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INCREASED EXPRESSION OF mRNA FOR HEPATOCYTE GROWTH FACTOR-LIKE PROTEIN DURING LIVER REGENERATION AND INFLAMMATION

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To assess potential roles of hepatocyte growth factor-like (HGFL) protein as a growth factor and as a cytokine, we determined the expression of mRNA for (HGFL) protein during liver regeneration and inflammation. Expression of mRNA for HGFL protein in the regenerating liver was upregulated to 93% and 64% above controls at 1 h following partial hepatectomy and carbon tetrachloride treatment of rats, respectively. Similarly, rat liver mRNA for HGFL protein was upregulated to 78% above controls at 24 h after injection of thioglycollate (inducing activation of peritoneal macrophages) and to 118% at 48 h after injection of turpentine (inducing the acute phase response). These results support a potential role for HGFL protein in the early phase of liver regeneration and as an inflammatory mediator.

We have cloned and characterized the gene and cDNA coding for human and mouse hepatocyte growth factor-like (HGFL) protein (1,2). The translated amino acid sequence of the cDNA coding for HGFL protein predicts an open reading frame of 711 amino acids composed of 4 kringle domains followed by a serine protease-like domain. Two functions have been proposed for HGFL protein. The first is as a regulator of cell growth, based on its structural homology to hepatocyte growth factor (HGF). HGF is a secreted protein that consists of an α-subunit of 69 kDa and a β-subunit of 34 kDa (3). Although HGF was initially characterized as a potent mitogen to hepatocytes (4-6), it is now known that it has multiple functions, eliciting different biological responses in a tissue- or cell-specific manner (reviewed in ref 7). For example, HGF has been found to be identical to scatter factor, a protein secreted from certain fibroblasts that causes morphologic changes and dissociation of epithelial cells and has antiproliferative effects on hepatocellular carcinoma, melanoma, and squamous carcinoma cells. Messenger RNA for HGF has been identified in various organs, and in the liver it is present in nonparenchymal fat-storing cells (8,9). The expression of HGF mRNA increases during liver regeneration induced by partial hepatectomy or carbon tetrachloride treatment, achieving highest levels of expression before the peak of DNA synthesis (10,11). These data suggest that HGF produced by liver nonparenchymal cells may act on hepatocytes in a paracrine fashion stimulating liver growth. Although localization

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of expression of mRNA coding for HGFL protein has been established in the hepatocyte (12), its expression during liver regeneration has not been studied.

The second proposed function for HGFL protein is as an inflammatory mediator, based on the identity of its translated amino acid sequence with that of the partial amino acid sequence of macrophage stimulating protein (MSP; 13). MSP is involved in the activation of macrophages, inducing chemotaxis and phagocytosis in peritoneal macrophages (14,15). Thus, it is possible that HGFL protein may modulate inflammatory responses through activation of macrophages.

In this paper, we assess whether the level of expression of mRNA for HGFL protein is increased during liver regeneration and inflammation. Based on the suggested role of HGFL protein as a potential growth regulator, we measure the level of expression of mRNA for HGFL protein during liver regeneration induced by 70% partial hepatectomy or carbon tetrachloride treatment. We also determine the level of expression of mRNA for HGFL protein as a potential inflammatory mediator in two models of extrahepatic inflammation: activation of peritoneal macrophages by administration of thioglycollate medium and induction of acute phase response by administration of turpentine.

MATERIALS AND METHODS

Animals and Experimental Procedures. Adult male Sprague-Dawley rats weighing 200-250 grams were purchased from Charles River Laboratories. Rats were randomly assigned to one of four groups: partial hepatectomy, carbon tetrachloride treatment, thioglycollate medium treatment, and turpentine treatment. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Children's Hospital Research Foundation.

Partial Hepatectomy. Rats underwent 70% partial hepatectomy as described previously (16). In control (sham-operated) animals, the abdominal cavity was opened, the liver exposed, and then placed back into the abdomen without further manipulation. At set intervals, animals were anesthetized and the regenerating liver lobes were removed, snap frozen in liquid nitrogen for RNA isolation, and kept at -70°C until the time of analysis. *In situ* hybridization analysis of segments of regenerating liver was done as previously described (12).

Carbon Tetrachloride, Thioglycollate Medium, and Turpentine. In the other three experimental protocols, groups of rats received either carbon tetrachloride (Aldrich Chemical Company, Inc.) at a dose of 0.2 ml/100 gm body weight intraperitoneally, thioglycollate medium (Difco Laboratories) at a dose of 1 ml/100 gm body weight intraperitoneally, or turpentine (Parks Corporation) at 0.5 ml/100 gm body weight subcutaneously in the hind legs. Control rats received the appropriate equivalent volume of 0.9% NaCl. Rats were sacrificed at specific time points after injection, the liver specimens were snap frozen in liquid nitrogen for RNA isolation, and stored at -70°C until the time of analysis.

Plasmid and Probes. The probe used for Northern analysis was a 650 bp *Eco* RI fragment isolated from a cDNA coding for rat HGFL protein. The DNA sequence of this fragment is 91.4% identical to nucleotides 833-1519 in the mouse cDNA coding for HGFL protein (2; unpublished data). A 700 bp *Xba* I-*Pst* I fragment coding for the 5' end of the cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ATCC, catalog # 57090) was used to control for loading efficiency in Northern analysis for experimental models involving carbon tetrachloride, thioglycollate, and turpentine injection. All fragments were random-primer labeled using ^{32}P-αdCTP (Dupont NEN). An oligonucleotide complementary to rat 18S ribosomal RNA (17) was end-labeled with ^{32}P-γATP (NEN Dupont) using T4 polynucleotide kinase (Bethesda Research Laboratories) and used to control for loading efficiency in the Northern analysis for partial hepatectomy experiments. For synthesis of sense and antisense probes for *in situ* hybridization analysis, the plasmid pBluescript SK (+/-) containing the cDNA coding rat for HGFL protein was linearized with *Xba* I or *Hind* III to allow transcription from the T3 or T7 RNA polymerase promoters. ^{35}S-labeled sense and antisense RNAs were synthesized using a commercially available kit (NEN Dupont; Stratagene).

Northern Analysis. Total RNA was isolated from rat liver and subjected to Northern analysis as previously described (12,18). The amount of hybridization was quantified using a

Molecular Dynamics PhosphorImager (Sunnyvale, CA). Results are expressed as a percentage of the mRNA levels observed in partially hepatectomized or treated animals over sham-operated or saline treated control rats after specific hybridization is adjusted for variability in RNA loading.

***In Situ* Hybridization Analysis.** Cryostat sections (6-8 μm) of embedded tissues were transferred onto silane coated glass slides (Sigma). Sections were hybridized with radiolabeled sense and antisense RNA for HGFL protein as previously described (12).

RESULTS

Partial Hepatectomy. The level of expression of mRNA for HGFL protein in the liver was analyzed at various time points following 70% partial hepatectomy. For each time point, one rat was included as a sham-operated control and two rats underwent partial hepatectomy. Messenger RNA for HGFL protein increased at 1 hour after partial hepatectomy compared to sham-operated (control) rats and returned to control levels of expression by 2-4 h (Figure 1A). To further assess whether this increase reached statistical significance, 6 additional rats underwent 70% partial hepatectomy while 4 underwent sham operation. All rats were sacrificed at 1 h. Northern analysis of regenerating liver samples showed an increase in mRNA to 93% above the level observed in sham operated rats (Figure 1A; 0.71±0.29 vs 0.37±0.2, respectively, mean±SD, Student's t test, P = 0.04).

The level of expression of mRNA for HGFL protein was also evaluated in regenerating liver at the cellular level. *In situ* hybridization of sections of regenerating liver using an antisense radiolabeled RNA probe for rat HGFL protein showed the signal restricted to hepatocytes (Figure

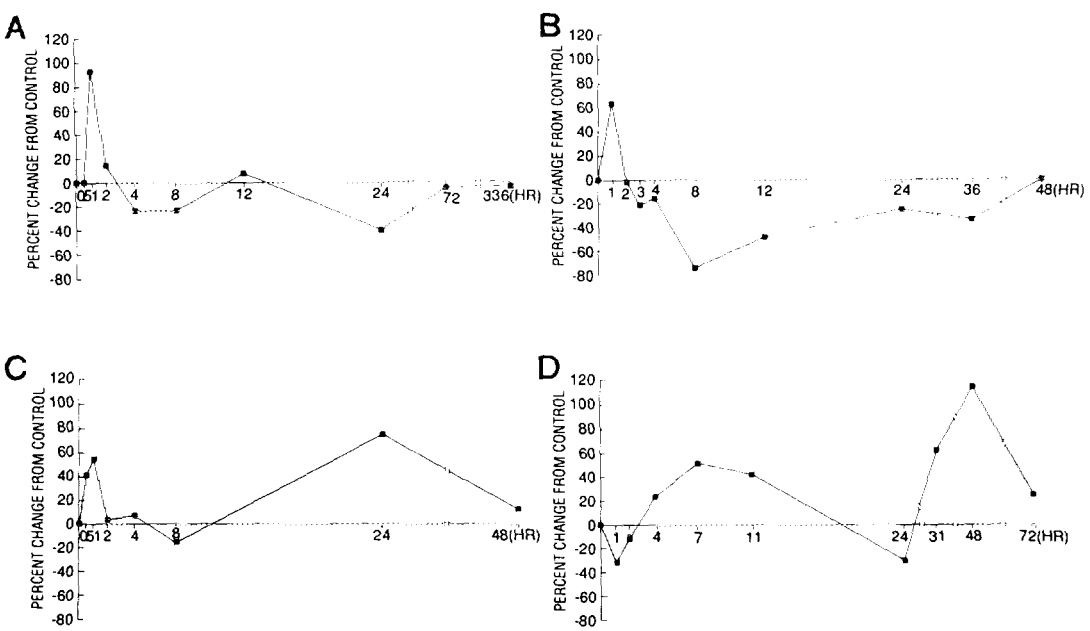

Figure 1.
Expression of mRNA for rat HGFL protein in the liver at various time points after (A) 70% partial hepatectomy, (B) carbon tetrachloride injection, (C) thioglycollate medium injection, and (D) turpentine injection. Values are expressed as percent change from sham-operated controls (A) or saline-injected controls (B,C,D), after specific hybridization is adjusted for variability in RNA loading.

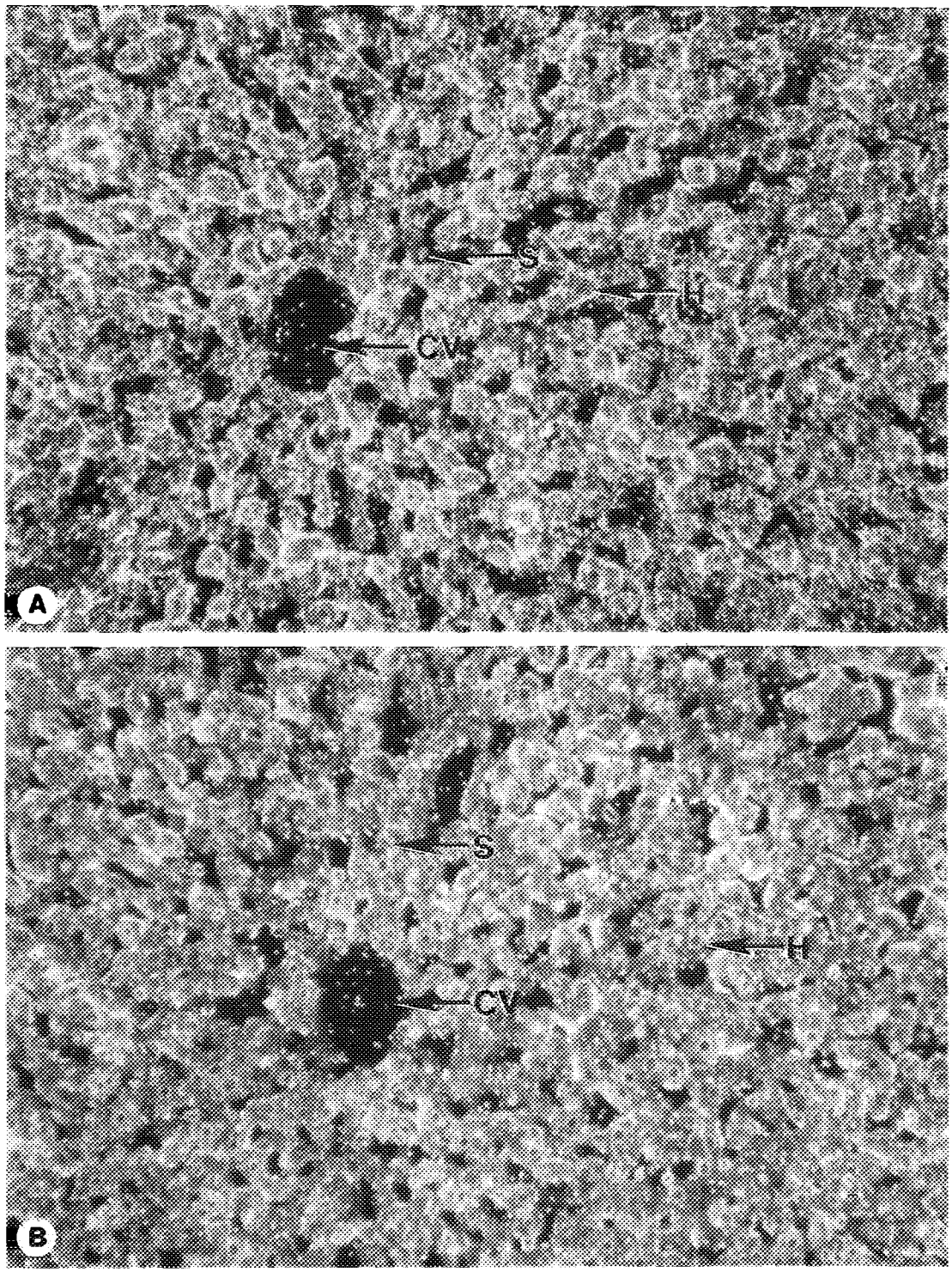

Figure 2.
Localization of mRNA for HGFL protein in the liver during liver regeneration. Darkfield photomicrograph of a section of regenerating liver hybridized with (A) antisense and (B) sense ^{35}S-labeled RNA for rat HGFL protein after 2 weeks of exposure (magnified 96x). H = hepatocytes; S = sinusoidal cells; CV = central vein.

2). There was no differential expression of mRNA for HGFL protein along the hepatic lobule in regenerating liver or in the liver of sham-operated controls (photomicrograph not shown).

Carbon Tetrachloride. Administration of carbon tetrachloride at a dose of 0.2 ml/100 gm body weight causes hepatocellular necrosis and is followed by liver regeneration in the presence of an inflammatory infiltrate (19). The level of expression of mRNA for HGFL protein in the liver was analyzed at various time points after intraperitoneal administration of carbon tetrachloride. For each time point, one rat was included as a control and received 0.9% NaCl and two rats received carbon tetrachloride. Messenger RNA for HGFL protein increased in the liver at 1 hour after carbon tetrachloride injection to 64% above control rats (Figure 1B). This increase, however, was followed by a decrease to levels below controls at 2 h, and a subsequent return to control levels by 48 h.

Thioglycollate Medium. Administration of thioglycollate medium has been shown to induce phenotypic changes in peritoneal macrophages, such as the secretion of plasminogen activator and other proteases (20). The level of expression of mRNA for HGFL protein in the liver was analyzed at various time points after intraperitoneal administration of thioglycollate medium to determine if the expression is influenced by activation of peritoneal macrophages. For each time point, one rat was included as a control and received 0.9% NaCl and two rats received thioglycollate medium. Two peaks of expression of mRNA for HGFL protein in the liver were observed at 1 h and 24 h after injection of thioglycollate medium (54% and 78% above control levels, respectively; Figure 1C).

Turpentine. Administration of turpentine causes localized tissue injury and induces an acute phase response, characterized by systemic metabolic changes and the secretion of liver-specific plasma proteins (21). The level of expression of mRNA for HGFL protein in the liver was analyzed to determine if increased expression is seen during an acute phase response. Rats treated with turpentine were sacrificed at different time points after injection. Two peaks were observed in the level of expression of mRNA for HGFL protein after turpentine injection: an early peak of 53% above controls at 7 h and a later, higher peak of 118% above controls at 48 h (Figure 1D).

DISCUSSION

This study describes the increased expression of mRNA for HGFL protein during liver regeneration and inflammation. HGFL protein has been identified in plasma of healthy humans with a molecular weight of 90 kDa and is synthesized by hepatocytes as determined *in situ* hybridization in mouse embryos and adult mice (1,12,13). Based on these data, we have suggested that HGFL protein is secreted by hepatocytes and exerts a potential autocrine effect on hepatocytes and endocrine effect in extra-hepatic target cells.

Although the specific function of this protein is not known, two potential roles have been suggested. The first, as a mitogen to hepatocytes, is based on the striking structural homology HGFL protein shares with HGF (1). Liver HGF mRNA is upregulated in models of liver regeneration, such as 70% partial hepatectomy and carbon tetrachloride injection, with maximal expression at 10-24 hours after 70% partial hepatectomy and at 24-36 hours after carbon tetrachloride injection (10,11). In an attempt to assess a potential role for HGFL protein in liver

regeneration, we determined the level of expression of mRNA for HGFL protein in the liver in these two models of liver regeneration. Our results from Northern analysis of liver RNA isolated from rats subjected to 70% partial hepatectomy show an upregulation at 1 hour to approximately 93% above the levels found in sham-operated controls (Figure 1A). The early upregulation of mRNA for HGFL protein does not suggest a direct link to DNA synthesis; instead, it suggests that the protein may participate in the early signaling events that prime hepatocytes to enter the cell cycle. This may occur through a direct autocrine effect of HGFL protein or indirectly through a paracrine stimulation of neighboring non-parenchymal cells to release factor(s) that prime hepatocytes to enter the cell cycle.

A similar profile of early upregulation of mRNA coding for HGFL protein in regenerating liver is seen following carbon tetrachloride injection (Figure 1B). Again, the dissociation between the time of maximal mRNA expression and the time of peak DNA synthesis after carbon tetrachloride injection (approximately 48 hours; 22) does not support a direct link with DNA synthesis, but more likely suggests a potential role in priming hepatocytes to enter the cell cycle. In contrast to the modulation of mRNA levels following a partial hepatectomy, the early upregulation of mRNA for HGFL protein following carbon tetrachloride treatment is followed by a decrease to levels below controls at 2 h and a return to control levels at 48 h after treatment. This may be a result of inhibition of protein synthesis by hepatocytes (23) or to hepatocyte death that is seen after carbon tetrachloride injection. Since only hepatocytes express mRNA for HGFL protein, extensive hepatocyte death would cause a decrease in the level of specific expression since the total RNA was obtained from whole liver, which includes unaffected non-parenchymal cells. It is also known that carbon tetrachloride injection induces liver regeneration in the presence of an inflammatory process (24). This is different from the model of liver regeneration induced by 70% partial hepatectomy, which constitutes a mechanical stimulus to liver regeneration without a concomitant inflammatory process. As such, we speculate that the early upregulation of mRNA for HGFL protein may be important in the activation of Kupffer cells and in the modulation of this inflammatory process. This speculation is in accordance with the second proposed function for HGFL protein as a cytokine to macrophages, based on the identity between the translated amino acid sequence of HGFL protein and the partial amino acid sequence of MSP (13).

MSP is a cytokine to peritoneal macrophages. Human MSP has been shown to induce morphologic changes and migration of mouse peritoneal macrophages in the presence or absence of a chemotactic stimulus (15). MSP also induces phagocytosis by stimulating peritoneal macrophages to ingest opsonized erythrocytes (14). MSP has been isolated to homogeneity and the partial amino acid sequence shows similarity with the translated amino acid sequence for HGFL protein (13). More recently, the cDNA coding for human MSP has been cloned (25). Comparison of this cDNA with the published cDNA for human HGFL protein suggests that these two proteins are identical (1). We assessed the expression of mRNA for HGFL protein after chemical activation of peritoneal macrophages by intraperitoneal injection of thioglycollate medium (Figure 1C). This experimental model is known to induce phenotypic changes in macrophages, such as secretion of plasminogen activator and other proteases (20). Northern analysis of liver RNA isolated from rats injected with thioglycollate medium shows an early increase at 1 hour and a later peak at 24 hours after injection to 85% above controls. The second peak is later than the peaks seen in the two

models of liver regeneration and might be related to the fact that this chemical stimulus is directed to peritoneal macrophages (extrahepatic stimulus) and may represent an endocrine effect of HGFL protein.

Lastly, we assessed whether the expression of mRNA for HGFL protein is modulated by turpentine injection. Administration of turpentine causes a localized tissue injury and induces an acute phase response. The acute phase response represents the systemic consequences of a localized tissue injury (infection, chemical injury, neoplastic growth, or trauma) and is associated with an increase in expression of several plasma proteins (26). Most of these acute phase proteins are synthesized exclusively in the liver and have been suggested to possess antiproteolytic and opsonization effects and to participate in immunomodulation. The increased liver expression of mRNA for HGFL protein noted following turpentine injection suggest that the protein is part of the acute phase response (Figure 1D). Whether HGFL protein functions in this response as an immunomodulator remains to be determined.

In summary, the early increase in expression of mRNA for HGFL protein during liver regeneration after 70% partial hepatectomy and carbon tetrachloride injection supports a potential role in the initial stages of liver regeneration, possibly serving to prime hepatocytes to enter the cell cycle. Likewise, increased mRNA expression following chemical activation of peritoneal macrophages and during the development of an acute phase response supports a potential role for HGFL protein as an inflammatory mediator. Further direct functional evidence as a regulator of cell proliferation and modulator of inflammation will be possible with the expression and purification of HGFL protein.

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REFERENCES

1. Han, S., Stuart, L.A., and Degen, S.J.F. (1991) Biochemistry 30, 9768-9780.
2. Degen, S.J.F., Stuart, L.A., Han, S., and Jamison, C.S (1991) Biochemistry 30, 9781-9791.
3. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashirok, and Shimizu, S. (1989) Nature 342, 440-443.
4. Michalopoulos, G., Houck, K.A., Dolan, M.L., and Luetteke, N.C. (1984) Cancer Res. 122, 1450-1459.
5. Nakamura, T., Nawa, K., and Ichihara, A. (1984) Biochem. Biophys. Res. Commun. 122, 1450-1459.
6. Russell, W.A., McGowan, J.A., and Bucher, N.L.R. (1984) J. Cell. Physiol. 119, 183-192.
7. Michalopoulos, G.K., and Zarnegar, R. (1992) Hepatology 15, 149-155.
8. Tashiro, K., Hagiya, M., Nishizawa, T., Seki, T., Shimonishi, M., Shimizu, S., and Nakamura, T. (1990) Proc. Natl. Acad. Sci. USA 87, 3200-3204.
9. Schirmacher, P., Geerts, A., Pietrangelo, A., Dienes, H.P., and Rogler, C.E. (1992) Hepatology 15, 5-11.

10. Kinoshita, T., Hirao, S., Matsumoto, K., and Nakamura, T. (1991) Biochem. Biophys. Res. Commun. 177, 330-335.
11. Zarnegar, R., DeFrances, M.C., Kost, D.P., Lindroos, P., and Michalopoulos, G.K. (1991) Biochem. Biophys. Res. Commun. 177, 559-565.
12. Bezerra, J.A., Witte, D.P., Aronow, B.J., and Degen, S.J.F. (1993) Hepatology 18, 394-399.
13. Bezerra, J.A., Han, S., Danton, M.J.S., and Degen, S.J.F. (1993) Protein Sci. 2, 666-668.
14. Skeel, A., Yoshimura, T., Showalter, S.D., Tanaka, S., Appella, E., and Leonard, E.J. (1991) J. Exp. Med. 173, 1227-1234.
15. Leonard, E.J., and Skeel, A. (1976) Exp. Cell Res. 102, 434-438.
16. Higgins, G.M., and Anderson, R.M. (1931) Arch. Path. 12, 186-219.
17. Mann, E.A., and Lingrel, J.B. (1991) Biochem. Biophys. Res. Commun. 174, 417-423.
18. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
19. Proctor, E., and Chatamra, K. (1982) Gastroenterology 83, 1183-1190.
20. Unkeless, J.C., Gordon, S., and Reich, E. (1974) J. Exp. Med. 139, 834-850.
21. Schreiber, G., Aldred, A.R., Thomas, T., Birch, H.E., Dickson, P.W., Tu, G.-F., Heinrich, P.C., Northemann, W., Howlett, G.H., DeJong, F.A., and Mitchell, A. (1986) Inflammation 10, 59-66.
22. Lindroos, P.M., Zarnegar, R., and Michalopoulos, G.K. (1991) Hepatology 13, 743-749.
23. Gravela, E., Albano, E., Dianzani, M.U., Poli, G., and Slater, T.F. (1979) Biochem. J. 178, 509-512.
24. Fausto, N., and Mead, J.E. (1989) Lab. Invest. 60, 4-13.
25. Yoshimura, T., Yuhki, N., Wang, M.-H., Skeel, A., and Leonard, E.J. (1993) J. Biol. Chem. 268, 15451-15468.
26. Gerok, W. (1992) In Extrahepatic Manifestations in Liver Diseases. (R. Schmid, W. Gerok, L. Bianchi, and K.P. Maier, Eds.), pp. 362-379, Kluwer Academic Publishers, Boston.