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Expression of Hepatocyte Growth Factor mRNA in Regenerating Rat Liver After Partial Hepatectomy

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SUMMARY. Hepatocyte Growth Factor (HGF) is a potent complete mitogen for primary cultures of hepatocytes *in vitro*. There is strong evidence that this novel growth factor may mediate hepatocyte regeneration after liver damage. We have shown previously that the amount of immunoreactive HGF markedly increases in the serum of rats soon after partial hepatectomy or CCl_4 administration. In the present paper, we demonstrate that the level of HGF mRNA in rat liver also dramatically increases from 3 to 6 hours post hepatectomy, peaks at 12 hr and gradually returns to undetectable levels by 72 to 96 hours post hepatectomy. In separate experiments, DNA synthesis (*in vivo*) was determined in rat liver remnants after partial hepatectomy. DNA synthesis peaked 24 hr after hepatectomy, 12 hr after the peak of HGF mRNA expression. These results suggest that HGF may be one of the major early signals that triggers hepatocyte proliferation during liver regeneration.

Hepatocyte growth factor (HGF) also known as Hepatopoietin A was originally identified in the serum of hepatectomized rats as a potent mitogen for normal adult rat hepatocytes in culture (1, 2). HGF has been purified to homogeneity by several laboratories (3-7), and its primary structure has been deduced from its cDNA clone (8-10). We have shown that HGF is present in a variety of rabbit and rat tissues and have also shown that HGF is mitogenic for epithelial cells such as kidney proximal tubule epithelial cells, mouse keratinocytes, human melanoma cells and liver non-parenchymal cells in culture (11, 12). It has also been shown by others that HGF is mitogenic for melanocytes, endothelial cells, and mammary and bronchial epithelial cells (13). Recently, we described for the first time that HGF exerts its biological effect on hepatocytes through a unique cell surface receptor (14). Despite significant progress concerning the biochemical properties of HGF, its physiological role *in vivo* remains unclear. Others have shown that the amount of circulating HGF in rat plasma as well as its mRNA expression increase after treatment with the hapatotoxin CCl_4 (carbon tetrachloride) (16-19). We corroborate these findings by demonstrating that the amount of immunoreactive HGF in rat plasma increases after CCl_4 administration and after 2/3 partial hepatectomy (15). HGF levels are also seen to increase in the plasma of patients with fulminant hepatic failure (3, 5, 20). The expression of HGF mRNA in liver after injury by hepatotoxins or hepatectomy has been investigated by several researchers. Kinoshita, et al., have

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shown that HGF mRNA dramatically increases in livers of rats 5 to 10 hours after administration of CCl_4 or 24 hours after administration of D-galactoseamine, and it persists up to 40 hours post treatment (16). Okajima, et al., reported a similar finding which showed a significant increase in the amount of rat liver HGF mRNA after CCl_4 and D-galactosamine administration to rats, but not after 2/3 partial hepatectomy. (They studied only two time points, namely 24 and 48 hours post hepatectomy [18].) Noji, et al., demonstrated an increase in HGF transcript in liver after treatment of rats with CCl_4 using *in situ* hybridization. However, they mentioned that they did not detect any significant change in HGF mRNA in partially hepatectomized rat livers (17). Meanwhile, Selden, et al., (19) have seen a transient *increase* in the rat liver HGF mRNA level which is detectable 2 to 10 hours post hepatectomy and returns to normal levels by 24 hours. Because such discrepancy in the literature concerning HGF mRNA expression in liver after partial hepatectomy is found, our present studies were undertaken to clarify this ambiguity.

In the present paper, we demonstrate that the amount of HGF mRNA increases in rat liver dramatically as early as 3 hr after 2/3 partial hepatectomy. The peak of HGF mRNA expression (12 hr after operation) precedes the peak of hepatocyte DNA synthesis in operated animals by least 12 hr which strongly indicates that HGF gene expression is involved in hepatocyte proliferation and liver regeneration.

MATERIALS AND METHODS

HGF Cloning-A human placental cDNA library (λgt11 from Clontech Laboratories, Inc., Palo Alto, CA) was screened according to the standard procedures (21) using a synthetic oligonucleotide (39-mer with 16-fold degeneracy) corresponding to the amino terminal sequence of the light chain of HGF which we reported earlier (7) and a 57 anti-sense oligomer corresponding to the nucleotide sequence 309-366 (5'TTTGTTTTCATAGAGGTCAAATTCATGGCCAAATTCTTT TTTCACTCCACTTGACAT3') of the heavy chain of human HGF (Miyazawa, et al.) as probes. The probes were 5'-end labeled using T_4 kinase (Gibco/BRL, Gaithersburg, MD) and $\gamma(^{32}P)$ dATP. Twenty strongly hybridizing positive plaques were isolated and purified from 1 x 10^6 independent clones after three rounds of screening and plaque purification. DNA from these positive clones was prepared and analyzed by restriction enzyme analysis (EcoR I). One clone, designated clone 14 A which contained the largest insert of about 3 Kb, was selected for further analysis. This clone was digested with BamH I and Dra I which cut HGF at nucleotide -23 at the 5' end and at nucleotide 2262 at the 3' end, respectively (published sequence of Miyazawa, et al.). The digested products were resolved on 1% agarose gel. The band migrating at about 2.3 Kb was cut out and purified by Gene Clean II (Bio 101, La Jolla, CA). A BamH I linker (Pharmacia LKB Biotechnology, Piscataway, NJ) was then ligated to the 3' blunt end produced by Dra I, digested with BamH I and then subcloned into the BamH I site of pSPORT plasmid (Gibco/BRL, Gaithersburg, MD). The DNA from this subclone was then subjected to restriction enzyme mapping (EcoR I, Hind III, Kpn I, and Pst I) and double stranded DNA sequencing by the dideoxy chain termination method using the T7 sequencing kit from Pharmacia. The results confirmed that the cloned DNA contained the entire coding region of human HGF (nucleotide -23 to 2262).

Partial Hepatectomy and Measurement of DNA Synthesis in Rat Liver Remnants-Two-thirds partial hepatectomy and measurement of DNA synthesis in rat liver remnants after hepatectomy was carried out as described previously (15).

RNA Extraction and Northern blot-Total RNA was extracted from normal and partially hepatectomized rat livers and normal human placenta using RNAzol B solution according to the manufacturer's recommendation (Cinna/Biotecx, Friendswood, TX). Thirty micrograms of total RNA from each sample as determined at absorbance 260 nm was fractionated on 1% agarose/formaldehyde gel, transferred to nitrocellulose paper, immobilized by UV cross-linking and probed with the 2.3 Kb human HGF cDNA (BamH I fragment cloned into pSPORT plasmid described above). Blots were prehybridized for 2 hr at 65° C in hybridization solution consisting

of 6X SSC (1X SSC: 0.15 M NaCl and 0.015M Sodium Citrate, pH 7.0), 5X Denhardt's Solution (50X Denhardt's Solution: 1% Ficoll, 1% Polyvinylpyrrolidone, and 1% BSA) 10% dextran sulfate, 0.5% SDS, and 100 μg/ml denatured Salmon Sperm DNA. HGF cDNA probe was then added ($\alpha(^{32}P)$dCTP multiprime-labeled--Amersham, Arlington Heights, IL) at a concentration of 2 ng/ml (2 x 10^6 cpm/ml) in fresh hybridization solution. Hybridization was carried out overnight at 65° C. Blots were washed three times in 2X SSC at room temperature for 10 min each and twice in 0.2X SSC, 0.5 % SDS at 65° C for 20 min each. Blots were exposed to XAR X-ray film (Eastman Kodak, Rochester, NY) 1 to 3 days at -70° C.

RESULTS AND DISCUSSION

Liver regeneration can be achieved experimentally by surgical removal of part of the liver (also known as partial hepatectomy), or chemically by administration of hepatotoxins such as CCl_4. It was shown more than a decade ago that during liver regeneration blood-borne soluble factor(s) transmit mitogenic stimuli (23-25). Since then, significant progress has been made in identifying such substances. A good candidate as the physiological inducer of liver regeneration is HGF (for review see 26).

Hepatocyte growth factor (HGF) is a potent mitogen for primary cultures of rat hepatocytes and has been purified and characterized from human plasma and rat platelets (1-7). Its primary structure has been deduced from its cDNA clone (8, 9) which shows more than 90% nucleotide sequence homology to that of rat (10, 18). Previously, it has been shown by us that the amount of immunoreactive (HGF) increases in plasma of rats treated with CCl_4 or of rats that have undergone 2/3 partial hepatectomy (15). Immunohistological staining of the CCl_4-treated rat livers indicated strong immunoreactivity of damaged hepatocytes with anti-HGF antiserum. Levels of HGF also rise in sera of patients with fulminant hepatic failure or other liver diseases such as acute and chronic hepatitis and cirrhosis (3, 5, 20). Several lines of evidence suggest that HGF may be mediating hepatocyte regeneration in vivo.

In this paper, we investigated the level of HGF mRNA in regenerating liver of partially hepatectomized rats. To accomplish this, we isolated and cloned a human HGF cDNA corresponding to the entire coding region of human HGF (2.3 Kb) from a human placenta cDNA library as described under Materials and Methods. The authenticity of our cDNA clone as HGF was ascertained by restriction enzyme mapping and double stranded DNA sequencing. In addition, the HGF cDNA was used as a probe to detect HGF mRNA in human placenta by northern blotting. A 6 Kb band originally reported by Miyazawa et al (8) was detected (Fig 1). We recently showed that placenta contains a substantial amount of bioactive HGF (22). The 2.3 Kb HGF cDNA was then used to evaluate the level of HGF mRNA in regenerating rat liver after 2/3 hepatectomy. A dramatic increase in HGF mRNA was noted in regenerating livers after operation. The increase in HGF mRNA is transient; it increases more than 15-fold by 6 hours post hepatectomy, peaks at 12 hours (more than 27 fold) and gradually returns to normal levels after 72 hours post hepatectomy (see Figs. 2 and 3). In addition to the 6.0 Kb band which is the reported size of the HGF mRNA (8-10), we also detect a prominent 2 Kb band which may represent a different spliced form of HGF mRNA. We also measure DNA synthesis activity (*in vivo*) in the liver remnants of rats that underwent partial hepatectomy. It is of interest to note that DNA synthesis peaks at 24 hours post hepatectomy (Figure 3), 12 hours after the peak of HGF mRNA synthesis (and/or its stabilization).

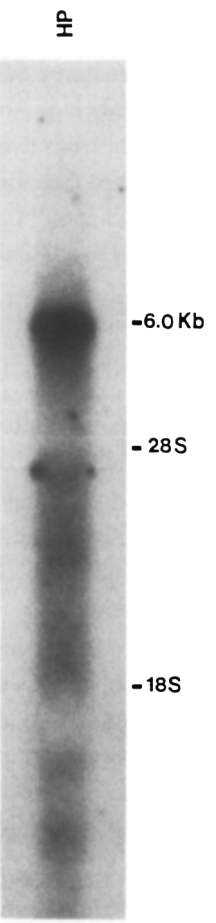

Figure 1. *The hybridization of 2.3 Kb HGF cDNA probe to a 6 Kb mRNA band prepared from human placenta.* Total RNA was extracted from human placenta and analyzed by northern blot using the 2.3 Kb human HGF cDNA as a probe as described under Materials and Methods.

Our results confirm the findings of Selden, et al. (19), who also detected a significant rise in HGF mRNA levels which peaks by 10 hours post hepatectomy. However, we have done a more detailed kinetic analysis of HGF mRNA expression after hepatectomy and show that HGF mRNA levels are elevated up to 48 hours. On the other hand, it is possible that Okajima, et al., (18) were unable to detect changes in HGF mRNA levels after hepatectomy due to the fact that they analyzed HGF mRNA starting 24 hours after surgery, a time when HGF mRNA levels are declining.

We have shown that the levels of plasma HGF increases in rats within 1 to 6 hours after CCl_4 or hepatectomy and that the CCl_4 damaged hepatocytes 24 hours after administration are strongly immunoreactive with anti-HGF antiserum as determined by immunohistological staining (15). The rapid elevation of HGF in plasma (1-6 hr) may be due to the release of this growth factor already present in the liver extracellular matrix (27) or other tissues which we have shown to contain substantial amounts of HGF. These include pancreas, salivary glands, small intestine, thyroid, and brain (11). Therefore, it is conceivable that HGF from these tissues (in an endocrine fashion) as well as HGF synthesized by the liver (non-parenchymal cells such as endothelial cells and Kupffer cells (17) in a paracrine fashion) triggers hepatocyte proliferation.

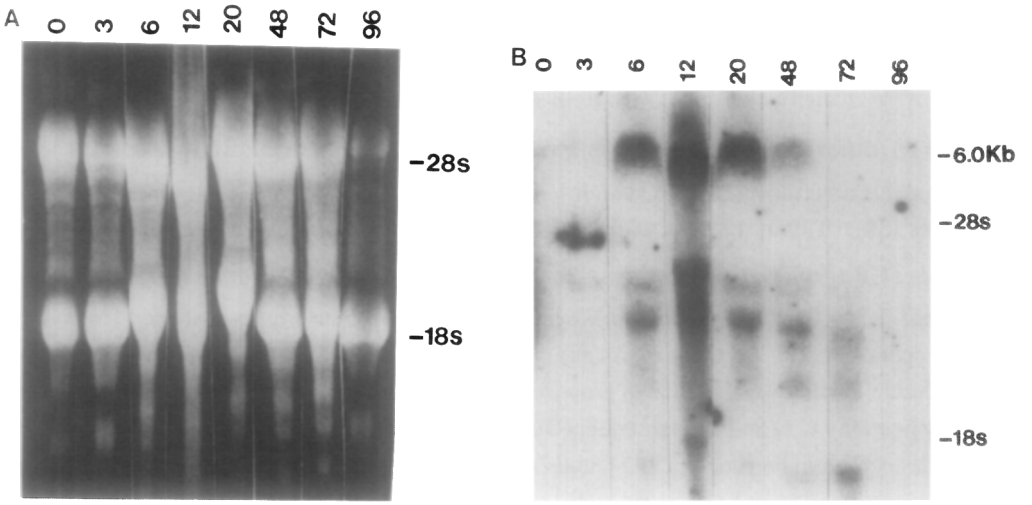

Figure 2. *Analysis of HGF RNA expression in regenerating rat liver after 2/3 hepatectomy by northern blot.* Total RNA was extracted from regenerating rat liver that underwent 2/3 partial hepatectomy at various times and analyzed by northern blot as described under Materials and Methods. (Numbers on top of Fig 2A indicate the time post hepatectomy in hours; time point 0 represents the RNA sample from control unoperated rat liver.) Thirty µg of total RNA from each time point was electrophoresed in a 1% agarose formaldehyde gel, stained with ethidium bromide (Fig. 2A), transferred to nitrocellulose paper, immobilized by UV cross-linking and hybridized to $\alpha(^{32}P)$dCTP multiprime-labeled 2.3 Kb fragment of HGF cDNA corresponding to the entire coding region of human HGF (2 x 10^6 cpm/ml). Hybridization was performed in 6X SSC, 5X Denhardt's Solution, 0.5% SDS, 10% dextran sulfate, 100 µg/ml Salmon Sperm DNA at 65° C. Blots were washed in 2X SSC for 30 min at room temperature and twice in 0.2X SSC, 0.5% SDS at 65° C for 20 min each. The blots were exposed at -70° C for 1 to 3 days (Fig 2B).

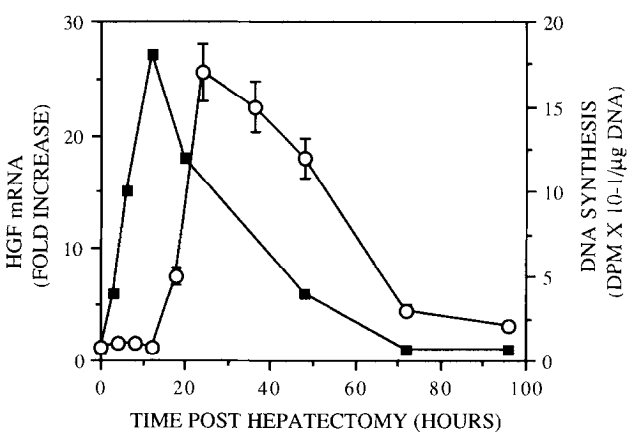

Figure 3. *Relative amount of HGF mRNA and DNA synthesis activity in regenerating rat liver after 2/3 partial hepatectomy.* The autoradiogram shown in Fig 2B was analyzed by an LKB Ultrascan XL Enhanced Laser Densitometer. Relative HGF mRNA amount is presented as fold increase (shown as solid squares) in the 6 Kb band as compared to that of control rat liver using the computed area under the peak for each time point. Total hepatic DNA synthesis in the rat liver remnants at various times after partial hepatectomy was determined by injecting ^{3}H-Thymidine into these rats and determining the amount of incorporated ^{3}H-Thymidine into DNA (shown as open circles) as described previously (15).

Further evidence which supports that HGF synthesized by the liver may be responsible for hepatocyte proliferation is the finding that the kinetics of the expression of two other hepatocyte mitogen mRNA, namely aFGF (acidic fibroblast growth factor) and TGFα (transforming growth factor type α), differ from that of HGF. The transcripts of both of these mitogens peak at 24 hours after partial hepatectomy (28, 29) which coincides with the peak of DNA synthesis in the hepatocytes (30). This is 12 hr later than the peak of HGF mRNA expression. Interestingly, aFGF and TGFα mRNA expression precedes the peak of DNA synthesis of *liver non-parenchymal cells* (30). Furthermore, the elevation of the aFGF transcript is sustained for up to 7 days post operation.(29) (This, again, is not observed in HGF mRNA expression which returns to undetectable levels by 3 days.) We propose that HGF elevation in the plasma one hour after partial hepatectomy (15) and the subsequent increase in its mRNA expression at 12 hr causes the DNA synthesis peak in hepatocytes at 24 hours after partial hepatectomy. Other mitogens, such as aFGF and TGFα though perhaps partly responsible for inducing DNA synthesis in hepatocytes, are the major mitogenic stimuli for the *later round* of DNA synthesis observed in the non-parenchymal cells which peaks at 48 hours after hepatectomy (30). We are currently attempting to determine the precise cellular source of HGF mRNA in liver after partial hepatectomy.

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