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Human Serum does contain a high molecular weight hepatocyte growth factor: studies pre- and post-hepatic resection

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Levels of a high molecular weight hepatotrophin were measured in human serum taken from patients before and 24 hours after undergoing major hepatic resection. In in-vitro rat hepatocyte cultures a 'hepatotrophin' enriched fraction of human serum induced the incorporation of tritiated thymidine into DNA in both pre and post-operative patients. Levels after hepatic resection were 2-3 fold higher than those achieved at the same protein concentration before operation in the same patient. The hepatotrophic factor had an apparent molecular weight of approximately 150,000 daltons, and was an anionic protein. © 1986 Academic Press, Inc.

The liver regenerates very rapidly after massive hepatic resection (1). Several potential mechanisms have been proven. Numerous substances said to have a 'trophic' effect on the liver have been described (2), including amino acids (3), a number of polypeptides such as insulin and epidermal growth factor (4), and larger proteins (5). Recently two reports of hepatotrophic factors have described proteins of molecular weight greater than 120,000 daltons (6) and approximately 150,000 daltons (7) in rat serum after a 67% partial hepatectomy. Michalopoulos et al showed a small hepatotrophic substance (MW 3000 daltons) to be present in human serum but could find no evidence of the larger protein liver cell growth factor. Nakamura et al described a high molecular weight hepatotrophic factor in rat serum, but did not find any evidence of such a factor in rabbit or human serum when tested in an in-vitro rat hepatocyte culture system.

This study reports the existence of a large molecular weight hepatocyte growth factor, notably in serum take from patients 24 hrs after a massive hepatic resection, but also present in normal serum.

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Materials and Methods

Materials: Collagenase was obtained from Boehringer Manheim (BCL). Collagen was prepared from rat tail tendon. These culture reagents were purchased from Gibco. Hypnorm was administered to the rats at 1 ml/kg (Janssen Pharmaceuticals). Sephadex G200 and Heparin Sepharose CL6B were obtained from Pharmacia Ltd, Milton Keynes, UK, and prepared as described by the manufacturer. 6-3H-thymidine was obtained from Amersham, UK, at 1mCi/ml (20 Ci/mmol). August in-bred rats (male 200-250 g(spf)) were obtained from the MRC laboratories, Mill Hill, UK. Hepatocytes were always prepared in the morning from animals fed ad libitum.

Chromatography: Human serum (20ml) was separated on a Sephadex G200 column (6 x 75 cm) in phosphate buffered saline (PBS) at pH 7.3., at 4°C. Sephadex G200 separates protein from 10,000 to 300,000 daltons. The column was calibrated with IgG (MW approx. 160,000) and the fractions of serum corresponding in size to this fraction were pooled and concentrated to a small volume on an Amicon diafiltration system using a PM10 membrane. The concentrated protein fraction was applied to a Heparin-sepharose CL6B affinity column (1 x 5 cm) as described by Nakamura et al. Briefly the sample was loaded in 10 mM phosphate pH 7.0, containing 0.15M NaCl and washed with 20 volumes of starting buffer at 7 ml/hr. The salt concentration was increased to 0.3M NaCl and a further 10 volumes of buffer collected. Finally the active fraction was eluted using 1.5M NaCl. 2 ml fractions were collected and monitored for protein at 280 nm. The protein containing fractions of the 1.5M NaCl eluate were pooled and diafiltrated with Williams E medium without FCS to provide the factor in a physiological medium. Rat serum taken 24 hrs after a 67% partial hepatectomy was prepared exactly as above.

Liver perfusion: Rat liver was perfused in situ via the portal vein by a modification of the classical method of Berry & Friend (8). After 5 min of perfusion in Calcium free buffer, the Kreb-Hensleit buffer was supplemented with 0.05% collagenase in the presence of 5 mM $CaCl_2$. Differential centrifugation at 50 g separated viable parenchymal cells from Kupffer cells and dead parenchymal cells to produce a final yield of approximately 250 million viable cells. Viability was tested using trypan blue dye exclusion and phase contrast microscopy, and always exceeded 80%.

Cell cultures: Tendons were removed from rat tails and stirred for 3 days in 1% acetic acid. The viscous solution was centrifuged at 3000g for 2 hrs to remove particulate matter. The resulting supernatant was used to coat petri dishes with collagen. 1 ml of collagen solution was added to each petri dish and allowed to stand at room temperature for 5 min. The collagen was decanted and the dishes washed with sterile Hanks medium twice until pH neutrality was reached. The dishes were sterilzed by exposure to UV light for 15 min, in sterile normal saline (0.155M). Cells were plated in collagen petri dishes (3.5 cm diameter) at 750,000 cells per ml in 1 ml of Williams E medium containing 5% FCS, Penicillin (100 µg/ml), Streptomycin (100 µg/ml) and Gentamicin (100 µg/ml), and Dexamethasone 10^{-8}M. The cells were incubated in a humidified 37°C incubator under 95% O_2, 5% CO_2 for 2 hrs. Phase contrast microscopy revealed flattened hepatocytes assuming a hexagonal appearance after this time. The medium was changed and the 'factors' under test were added. A positive control of epidermal growth factor (20 ng/ml) and insulin (10^{-7}M) was added in each experiment. Cells in medium and dexamethasone alone were considered unstimulated, and some of the positive control dishes were subjected to a DNA synthesis inhibitor, Mitomycin C, 30 min prior to tritated thymidine addition. 12 hrs after addition of the factor tritiated thymidine was added (3.7 µCi/plate) in 37.5 µl medium. 18 hrs later cells were harvested for thymidine incorporation.

Cell Harvesting procedure: The dishes were washed twice with 2 ml of cold PBS. 1 ml of ice cold 10% Trichloroacetic acid (TCA) was added to the cells for 5 mins to precipitate the protein. The cells were washed with cold PBS and solubilized with 1 ml of 1M KOH at 37°C for 1 hr. The

solutions were transferred to glass tubes, neutralized with 6N HCl, and treated with ice cold TCA (10%) for 30 min on ice. The cloudy solutions were centrifuged at 3000g for 10 mins and the precipiates washed with 5% TCA. DNA was solubilized by the addition of 10% TCA at 90°C for 15 min. The solutions were centrifuged and the supernatants containing the radio-labelled solubilized DNA were counted in a β-scintillation counter for radioactivity. The protein was solubilized by the addition of 4M KOH, and determined by the Lowry procedure. DNA assays were performed using the fluorimetric diamidino-phenylindole-HCL DNA complexing technique (9). Results were expressed as dpm/mg protein or dpm/μg DNA.

Preparation of human serum

30-40 ml of blood was taken into glass bottles and allowed to clot at 4°C overnight prior to the serum preparation. Blood was collected from 2 patients prior to a substantial hepatic resection (extended right hepatic lobectomy for tumour) and 24 hrs post operation. Healthy volunteers provided control serum. Each sample was subjected to the chromatographic steps described and the semi-purified factor from each time point tested in triplicate in the same tissue culture experiment. The factor was tested for activity at 3 doses, 67 μg/ml, 160 μg/ml and 320 μg/ml.

Results

The hepatotrophin enriched fraction from rat serum was found to stimulate 3H-thymidine incorporation into DNA in in-vitro rat hepatocyte culture, reaching a peak at a dose of 81.25 μg protein/ml, confirming the results of Nakamura et al.

In human serum semi-purification of the bioactive fractions by gel filtration on Sephadex G200 followed by affinity chromatography on heparin-sepharose with stepwise salt elution yielded a preparation of protein concentration approximately 1 mg/ml. Total protein yield in this fraction was approximately 2 mg from 20 ml human serum.

This fraction similarly induced tritiated thymidine incorporation into DNA. The fraction was tested in triplicate tissue culture dishes at three doses of 67, 160 and 320 μg/ml. In both subjects hepatotrophic activity was evident in the pre- and post-operative samples at all three doses, with enhanced activity in the post-operative sample. This activity is manifest when expressed as dpm 3H-thy in DNA/mg protein (Fig 1) or per μg total DNA (Fig 2). Results expressed in this way indicated DNA synthesis by the salvage pathway; confirmation of thymidine incorporation into nuclear DNA was obtained autoradiographically.

Availability of human liver cell growth factor is by its nature limited hence even at the highest dose tested in the post-operative serum

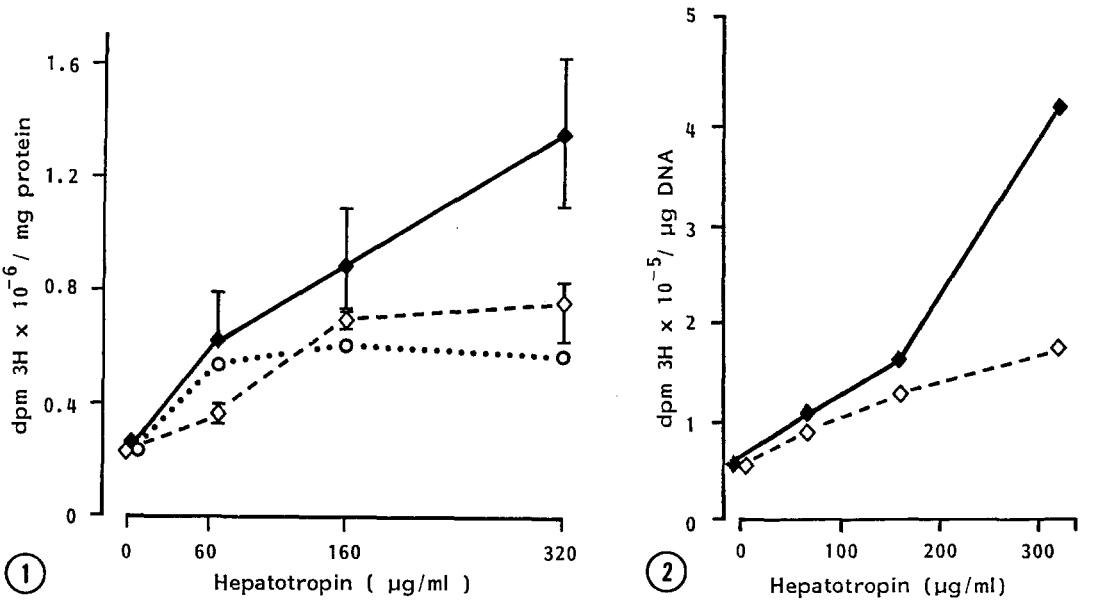

Fig 1

Tritiated thymidine incorporation into DNA in response to increasing concentrations of hepatotropin. Results expressed per mg protein. ◇ patient 1 prior to operation; ◆ patient 1 24 hr post operation; o normal control. Bars indicate ranges within each dose.

Fig 2

Tritiated thymidine incorporation into DNA in response to increasing concentrations of hepatotropin. Results expressed per ug DNA. ◇ serum from patient 2 prior to operation; ◆ patient 2 24 hr post operation.

the maximum stimulation may not have been reached, but it is at least five-fold above basal levels. The hepatotrophic activity of human serum taken pre-operatively is less striking, but still shows a dose dependent increase. At the highest dose tested there is a 2-3 fold increase in DNA synthesis post-operatively (0.814×10^6 and 1.454×10^6 dpm 3H DNA/mg protein) as compared with pre-operative values (0.359×10^6 and 0.760×10^6 dpm 3H DNA/mg protein). In comparison with unstimulated hepatocytes, the positive control used in this study, of insulin 10^{-7}M and epidermal growth factor at 20 ng/ml, caused a 10-20 fold increase in DNA synthesis. DNA incorporation into hepatocytes in the presence of hepatotrophic factors from normal human serum showed similar kinetics to those with fractions from pre-operative patient serum. Human 'Hepatotropin' when added to tissue cultures together with insulin (10^{-7}M) acts in a

Table 1 DNA Synthesis
Incorporation of 3H-thymidine into DNA

	dpm 3H x 10^{-6}/ mg protein
Dexamethasone (Dx) 10^{-8}M	0.158
Dx + Insulin (Ins) 10^{-7}M	0.670
Dx + Hepatotropin (Hep) 160µg/ml	0.399
Dx + Insulin 10^{-7}M + Hepatotropin 160µg/ml	1.29
Dx + Hep 320µg/ml pre-operation	0.359
Dx + Hep 320µg/ml 24hr post-operation	0.814
Dx + Ins + Epidermal growth factor 20ng/ml	2.36

synergistic rather than additive manner with regard to DNA synthesis. Table 1 shows the thymidine incorporation in a single experiment. The 'Hepatotropin' was found to be stable to storage at -20°C and -70°C. Frozen and thawed samples showed no decrease in activity as compared with fresh samples.

Discussion

In this study we have shown that human serum contains a high molecular weight liver cell growth factor which is active in in-vitro primary rat liver cell culture. This observation is not in agreement with Michalopoulos et al or with Nakamura et al despite the essentially identical experimental procedures used in the latter report, however, these authors only looked at normal human serum. The convincing demonstration of this factor in this study derived from the opportunity of studying serum from patients under going partial hepatectomy, and massive hepatic resection in man is an infrequently performed operation.

We considered the possibility that the hepatotrophic activity present after resection reflected a tumour product released into the circulation from the resected tumour at surgery, as both serum specimens came from patients undergoing hepatic resection for this condition. However, the

cells of origin of the two tumours (one primary hepatocellular carcinoma, one metastatic colonic adenocarcinoma secondary) make this extremely unlikely.

The concentration of 'Hepatotrophin' required for maximum activity assuming an approximate molecular weight of 150,000 is 0.54 μM. For a conventional growth factor this concentration might be considered pharmacological (cf EGF 3nM), however, most conventional growth factors described to date are small peptides, and preliminary polyacrylamide electrophoresis of the semi-purified preparation revealed 2 major and 3 minor components; hence a physiological role for this factor should be not ruled out. Previous studies in our institution on the effect of partial hepatectomy on ectopically implanted liver cells in rat spleen indicate the presence of systemic hepatotrophic stimulatory activity since the rate of proliferation of hepatocytes in the spleen doubled 24 hr after partial hepatectomy as compared with sham operated controls (10, 11).

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