Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 133-139

Partial Purification and Characterization of Hepatocyte Proliferation Stimulatory Factor from Liver of Rats Treated with D-Galactosamine

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Received November 17, 1987

Summary: The partial purification and characterization of a hepatocyte proliferation stimulatory factor (HPSF) isolated from the liver of D-galactosamine-treated rats are described. The HPSF was a heat-labile, acid-stable and trypsin-sensitive protein. The partially purified HPSF stimulated DNA synthesis and increased the labeling index of parenchymal hepatocytes at 5 μg/ml and maximally at 50 μg/ml. The effect of HPSF in stimulating DNA synthesis was synergistic with that of insulin plus epidermal growth factor (EGF). The HPSF was scarcely detected in normal rat liver. The results obtained indicate that this HPSF is distinct from insulin, multiplication-stimulating activity (MSA), EGF and other hepatocyte growth factors previously reported, and suggest a plausible role for HPSF in the regeneration of liver tissue following hepatotoxic damage.

The liver has a capacity to regenerate its tissue rapidly and to restore its functions after partial removal or necrosis due to various hepatotoxins. The mechanism of hepatic regeneration after partial hepatectomy has been extensively studied and several hepatocyte growth factors have been isolated and characterized from serum and liver tissue (1-5, 8, 13, 14).
On the other hand, there are only two papers describing the presence of hepatocyte growth factors in serum of rats treated with hepatotoxin (9, 10), and there are no reports on the presence of hepatocyte growth factor in the liver of rats treated with D-galactosamine, which is commonly used to induce hepatitis-like injury in rat liver (17).

0006-291X/88 $1.50

In this paper, we describe the purification and characterization of a hepatocyte proliferation stimulatory factor (HPSF) from the liver of rats treated with D-galactosamine, which were done as part of a study to investigate the mechanism of liver regeneration after diffuse liver cell necrosis.

MATERIALS AND METHODS

Chemicals Methyl-[^{3}H] thymidine (18.2 Ci/mmol) was obtained from New England Nuclear (Boston, Mass); insulin, trypsin and soybean trypsis inhibitor from Sigma (St. Louis, Mo); mouse epidermal growth factor (EGF) and multiplication-stimulating activity (MSA) from Collaborative Research (Waltham, Mass); heparin-Sepharose CL-6B from Pharmacia (Uppsala, Sweden); DEAE from Whatman (Clifton, NJ); collagenase type 1 and D-galactosamine hydrochloride from Wako Pure Chemical Industries (Osaka, Japan); and Sakura NR-M2 emulsion for autoradiography from Konishiroku Photographic Industries (Tokyo, Japan).

Primary culture of parenchymal hepatocytes from adult rat liver Parenchymal hepatocytes were isolated from male Wistar rats, weighing about 200 g, by perfusing the liver in situ with 0.05% collagenase using the method of Seglen (11). The cells were plated in 35-mm Falcon dishes (4×10^5 cells/dish) and cultured as monolayers at 37℃, in a humidified atmosphere of 5% CO_2 in air, essentially as described by Tanaka et al. (12). The culture medium was 5% FCS-WEN (Williams medium E supplemented with 5% fetl calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 40 mM ℓ-glutamine) containing 1 μM dexamethasone and 1 nM insulin. The medium was changed 4 h after seeding to 5% FCS-WEM supplemented with 1 μM dexamethasone.

Assay of DNA synthesis Test samples or insulin (0.1 μM) and EGF (100 ng/ml) was added to rat hepatocyte cultures 24 h after plating. The culture medium was changed to serum-free WEM 22 h after the addition of these growth factors and them pulse-labeled with [^{3}H] thymidine for 2 h at 37℃, followed by washing 3 times with cold phosphate-beffered saline (PBS). After the cells had been fixed with 10% trichloroacetic acid, the anchored cells were solubilized with 1.0 ml of 0.1 N NaOH, 0.1% sodium dodecyl sulfate. Aliquots of this solution were used for measurement of radioactivity and protein.

Autoradiography Hepatocytes were cultured on Lux Thermonox coverslips which were immersed in dishes, and labeled with [^{3}H] thymidine (4 μCi/ml, 18.2 Ci/mmol) between 22 and 46 h after plating. The coverslips bearing the cells were then washed 5 times with cold PBS, fixed in 8% neutral formalin (pH 7.4) and mounted on microscope slides. These were the dipped in Sakura NR-M2 emulsion and stored in the dark for 5 days. After development, the cells were stained with hematoxylin and eosin.

Preparation and partial purification of HPSF Male Wistar rats weighing 200 g were given food and water ad libitum. D-Galactosamine hydrochloride (D-GalN) was dissolved in saline immediately before use and adjusted to pH 7.0 with 6 N NaOH. D-GalN (1000 mg/kg body weight) was administered intraperitoneally after overnight fasting. At 24 h after D-GalN administration, livers were removed after perfusion with 100 ml of cold physiological saline solution in situ via the portal vein, cut into pieces and homogenized in a 4-fold volume of ice-cold buffer (0.25 M sucrose, 20 mM Hepes, pH 7.4) with a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 10,000 x g for 10 min and the supernatant was then centrifuged at 100,000 x g for 1 h. The supernatant thus obtained was used as a crude HPSF preparation and as a starting material for further purification. Crude HPSF preparation obtained 24 h after D-GalN administration was found to show the most marked stimulatory activity for DNA synthesis in adult rat hepatocytes (Fig. 1). Details of the purification

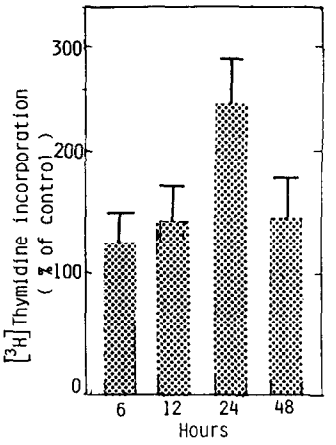

Fig. 1. Time course of the production of HPSF activity in the liver after D-GalN administration.

procedures are described in Results and in the legends of the figures and tables. All test samples were sterilized by filtration through a 0.22-μm membrane filter.

Other assay methods The protein concentration in the solubilized cells was determined using the method of Lowry et al. (15). Other proteins were determined using Bradfold reagent (16) purchased from BioRad.

RESULTS

Partial purification of HPSF The crude HPSF preparation was fractionated with ammonium sulfate at a saturation of between 30% and 40%. The precipitate was collected by centrifugation for 10 min at 10,000 x g, dissolved in 20 mM Hepes buffer (pH 7.4), and dialyzed for 24 h against the same buffer. The dialyzed preparation showed an 11-fold elevation in specific activity for DNA synthesis compared with the crude preparation. The ammonium sulfate fraction was then applied to a heparin-Sepharose column (1 x 10 cm) equilibrated with 20 mM Hepes buffer (pH 7.4). The HPSF activity was collected in the flow-through fraction (heparin fraction) and dialyzed against 50 mM Tris-HCl buffer (pH 7.4). This fraction was applied to DEAE cellulose column (2 x 20 cm) equilibrated with the same buffer, and the column was washed extensively with the buffer until the 280-nm absorbance of the eluate reached the baseline level. The HPSF activity retained on the column was eluted with a stepwise NaCl gradient in the same buffer between 0.1 M and 0.2 M (Fig. 2). The active fraction was dialyzed against 20 mM Hepes buffer (pH 7.4) and

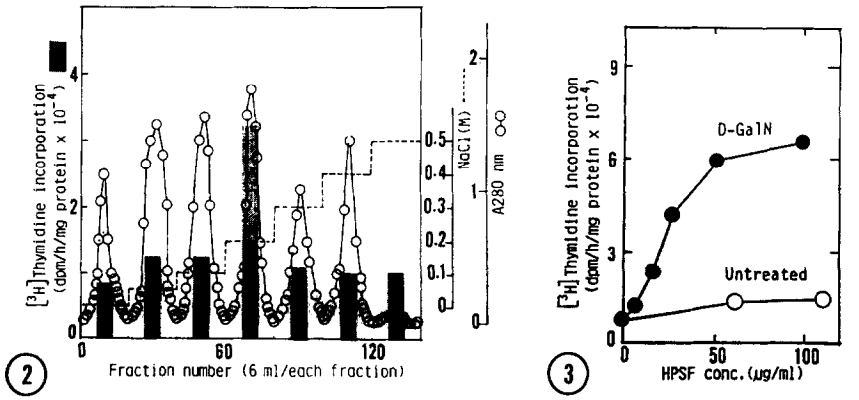

Fig. 2. Flow-through fraction obtained from a heparin-Sepharose CL-6B column applied to a DEAE-cellulose column in Tris-HCl (50 mM, pH 7.4) and eluted with a stepwise NaCl gradient (0.0-0.5 M).

Fig. 3. Dose-response curves showing stimulatory activity on DNA synthesis of HPSF obtained by DEAE-cellulose chromatography from untreated livers and from D-GalN-treated livers.

concentrated on an Amicon Diaflo PM-10 ultrafiltration membrane. This partially purified HPSF preparation, about 300 times purer than the crude HPSF, was used throughout the experiment. Figure 3 shows the dose-response curve of HPSF for DNA synthesis, which indicates that a stimulatory effect occurred at 5 μg/ml and was maximal at 50 μg/ml. When the liver of normal rats was subjected to the same purification steps, the preparation corresponding to the HPSF preparation showed only slight stimulatory activity for DNA synthesis, far less than that of the HPSF obtained from D-GalN-treated rats.

Characterization of HPSF When the HPSF preparation (1 mg/ml) was heated for 10 min in a boiling water bath or for 30 min at 56℃, the HPSF activity in the supernatant was completely lost. However, the HPSF activity was relatively stable to acid: when the factor was incubated with 1 M acetic acid for 12 h at 4℃, 85% of the activity remained. The HPSF preparation was then treated with trypsin (10 μg/ml) for 2 h at 37℃, and the reaction was stopped by the addition of soybean trypsin inhibitor (100 μg/ml). This treatment completely inactivated the HPSF activity. The HPSF thus appeared to be a heat-labile, acid-stable and trypsin-sensitive protein (Table 1).

Table 2 shows the biological properties of HPSF. The HPSF alone markedly stimulated DNA synthesis in adult rat hepatocytes and showed a greater

Table 1.
Effects of various treatments on the activity of HPSF partially purified by DEAE-cellulose chromatography
Details of treatments are described in Results

Treatment	DNA synthesis*	(% activity)
No addition	14.4	
Untreated	172	(100)
Heat		
100℃, 10 min	7.0	(0)
56℃, 10 min	11.0	(0)
1 N Acetic acid		
12 h, 4℃	146	(85)
Trypsin		
10 μg/ml, 2 h, 37℃	13.8	(0)

*dpm/h/mg protein x 10^{-3}
Value are means for triplicate dishes

stimulatory effect than insulin plus EGF, although the protein concentration of HPSF used was higher than those of insulin of EGF. Insulin (10^{-7} M), MSA (200 ng/ml) and EGF (100 ng/ml) were each used at the maximal effective dose. The increase in DNA synthesis after addition of various growth factors was found to correlate well with that of the labeling index.

DISCUSSION

The mammalian liver possesses a remarkable capacity for regeneration in response to a variety of stimuli. Studies on mammalian liver regeneration have revealed the existence of putative hepatocyte proliferation factors (1-10, 13, 14). In the present study, a proliferation factor for adult rat

Table 2.
Effect of HPSF, insulin, MSA and EGF on DNA synthesis and labeling index of adult rat hepatocytes in primary culture

Hormones	KNA synthesis*	Labeling index (%)
No addition	16.1	0.3
Insulin (10^{-7} M)	27.9	0.6
MSA (200 ng/ml)	24.1	
EGF (100 ng/ml)	117	4.9
HPSF (50 μg/ml)	225	12.3
Insulin, EGF	143	8.9
Insulin, HPSF	274	
EGF, HPSF	416	
Insulin, EGF, HPSF	457	22.2

*dpm/h/mg protein x 10^{-3}
Values are means for triplicate dishes

hepatocytes in primary culture was isolated and partially purified. This factor was found in the liver of rats treated with D-GalN and was tentatively designated HPSF.

Stimulation of DNA synthesis by the HPSF was several times higher than that by other growth factors, although the amount of HPSF protein used was much higher because of the fact that the preparation was only partially purified. The effect of HPSF was synergistic with that of insulin or EGF. These data suggest that this HPSF differs from insulin, MSA and EGF.

In a preliminary experiment, we subjected the crude HPSF preparation to gel filtration on a Sephadex G-100 column and the molecular weight of HPSF was found to be about 120 kDa(data not shown). HPSF was a heat-labile, acid-stable and trypsin-sensitive protein (Table 1). The factor purified by Goldberg (13) from the liver of partially hepatectomized rats was heat-stable protein with a molecular weight of'38 kDa. Another factor purified by LaBrecque et al. (8) from the liver of weaning rats was a heat-stable protein showing a molecular weight of 12-18 kDa.

Recently, Nakamura et al. (6) purified a hepatocyte growth factor from rat platelets, which was a heat- and acid-labile protein with a high affinity for heparin, and suggested that this was not produced in the liver. HPSF seems to differ from this factor because the HPSF is acid-stable and shows no affinity for heparin. Two papers have reported the presence of hepatocyte growth factor in sera of rats treated with hepatotoxin. Leevy et al. (9) reported that in plasma, a heat-stable substance was present during initiation of the regenerative response after CCl_4 intoxication. Morley et al. (10) reported that serum obtained between 6 and 48 h after administration of thioacetamide to rats stimulated hepatic DNA synthesis in mice and rats. Both of these reported factors were present in the blood of rats and were heat-stable. Moreover, they seem to be different from HPSF not only with regard to stability against heat or acid, but also in their affinity for heparin, localization and molecular weight.

The results obtained here suggest that an autostimulatory factor may play an important role in the mechanism of liver regeneration after hepatotoxic injury. Further purification and characterization of HPSF are currently in progress.

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