first minute of perfusion, HGF activity was determined in the perfusate, increased gradually with time and reached a maximum level after 5 min (Fig. 1). The LDH activity in the perfusate due to release from cells was negligible during the first 10 min and increased rapidly after 15 min of perfusion. This finding indicates that HGF is present in normal adult rat liver, and that the HGF in the perfusate apparently is not due to secretion and /or release from cells, but to elution from the storage in the ECM in the subendothelial space (Disse's space). Since there is little the hepatocyte proliferation in normal adult rat liver, there is a possibility that the HGF in normal adult liver is not involved in the stimulation of hepatocyte proliferation but plays a role in other function(s).

When the perfusate obtained during the first 10 min was diluted and applied to a previously equilibrated Heparin-Sepharose CL-6B column and eluted with 120 ml of a gradient of 0.15-3.0 M NaCl in 10 mM phosphate buffer, a single growth factor peak was eluted from the column at about 0.65 M NaCl (Fig. 2) similar to the results obtained for NPC-derived HGF. Moreover, the HGF in liver appeared to be related to the NPC-



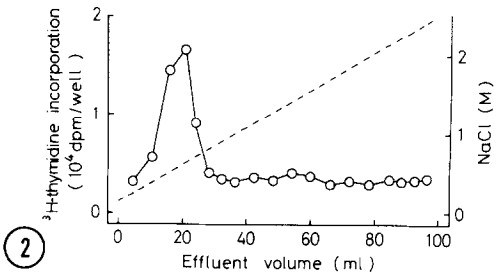


Fig. 1. Time course of HGF elution from normal adult rat liver by perfusion with Hanks' solution containing 1 M NaCl. Symbols: open circles, HGF potency; closed circles, LDH activity; closed triangles, protein content.

Fig. 2. Heparin-Sepharose chromatography of HGF in the perfusate. The broken line represents the concentration of NaCl.

derived HGF by the criteria of (i)specificity for hepatocyte growth, (ii)having Mr value of about 100 KDa, and (iii)heat- and acid-sensitivities (manuscript in preparation).

The HGF content of liver in rats of various ages. The potential for liver regeneration is known to decrease as the aging process progresses (12). We investigated whether or not the HGF content of the liver also decreases with age. The livers were perfused for 5 min at 4 °C with Hanks' solution containing 1 M NaCl. The HGF content of the liver barely decreased with age (Table 1). This indicates that intracellular

Table 1. HGF content of livers in rats of various ages

Age	Animal number	Body weight (g)	Liver weight (g)	HGF content (units/g of liver)	HGF content (units/liver)
3 weeks	1	70	3.69	453	1637
	2	70	3.55	341	1211
10 weeks	3	220	7.17	336	2406
	4	240	8.68	234	2031
	5	240	7.68	465	3752
8 months	6	350	10.40	313	3254
	7	360	9.58	279	2674
	8	360	10.69	376	4019
21 months	9	410	12.52	396	4954
	10	410	12.20	332	4055
	11	430	11.67	262	3062

transduction systems after interaction of HGF with hepatocytes become impaired during the aging process and supports the previous finding (13) that 22-month-old rat hepatocytes can traverse from G_0 to G_1 phase of the cell cycle but are blocked from entering the S phase after the additon of epidermal growth factor.

The HGF content of liver in CCl_4-treated rats. After CCl_4 administration, there is an increase in NPC due to the proliferation of Kupffer cells (14) and the arrival of circulating mononuclear macrophagic cells (15), and in the level of HGF mRNA in Kupffer cells (16). These phenomena will result in an increase in HGF content after CCl_4 administration. The HGF content of the serum increased temporarily 24 h after CCl_4 injection, and then decreased. The HGF content of the liver, on the other hand, dropped 24 h after injection of the hepatotoxin, and then started to increase and reached a slight higher level than normal during 2 to 3 days after CCl_4 injection (Table 2). The period of high HGF content in the liver agrees well with that of hepatocyte proliferation (3). This result indicates

Table 2. Changes in HGF content of serum and liver after CCl_4 injection

After CCl_4 injection	Animal number	Body weight (g)	Liver weight (g)	HGF content (units) Serum	Liver	Total
Non-injection	1	140	5.78	240	1907	2147
	2	170	5.97	242	2652	2894
1-day	3	130	6.25	1260	991	2251
	4	145	5.92	1093	971	2064
	5	130	5.02	1287	1015	2302
2-day	6	150	6.99	678	2730	3408
	7	155	4.93	1065	2755	3831
3-day	8	165	6.60	766	3861	4627
	9	165	5.75	455	1788	2243
5-day	10	160	6.73	411	1216	1627
	11	145	6.29	399	1538	1937
	12	140	6.45	183	1920	2103
7-day	13	165	7.01	254	2595	2849
	14	170	6.49	241	1981	2222
	15	150	6.59	370	2973	3343

The total serum HGF content was determined by regarding the total blood volume as 8% of the body weight.

that partial degradation of the ECM by liver injury may occur and cause release of HGF in the ECM into the blood, and then the secretion of both HGF and ECM components from NPC and/or hepatocytes may enhance tissue repair.

The results presented in this communication indicate that hepatocyte proliferation may be regulated not by HGF in the serum but by a local phenomenon through cell-cell and cell-matrix interactions:the NPC-derived HGF is sequestered in the ECM and its effect will be either neutralized or potentiated by other local factors that maintain control of the milieu interne.

REFERENCES

1. Michalopoulos, G., Houck, K.A., Dalan, M.L., and Luetteke, N.C. (1984) Cancer Res. 44, 4414-4419.
2. Nakamura, T., Nawa, K., and Ichihara, A. (1984) Biochem. Biophys. Res. Commun. 122, 1450-1459.
3. Gohda, E., Hayashi, Y., Kawaida, A., Tsubouchi, H., and Yamamoto, I. (1990) Life Sci. 46, 1801-1808.
4. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Nature 342, 440-443.
5. Miyazaki, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Araki, N., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y., and Kitamura, N. (1989) Biochem. Biophys. Res. Commun. 163, 967-973.
6. Yamamoto, N., Imazato, K., and Masumoto, A. (1989) Cell Structure and Function 14, 217-229.
7. Vlodavsky, I., Folkman, J., Sullivan, R., Friedman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987) Proc. Natl. Acad. Sci. USA. 84, 2292-2296.
8. Saksela, O. and Rifkin, B. (1990) J. Cell Biol. 110, 767-775.
9. Stow, J.L., Kjellan, L., Unger, E., Hook, M., and Farquhar, M.G. (1985) J. Cell Biol. 100, 975-980.
10. Kornberg, A. (1955) In Methods in Enzymology (S.P. Colowick and N.O. Kaplan, Ed) vol. 1, pp441-443, Academic Press, New York.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Bucher, N.L.R. (1963) Int. Rev. Cytol. 15, 245-300.
13. Sawada, N. (1989) Exp. Cell Res. 181, 584-588.
14. Heine, W.D. (1980) In The Reticuloendothelial System and Pathogenesis of Liver Disease, (H. Liehr and M. Grun Ed.) pp27-44, Elsevier, Amsterdam.
15. Rojkind, M. and Valadez, G. (1985) In Fibrosis, (D. Evered and J. Whelan Ed.) pp208-221, Ciba Foundation Symposium 174, London, Pitmann.
16. Kinoshita, T., Tashiro, T., and Nakamura, T. (1989) Biochem. Biophys. Res. Comm. 16, 1229-1234.