Hepatotropin mRNA expression in human foetal liver development and in liver regeneration

Clare Selden[1], Michael Jones[2], David Wade[3] and Humphrey Hodgson[1]

[1]*Gastroenterology Unit, Department of Medicine, Royal Postgraduate Medical School, Du Cane Rd, London W12 0NN, UK,* [2]*Department of Virology, R.P.M.S., London, UK* and [3]*MRC Lipoprotein Team, R.P.M.S., London, UK*

Received 28 June 1990; revised version received 12 July 1990

A 569 bp probe against the β-chain of hepatotropin was used to examine expression of RNA for this growth factor in human adult and foetal liver, foetal kidney and pancreas, and rat liver after partial hepatectomy. Low level expression of a 6 kb RNA occurred in human adult and normal rat liver. 70% hepatectomy increased expression, peaking at 10 h and returning to near normal levels 24 h after resection. The 6 kb band was strongly expressed in human foetal liver, as compared with adult, but not in foetal kidney or pancreas, suggesting a major role for hepatotropin in both foetal development and regeneration of the liver.

Liver; Fetal development; Messenger RNA; Regeneration

1. INTRODUCTION

Hepatotropin was first described in rat serum in 1984, by Nakamura et al. [1,2], as a growth factor which induced proliferation in cultures of adult rat hepatocytes in vitro and whose concentration rose after partial hepatectomy. We described a similar substance in normal human serum, and demonstrated a dramatic increase in its activity in the serum of patients who had undergone a hepatic resection for liver tumours [3]: a similar activity was described in serum of patients with fulminant hepatic failure [4]. At the same time Michalopoulos et al. described hepatopoietin A, a growth factor of >120 kDa, in rat serum and subsequently in rabbit and human serum [5]. These three high molecular weight growth factors, hepatotropin, hepatocyte growth factor and hepatopoietin A, now appear to be the same substance and in this paper will be referred to as hepatotropin for simplicity. Hepatotropin has been recently purified and cloned, and its primary structure deduced from the nucleotide sequence [6,7]. A 6 kb mRNA for hepatotropin was identified in human liver [7]. A role for hepatotropin in hepatocyte regeneration after CCl_4 induced damage was indicated by expression of a similar 6-kb RNA in rat liver 10 h after induction of damage [7].

We have constructed a 569 bp probe for the C-terminal end of the β-chain, using the published nucleotide sequence [6], to examine the expression of this RNA during regeneration after partial hepatectomy and during foetal development. Our results suggest a major role for hepatotropin in liver growth during embryonic development as well as in regeneration of adult liver after surgical and chemical injury.

Correspondence address: C. Selden, Gastroenterology Unit, Department of Medicine, Royal Postgraduate Medical School, Du Cane Rd, London, W12 ONN, UK

Abbreviations: MOPS, 3-[*N*-morpholino]-propanesulphonic acid; 20 × SSC, 3 M NaCl, 0.3 M sodium citrate

2. MATERIALS AND METHODS

2.1. *DNA probe preparation*

Two 20-mer oligonucleotides were synthesised on an Applied Biosystems oligonucleotide synthesizer, model 380B. Primer sequences were GCTTGGAATTCATGATGTCC and GCTGCAGACACACTTACTTC representing the ends of a 569 bp sequence from the hepatotropin cDNA which contained *Eco*RI and *Pst*I restriction sites. mRNA from human liver was prepared by the method of Chirgwin et al. [8]. The polymerase chain reaction (PCR) was used to amplify RNA previously treated with reverse transcriptase (super RT, Anglian Biotechnology Ltd), with 30 cycles at annealing temperature of 60°C, and Taq polymerase (Amplitaq, Perkin Elmer - Cetus). The PCR product was gel purified, ligated into Bluescribe plasmid (Stratagene, USA), cloned in *E. coli* TG2 bacteria. Plasmid DNA was purified utilizing caesium chloride ultracentrifugation. The PCR product was also cloned into M13 vectors in both orientations for sequencing of single stranded DNA; sequencing was achieved with the Taq polymerase Sequencing system (TAQuence, USB, Cleveland, OH, USA), 50 ng DNA were labelled with 50 μCi [^{32}P]CTP (Amersham PB10202, 370 MBq/ml, 3000 Ci/mmol) using random primer labelling [9].

2.2. *RNA filter hybridization*

RNA was prepared from human and rat liver by two methods, the guanidinium isothiocyanate/caesium chloride ultracentrifugation method of Chirgwin et al. [8] and the lauryl sarcosine/isopropanol/phenol chloroform method of Chomczynski et al. [10]. All solutions were made up with diethylpyrocarbonate-treated water and autoclaved. All glassware was baked at 200°C overnight. Total RNA was quantified by optical density measurements at 260 nm, assuming 1 absorbance unit = 40 μg RNA/ml. The ratio of absorbance at 260:280

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

nm was also assessed. For rat liver samples this was 1.7 ± 0.12 (SD), for human tissues it was 1.83 ± 0.15 (SD). Samples of total RNA were resolved on 1.5% agarose gels containing 6% formaldehyde, at 120 mA for 3 h, in a running buffer of 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0. RNA was transferred onto 0.2 μm Biodyne A biosilon membrane in 20 × SSC for 24 h and was fixed onto the nylon membrane by exposure to UV light at 254 nm for 12 min at a distance of 16 cm. Membranes were prehybridized for 15 min without probe and then hybridized with radiolabelled DNA overnight as described by Church and Gilbert [11]. Low stringency hybridization conditions were 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% bovine serum albumin and 7% sodium dodecyl sulphate (SDS), at 65°C using 2 × 10^6 dpm labelled probe/ml hybridization buffer. High stringency hybridization was achieved in 0.2 M sodium phosphate and 15% formamide. Blots were washed at high stringency in 40 mM sodium phosphate, 1% SDS, 1 mM EDTA, at 65°C with several changes. The filters were exposed to pre-flashed Kodak XAR-5 film at −70°C for up to 14d, using intensifying screens.

2.3. *Tissue samples*

Surgical specimens of adult human liver and specimens of foetal tissue obtained at termination of pregnancy, were studied with approval from the Royal Postgraduate Medical School Ethics Committee. Rat liver tissue was obtained under a Home Office licence (Animals - Scientific Procedures Act, 1986). Tissue was snap frozen and stored in liquid nitrogen until use. 70% partial hepatectomy was performed as described by Higgins and Anderson [12]. Rats were sacrificed at 15 min, 45 min, 2 h, 4 h, 10 h, 12 h and 24 h after surgery. Rats treated with carbon tetrachloride received a single dose of 75 mg/kg body weight in olive oil by gavage, and were sacrificed 10 h later.

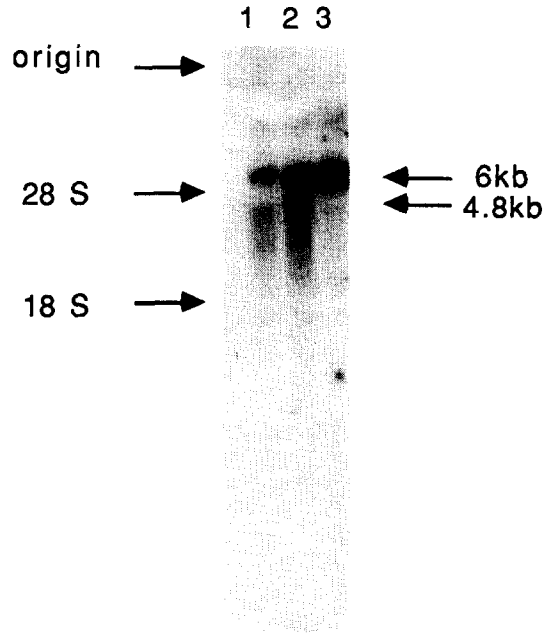

Fig. 1. Northern blot analysis of human liver RNA. 1, normal adult, 2, adult liver removed at time of resection for tumour; 3, foetal human liver, 16.2 week gestational age. 8.54 μg total RNA in each lane. Autoradiograph exposure 6d, −70°C. High stringency hybridization conditions as described in the text.

3. RESULTS

3.1. *Double stranded 569 bp DNA probe*

The 569 bp cDNA probe was shown by DNA sequencing to be homologous to the C-terminal portion of the β-chain of human hepatotropin described by Miyazawa et al. [6], nucleotides 1640–2209, and to have minor sequence differences from that described by Nakamura et al. [7].

3.2. *Northern Blot RNA analysis*

RNA preparation from rat and human liver yielded intact total RNA using either the caesium chloride method or the Chomczynski method. In subsequent studies the Chomczynski technique was used since it was more appropriate for small samples. The 569 bp probe hybridized with an approximately 6 kb human liver RNA under high stringency conditions, confirming the results of Nakamura using polyA+ human liver mRNA, and also hybridized to a smaller, approximately 4.8 kb, fragment.

3.3. *RNA expression in human adult and foetal tissue*

Fig. 1 shows the Northern blot analysis of adult human liver prepared from patients who underwent surgical procedures, and that of foetal human liver (16.2 weeks gestational age). The two RNA samples prepared from two adults show similar amounts of 6 kb RNA, but appear to show a size difference in the smaller fragment. At 16.2 weeks gestational age there is a marked increase in the expression of the 6 kb fragment, with proportionally less of the 4.8 kb fragment, as compared with adult human liver.

We investigated the time course of hepatotropin RNA expression in 5 specimens of foetal human liver ranging from 7.6–19.5 weeks gestational age. In all specimens there was strikingly enhanced mRNA expression compared to adult human liver (11 to 22-fold increase assessed by scanning densitometry of autoradiographs of the 6 kb band) (Fig. 2). Foetal pancreas demonstrated similar levels to normal adult liver whereas in foetal kidney the 6 kb mRNA species was only just detectable. An integrity agarose gel of the RNA samples showed all the liver, kidney and pancreas samples to be intact with a 2:1 ratio of 28:18S RNA, but the RNA prepared from foetal spleen was considerably degraded; despite evidence of the 6 kb RNA in this sample no conclusions could be drawn from splenic tissue. Ethidium bromide staining of integrity gels, performed in parallel, using equivalent concentrations of RNA determined by optical density at 260 nm demonstrated similar amounts of 28 and 18S fluorescence under 254 nm UV light in each sample. By densitometric scanning of photographed gels, the coefficient of variation in fluorescence of 28S ribosomal RNA between samples was 0.3.

3.4. *RNA expression in rat liver*

Using this probe we were able to detect the expression of mRNA in normal rat liver. In RNA prepared from normal rat liver, and from animals sacrificed at 15 min,

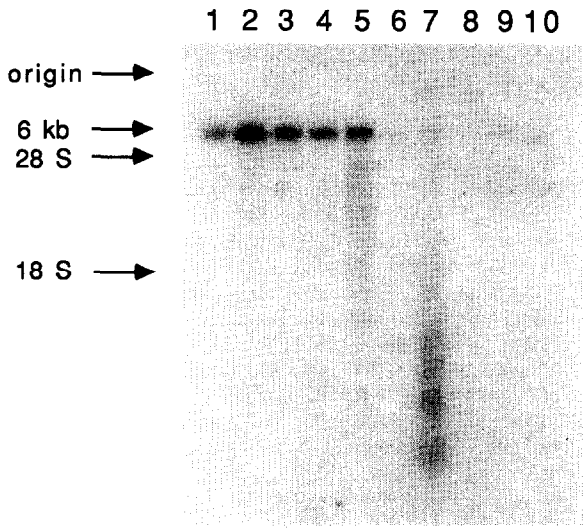

Fig. 2.Northern blot analysis of human liver RNA. 1-5 foetal human liver: 1, 7.6 wk gestational age; 2, 9.7 wk; 3, 12.6 wk; 4, 16.2 wk; 5, 19.5 wk; 6, foetal kidney 17.6 wk; 7, foetal spleen; 8, foetal pancreas 11.9 wk; 9, foetal pancreas 15.9 wk; 10, adult human liver. All lanes were loaded with 3.08 μg total RNA except 8 which had 0.87 μg. Autoradiograph exposure 14d, −70°C. High stringency hybridization conditions.

45 min, 2 h, 4 h, 10 h, 12 h and 24 h after partial hepatectomy, we studied the expression of the 6 kb mRNA. Maximum expression was observed 10 h after hepatectomy (Fig. 3), reaching a level at least 10-fold greater than that in normal rat liver (normal rat = 791 integral densitometric units, 10 h posthepatectomy = 8156 integral densitometric units, measured with a Joyce Loebl scanning densitometer). The earliest detectable increase in expression occurred 2 h post hepatectomy; by 24 h the RNA expression was reduced to near resting levels. Measuring hepatotropin RNA expression in rat liver after carbon tetrachloride administration we confirmed the reported elevation in 10 h, only investigating this time point. The level of expression was not as great as that seen after partial hepatectomy. The damaging effect of this high dose of CCl_4 was confirmed by substantial elevations of liver enzyme levels in plasma (alkaline phosphatase 780 IU/l, normal range 30–130 IU/l: aspartate amino transferase 678 IU/l, normal range 5–27 IU/l).

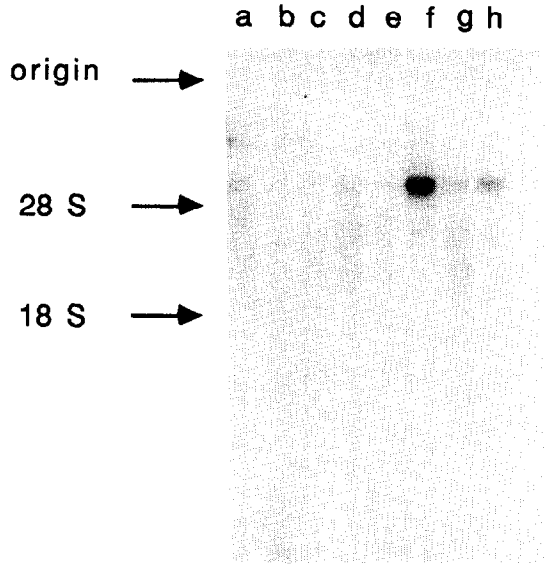

Fig. 3. Northern blot analysis of rat liver RNA. a, normal rat liver; b–g, liver after partial hepatectomy: b, 15 min; c, 45 min; d, 2 h; e, 4 h; f, 10 h; g, 24 h; h = rat liver 10 h after CCl_4 administration by gavage. 8.54 μg total RNA in each lane. Low stringency hybridization conditions as described in the text. Autoradiograph exposure 6d, −70°C.

4. DISCUSSION

This study reports enhanced expression of RNA for hepatotropin in human foetal liver. This implies for the first time that hepatotropin is involved in normal physiological liver growth during development, in addition to its involvement in liver regeneration after injury, suggesting similar mechanisms may be involved during normal embryonic development and liver regeneration after damage.

The previously reported evidence of hepatotropin mRNA expression in adult rat liver after damage followed the use of carbon tetrachloride. Kinoshita et al. demonstrated a peak at 10 h with persistent elevation of mRNA expression up to 24 h [13]. We demonstrated enhanced expression of hepatotropin RNA in the liver after surgical partial hepatectomy. The peak expression was also at 10 h but had returned to near normal levels by 24 h. This difference may be due to more prolonged damage induced by carbon tetrachloride. The findings after partial hepatectomy also confirm that RNA expression for this growth factor does not depend on exposure of liver cells to chemical injury.

Analysis of the kinetics of hepatotropin mRNA expression requires precise quantification of RNA; this is difficult since the recognised technique for quantitating changes in specific mRNA expression, by reference to expression of control 'housekeeping' genes, is not applicable to regenerating liver [14–16], as the levels of actin, tubulin, albumin etc. are altered during liver growth.

Previous reports have not commented on the presence of a second RNA species at approximately 4.8 kb in human liver, but this second mRNA is also evident in the Northern blot analysis of placental polyA+ RNA described by Miyazawa [6]. At present this second fragment has not been positively identified but it may be the product of a second related gene or could arise from alternative splicing of the hepatotropin gene. Studies are underway to clarify the importance of this smaller mRNA.

The exact source of hepatotropin synthesis is currently controversial. Recently Zarnegar et al. [17] developed antibodies to rabbit hepatopoietin A and studied its distribution in normal tissues from adult rabbits by immunohistochemistry, confirming their findings by preparing hepatopoietin A from the tissues which were immunologically positive. They localized the activity to several organs, namely pancreas, brain, salivary glands and Brunners glands, but not to spleen, thymus or kidney, and notably not to the liver. These findings differ from our results, measuring RNA expression rather than protein content, of hepatotropin RNA in normal adult human and rat liver, and from those of Kinoshita et al. who reported the expression of hepatotropin mRNA after carbon tetrachloride induced injury in the non-parenchymal cell population of the liver [13]. Zarnegar et al.'s immunohistochemical findings in normal tissues might be explained by only low level transcription of hepatotropin mRNA in non-proliferating liver, resulting in protein levels undetectable by this immunohistochemical technique. After partial hepatectomy the protein levels in the liver would increase as suggested by the increased mRNA expression demonstrated here.

Our results in foetal tissue also support a role for the liver as a major site for producing hepatotropin. The detectable expression in the foetal kidney, and levels similar to those of adult liver in foetal pancreas, predict a potential for hepatotropin synthesis in those organs.

The results of this study indicate a major physiological role for this hepatotrophic factor, in vivo, both in liver regeneration and in developmental liver growth. Further experiments will define its role relative to other stimuli to hepatocyte proliferation such as Transforming growth factor α and Heparin binding growth factor 1.

Acknowledgements: This work was supported by the Cancer Research Campaign.

REFERENCES

[1] Nakamura, T., Nawa, K. and Ichihara, A. (1984) Biochem. Biophys. Res. Commun. 122, 1450-1459.
[2] Nakamura, T., Teramoto, H., Ichihara, A., Kaise, N. and Nishino, T. (1987) FEBS Lett. 224, 311-316.
[3] Selden, C., Johnstone, R., Darby, H., Gupta, S. and Hodgson, H. (1986) Biochem. Biophys. Res. Commun. 139, 361-366.
[4] Nakayama, H., Tsubouchi, H., Gohda, E., Koura, M., Nagahama, J., Yoshida, H., Daikuhara, Y. and Hashimoto, S. (1985) Biomed. Res. 6, 231-237.
[5] Michalopoulos, G., Houck, K., Dolan, M.L. and Luetteke, N.C. (1984) Cancer Res. 4, 4414-4419.
[6] Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y. and Kitamura, N. (1989) Biochem. Biophys. Res. Commun. 163, 967-973.
[7] Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K. and Shimizu, S. (1989) Nature 342, 440-443.
[8] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
[9] Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266-267.
[10] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
[11] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991-1995.
[12] Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186-202.
[13] Kinoshita, T., Tashiro, K. and Nakamura, T. (1989) Biochem. Biophys. Res. Commun. 165, 1229-1234.
[14] Sobczak, J., Tournier, M., Lotti, A. and Duguet, M. (1989) Eur. J. Biochem. 180, 49-53.
[15] Armendariz-Borunda, J., Seyer, J.M., Kang, A.H. and Raghow, R. (1990) FASEB J. 4, 215-221.
[16] Johnson, A.C., Garfield, S.H., Merlino, G.T. and Pastan, I. (1988) Biochem. Biophys. Res. Commun. 150, 412-418.
[17] Zarnegar, R., Muga, S., Rahija, R. and Michalopoulos, G. (1990) Proc. Natl. Acad. Sci. USA., 87, 1252-1256.