

## Cell-specific Expression of Hepatocyte Growth Factor in Liver Upregulation in Sinusoidal Endothelial Cells after Carbon Tetrachloride

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### Abstract

The cellular origin of hepatocyte growth factor (HGF), a polypeptide implicated in liver regeneration, was examined in normal liver and in hepatic regeneration induced by carbon tetrachloride. In normal liver, HGF and its mRNA were abundant in lipocytes, with smaller amounts present also in sinusoidal endothelial and Kupffer cells. In regenerating liver, HGF gene expression increased exclusively in endothelial cells. HGF mRNA levels rose sixfold in these cells, peaking at 6 h after toxin administration and returning to near normal by 24 h. The rise in HGF mRNA was accompanied by a 5.4-fold increase in HGF secretion. CCl<sub>4</sub> did not alter HGF expression by either Kupffer cells or lipocytes; nor did it induce HGF expression by hepatocytes. Nonparenchymal liver cells contained two HGF transcripts: one predicting a full-length molecule of 728 amino acids; and the other encoding a functional five-amino acid deletion variant of HGF. The variant was less abundant than the full-length transcript, but increased in parallel with native HGF mRNA in response to CCl<sub>4</sub>. The response of nonparenchymal cells to HGF was examined by plating endothelial cells and lipocytes in the presence of recombinant human HGF. Under the conditions examined, the growth factor exerted neither mitogenic nor scatter factor activity on these cells. (*J. Clin. Invest.* 1993. 91:2244–2252.) Key words: hepatopoietin A • lipocyte • liver regeneration • Kupffer cell • scatter factor

### Introduction

Hepatocyte growth factor (HGF),<sup>1</sup> a 105-kD polypeptide with potent mitogenic activity toward hepatocytes in culture, has been implicated as a mediator of hepatic regeneration in vivo. The precise role of HGF during regeneration is under active investigation. Numerous reports attest to its effect on hepatocyte proliferation (1–4); others predict that HGF has a much broader range of activity on a variety of target cells. HGF is recognized to enhance DNA synthesis in epithelia other than

hepatocytes (5–8) as well as in some endothelial cells (9). In transformed cell lines, HGF does not promote growth, but instead induces migration and spreading (10, 11). The latter effect is referred to as “scatter factor” activity, named for a fibroblast-derived compound that is structurally identical to HGF (12). HGF can also affect culture morphology: Madin-Darby canine kidney (MDCK) cells, which ordinarily form compact aggregates in vitro, in the presence of HGF reorganize into an elaborate network of capillary-like tube structures (13). These observations suggest that HGF has the potential to serve a dual role during hepatic regeneration, restoring liver cell mass and promoting cell migration that may lead to architectural remodeling of the hepatic lobule.

To better define the role of HGF, a clearer understanding of its cellular source(s) and their responsiveness to a regenerative stimulus in liver is needed. Studies to date indicate that HGF mRNA is expressed constitutively in several extrahepatic organs (14–18) and is upregulated after liver injury or resection (16–18). Increases in HGF gene expression are accompanied by a rise in circulating HGF (19), suggesting an endocrine mechanism of action. HGF is also produced in the liver (16, 20–25), however, raising the possibility of paracrine or autocrine mechanisms of regeneration.

Little is known about the cellular regulation of HGF in liver. Indeed, there is still some debate about its cellular origin in normal and regenerating liver. Although there is general agreement that HGF and its mRNA localize exclusively to nonparenchymal liver cells, individual reports have placed the growth factor either in endothelial cells (21), Kupffer cells (21, 23) or lipocytes (24, 25). Actual production of HGF by liver cells has not been quantitated; nor have the number and size of their HGF transcripts been examined. The latter is particularly important in view of recent reports demonstrating a 1.3-kb variant of HGF mRNA in human placenta and fibroblasts, generated by truncation of the full-length 6-kb RNA (15, 26). This transcript encodes a peptide of 290 amino acids that binds competitively to the HGF receptor, but lacks mitogenic activity.

The present study addresses the issue of HGF production in liver and its regulation in a regenerative model. The findings indicate that in normal liver, HGF is produced by sinusoidal endothelial cells, Kupffer cells, and lipocytes. In carbon tetrachloride-induced liver regeneration, HGF synthesis and gene expression are upregulated selectively in endothelial cells. In both normal and regenerating liver, cellular HGF production parallels HGF gene expression.

### Methods

*Animal model of liver regeneration.* Adult male Sprague-Dawley rats were subjected to acute liver injury in the form of a single intragastric

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1. Abbreviations used in this paper: HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney (cells).

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dose of  $\text{CCl}_4$  (50% vol/vol in corn oil, 0.66 ml/kg). Treated animals were killed at 2, 4, 6, 12, 18, 24, and 48 h after  $\text{CCl}_4$  administration for preparation of liver cell isolates. Animals killed at 0 h received no treatment; in some experiments, control animals received corn oil alone and were killed 6 h later.

**Liver cell isolation.** Hepatocytes were isolated by *in situ* perfusion of the liver with collagenase (Boehringer Mannheim, Indianapolis, IN) as previously described (27). Crude cell suspensions were purified by centrifugal elutriation in a J2-21 centrifuge with a JE-6B rotor (Beckman Instruments, Palo Alto, CA) (27). Nonparenchymal liver cells, including sinusoidal endothelial cells, Kupffer cells, and lipocytes were isolated by sequential perfusion of the liver with Pronase (Boehringer Mannheim, Indianapolis, IN) and collagenase (28). Nonparenchymal cell suspensions were purified by density gradient centrifugation (Larex<sup>®</sup>; Larex International, Tacoma, WA) with or without centrifugal elutriation. All cell isolates from normal and  $\text{CCl}_4$ -treated liver were  $\geq 95\%$  pure, as assessed by specific histochemical markers (29).

**Quantitation of total RNA content in liver cells.** Total RNA was measured in liver cell samples by the method of Tolstoshev et al. (30). Briefly,  $5 \times 10^6$  cells from purified isolates were lysed in a solution of 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (wt/vol) sodium lauroyl sarkosinate, and 15% (vol/vol) 2-mercaptoethanol. After dilution to a final concentration of 1 M guanidine thiocyanate, nucleic acids were precipitated with 0.5 N perchloric acid. Pellets were resuspended and sonicated in 1 N NaOH. After 30 min at 37°C, samples were again precipitated with 0.5 N perchloric acid and RNA quantitated in the supernatant by the orcinol method (31). Yeast transfer RNA, treated in the same manner as test samples, was used to generate a standard curve. Results were expressed as micrograms of RNA per  $1 \times 10^6$  cells.

**RNAse protection (32).** Total RNA was extracted from individual liver cell isolates by the method of Chomczynski and Sacchi (33). RNA integrity was verified by formaldehyde-agarose gel electrophoresis, and by hybridization with a control cRNA encoding ribosomal protein S14 (34). HGF mRNA was quantitated by solution hybridization at 50°C, using 5–20  $\mu\text{g}$  of total cellular RNA and a  $^{32}\text{P}$ -labeled ( $[\alpha\text{-}^{32}\text{P}]$  CTP,  $> 800$  Ci/mmol) (Amersham Corp., Arlington Heights, IL) antisense cRNA transcribed from the rat HGF cDNA (14). The cDNA (gift of Dr. Paul Godowski, Genentech, Inc., South San Francisco, CA) is a 293-bp sequence encoding amino acids 154–250 that is capable of detecting native HGF mRNA as well as a five-amino acid deletion variant within the first kringle region. RNA hybrids were separated through 5% polyacrylamide-urea, and HGF mRNA quantitated by autoradiography and scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA).

**Northern analysis.** 20  $\mu\text{g}$  of total cellular RNA were electrophoresed through 0.8% (wt/vol) agarose/2.5 M formaldehyde and stained with ethidium bromide to confirm uniformity of sample loading. RNAs were then transferred to a nylon filter (Zeta-Probe; Bio-Rad Laboratories, Richmond, CA) and immobilized by baking at 80°C under vacuum. Hybridization was performed at 55°C, using  $^{32}\text{P}$ -labeled HGF cRNA as described above. Filters were washed once at room temperature and four times at 65°C. Bound probe was detected by autoradiography and quantitated by scanning densitometry.

**Immunocytochemistry.** Liver cells isolated as described above were suspended in medium 199 containing 10% calf serum and 10% horse serum, and plated on multichamber glass slides. After 14 h, endothelial cell and lipocyte cultures were supplemented with 5  $\mu\text{M}$  monensin to block protein secretion. Kupffer cells, which were killed by monensin, were maintained in plating medium. After an additional 3 h, all cultures were washed five times with PBS, pH 7.5 and fixed with acetone. They were then hydrated with PBS, incubated for 20 minutes in PBS containing 15% (vol/vol) goat serum, and for an additional hour with a chicken anti-rabbit HGF antiserum (gift of Dr. Reza Zarnegar, University of Pittsburgh) (1/100 in PBS). Control specimens were incubated with normal chicken IgG (50  $\mu\text{g}/\text{ml}$  in PBS). Primary antibody was followed by a biotinylated goat anti-chicken IgG and subsequently by avidin-biotin-peroxidase complex (both from Vector Laboratories,

Burlingame, CA). Antibody binding was detected with 3, 3'-diaminobenzidine. All slides were counterstained with methyl green and photographed with a microscope (Microphot-FXA; Nikon Corporation, Tokyo, Japan).

**Inhibition ELISA for HGF.** An inhibition ELISA for HGF was devised using the same chicken anti-rabbit HGF described above. Recombinant human HGF (gift of Dr. Paul Godowski, Genentech Inc.) (250 ng/ml) was adhered to microtiter plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) in 0.1 M sodium carbonate, pH 9.5. At the same time, human HGF standards and test samples, including liver cell homogenates and cell culture media, were diluted in PBS containing 1% (wt/vol) BSA and mixed with a standard volume of anti-HGF antiserum (1/3,000 in PBS/BSA). After adsorption for 16 h at 4°C, the antigen-antibody mixtures were transferred to the HGF-coated plates. The plates were incubated at room temperature for 1 h to permit binding of residual antibody to the surface-bound antigen; they were then washed five times with PBS containing 0.05% (vol/vol) Tween-20, and incubated further with biotinylated goat anti-chicken IgG (1/200 in PBS/BSA) and avidin-biotin-peroxidase (1/100 in PBS/BSA) for 1 h each, with interval washes between each step. After a final five washes in PBS/0.05% Tween-20, bound anti-HGF was detected colorimetrically using orthophenylenediamine as a substrate. Color development was stopped by addition of 4 N  $\text{H}_2\text{SO}_4$ ; absorbance was measured at 492 nm. Using these parameters, absorbance varied inversely with the amount of HGF in the test sample. The standard curve for the ELISA was linear over the range of 5–100 ng/ml. Serial dilutions of both cell culture medium and cell homogenates paralleled the slope of the standard curve.

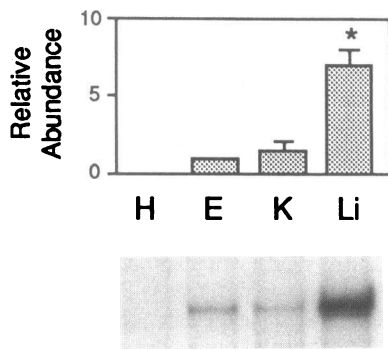
**Measurement of DNA synthesis.** Hepatocytes were plated at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> on multiwell tissue culture plates (Linbro<sup>®</sup>; Flow Laboratories, McLean, VA) in medium 199 containing 5% FCS. Endothelial cells and lipocytes were plated in similar medium but at a higher density ( $1 \times 10^5$  cells/cm<sup>2</sup>), because of their smaller size and poorer plating efficiency. 14 h after plating, all cultures were washed free of nonadherent cells and the medium replaced with medium 199 containing 5% FCS and 50 ng/ml of recombinant human HGF. Control cultures received the same medium but without HGF. After 48 h of incubation, cultures were again washed and labeled for 4 h with serum-free medium 199 containing 10  $\mu\text{Ci}/\text{ml}$  of [methyl- $^3\text{H}$ ]thymidine (45 Ci/mmol) (Amersham Corp.). DNA synthesis was measured as the amount of radiolabel incorporated into TCA-precipitable material, normalized to the total amount of DNA in each culture plate. DNA was measured fluorometrically (35).

**Assessment of scatter factor activity.** Lipocytes and endothelial cells were suspended in medium 199 containing 5% FCS and plated ( $1 \times 10^5$  cells/cm<sup>2</sup>) on either plain plastic dishes (lipocytes) or collagen-coated plastic (endothelial cells). 2 h after plating, nonadherent cells were removed and the medium replaced with medium 199 containing 5% FCS and 50 ng/ml of recombinant human HGF. Cultures were incubated for an additional 48 h, and the morphology of treated cells was compared to sister plates that did not receive HGF. MDCK cells, obtained from American Type Culture Collection (Rockville, MD), were used as positive controls for scatter factor activity. Subconfluent cultures were split 1:50 and plated in medium 199 containing 5% FCS. 1 d after seeding, test plates were supplemented with 50 ng/ml recombinant human HGF; scattering was assessed after 24 h. All cultures were photographed using an inverted phase microscope (Diaphot; Nikon Corporation, Tokyo, Japan).

**Statistical analysis.** All experiments were performed on cells from at least three separate animals. Numerical results were expressed as the mean  $\pm$  SEM. Results with  $P < 0.05$  by Mann-Whitney *U* test or Student's *t* test were considered significant.

## Results

**Cellular localization of HGF in normal rat liver.** The cellular origin of HGF in normal liver was examined by measuring



**Figure 1.** Relative abundance of HGF mRNA in normal liver cells. Graph depicts the relative abundance of HGF mRNA in hepatocytes (H), endothelial cells (E), Kupffer cells (K), and lipocytes (Li) from normal rat liver, as measured by RNase protection. Values represent mean  $\pm$  SEM for  $n = 3$  (signal for endothelial cells arbitrarily set at 1.0). \*  $P < 0.05$  vs. endothelial and Kupffer cells. A representative autoradiogram is shown (bottom). 20  $\mu$ g of total RNA were used for hybridization, with an exposure time of 5 h.

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HGF gene expression in purified liver cell isolates by RNase protection. Fig. 1 illustrates the relative abundance of HGF mRNA in four populations of normal liver cells: hepatocytes, sinusoidal endothelial cells, Kupffer cells, and lipocytes. As shown in the autoradiogram, all three nonparenchymal cell populations displayed clear hybridization signals for HGF. Lipocytes exhibited the strongest signal for the growth factor: these cells contained 7.4 and 4.6 times as much HGF mRNA per microgram of total RNA as endothelial and Kupffer cells, respectively ( $P < 0.05$ ). When HGF gene expression was normalized to cell number (Table I), lipocytes emerged even more clearly as the primary source of HGF mRNA in normal liver. The low level of HGF gene expression observed in endothelial and Kupffer cell isolates could not be attributed solely to lipocyte contamination, which was  $\leq 5\%$ . Additional experiments confirmed that endothelial and Kupffer cells themselves contained HGF (see below). In hepatocytes from normal liver, HGF mRNA was undetectable.

Cells expressing HGF mRNA also produced the protein. Endothelial cells, Kupffer cells, and lipocytes from normal liver were stained for HGF using a polyclonal anti-HGF antiserum. Fig. 2 demonstrates that all three cell populations contained immunoreactive HGF, confirming the results of the molecular hybridization studies. HGF staining was particularly intense in endothelial cells and lipocytes (Fig. 2, *a* and *e*), which were pretreated with monensin before fixation. Kupffer cells (Fig. 2 *c*), which could not withstand monensin treatment, stained more faintly. Control cells, incubated with non-immune IgG, exhibited no staining (Fig. 2, *b*, *d*, and *f*). Hepatocytes isolated from normal rat liver exhibited no HGF immunoreactivity (data not shown).

**HGF regulation during carbon tetrachloride-induced liver regeneration.** Regulation of HGF in the liver during regeneration was examined by measuring HGF gene expression and synthesis in individual cell populations at various intervals after  $\text{CCl}_4$ . HGF mRNA was examined in parenchymal as well as in nonparenchymal cells, because HGF has been identified within hepatocytes in this model of injury (23). HGF mRNA remained undetectable in hepatocytes after  $\text{CCl}_4$  administration (Fig. 3). In Kupffer cells and lipocytes, HGF mRNA was present but was unaffected by  $\text{CCl}_4$ . By contrast, in sinusoidal endothelial cells, HGF gene expression increased significantly, rising sixfold by 6 h after  $\text{CCl}_4$  and returning to near normal by 48 h. The change in HGF gene expression occurred well in advance of hepatic DNA synthesis, which peaks at 48 h in this model (19).

Production of HGF protein by sinusoidal endothelial cells also increased after  $\text{CCl}_4$ . Table II illustrates that endothelial cells isolated from regenerating rat liver secreted 2.3 times more HGF over 24 h than cells from normal rats or from animals treated with vehicle alone. Of note is that intracellular levels of HGF were similar in both normal and regenerating liver, suggesting that the majority of newly synthesized HGF was secreted.

**Characterization of HGF transcripts in nonparenchymal liver cells.** To determine whether liver cells expressed native or variant HGF species, the molecular size of their HGF transcripts was assessed by Northern analysis and RNase protection. The HGF cDNA used in these experiments derived from the first and second kringle regions, and thus would distinguish truncated as well as full-length HGF mRNAs. By Northern analysis, cells from normal and regenerating liver appeared to contain a single HGF transcript of  $\sim 6$  kb (Fig. 4). There was no evidence of either a 3.0- or a 1.3-kb transcript. RNase protection, however, revealed that nonparenchymal liver cells contained two forms of HGF mRNA (Fig. 5). The larger transcript corresponded to full-length HGF mRNA; the smaller transcript corresponded to a functional 15-bp deletion variant of HGF (36, 37). While the deletion variant was much less abundant than the larger HGF transcript (in a ratio of  $\sim 1:10$ ), both transcripts increased in parallel in response to  $\text{CCl}_4$  (Figs. 5 and 6).

**Effect of HGF on nonparenchymal liver cells.** Sinusoidal endothelial cells and lipocytes from normal rat liver were plated in primary culture in the presence or absence of recombinant human HGF. DNA synthesis and scatter factor activity were assessed after 48 h. The data in Table III illustrate that at a concentration of 50 ng/ml, HGF did not affect DNA synthesis by either endothelial cells or lipocytes. This is in contrast to

**Table I.** HGF mRNA Content in Normal Liver Cells as a Function of Cell Number

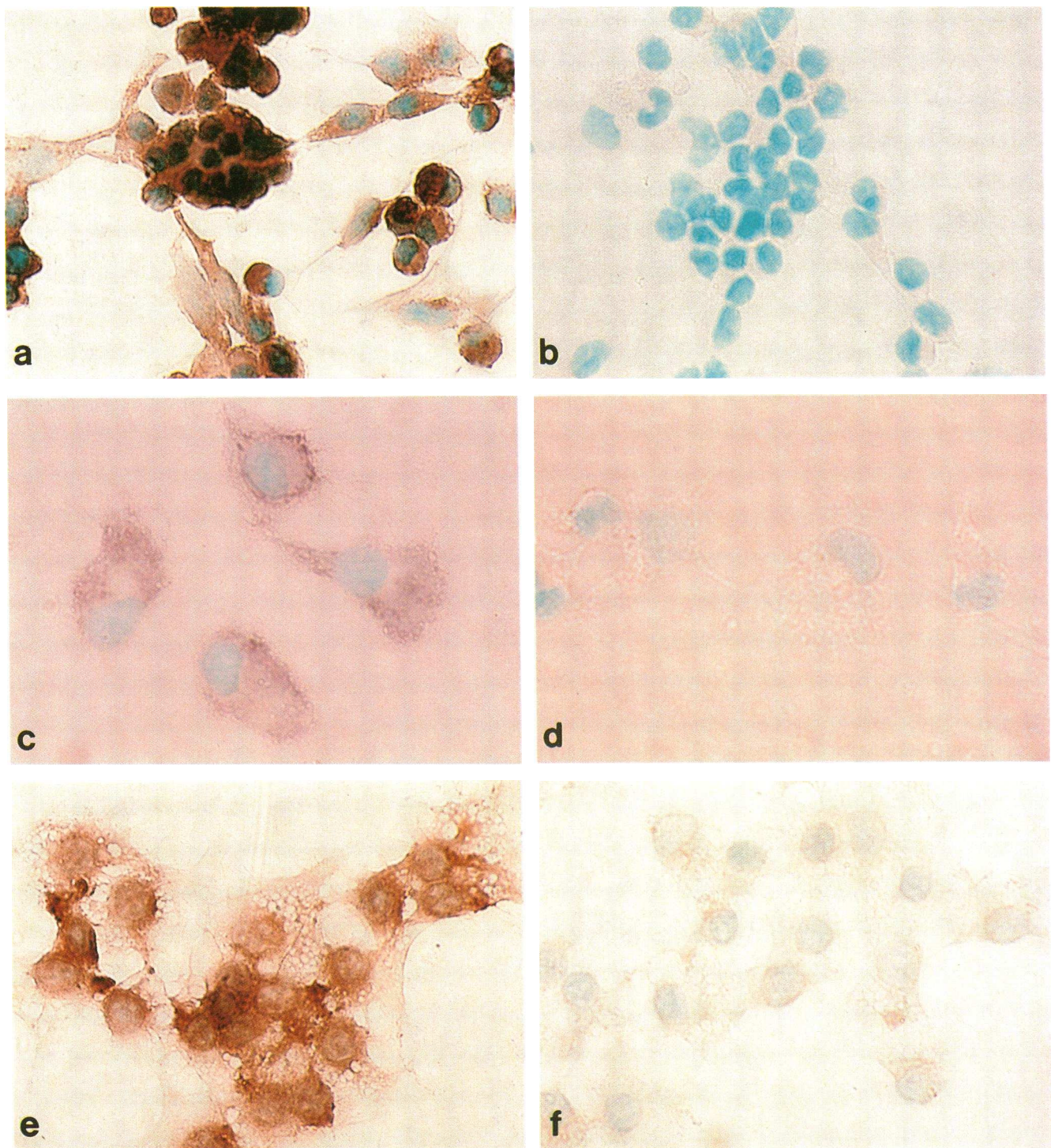
	Relative abundance of HGF mRNA** (U/ $\mu$ g total RNA)	Total RNA content†§ ( $\mu$ g/ $10^6$ cells)	Calculated HGF mRNA content (U/ $10^6$ cells)
Endothelial cells	1.0 $\pm$ 0	6.2 $\pm$ 0.5	6.2
Kupffer cells	1.6 $\pm$ 0.5	10.6 $\pm$ 0.1	17.0
Lipocytes	7.4 $\pm$ 1.0	14.7 $\pm$ 1.0	108.8

\* As compared to endothelial cells, assigned an arbitrary value of 1.0.

† Values represent mean  $\pm$  SEM, for  $n \geq 3$ .

§ Quantitated by the orcinol method (see Methods).



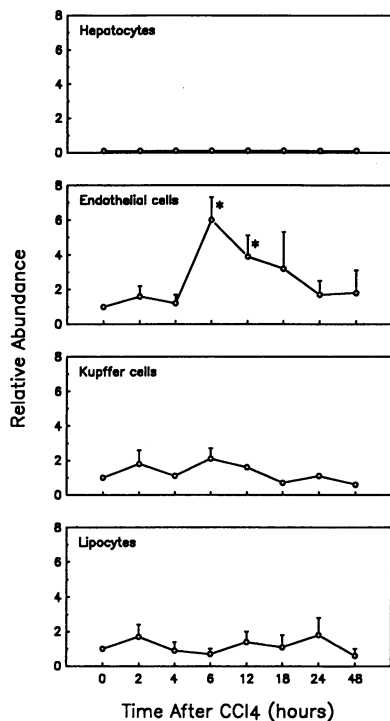


**Figure 2.** Immunocytochemical detection of HGF in nonparenchymal liver cells. Sinusoidal endothelial cells (*a* and *b*), Kupffer cells (*c* and *d*), and lipocytes (*e* and *f*) were isolated from normal rat liver and cultured for 14 h. HGF was detected after monensin pretreatment using a chicken anti-rabbit HGF (see Methods). HGF immunoreactivity is apparent in all three cell populations (*a*, *c*, and *e*). Parallel cultures, incubated with nonimmune chicken IgG (*b*, *d*, and *f*) exhibited no staining. Endothelial cells stain intensely because of their compact morphology at 14 h of culture. Kupffer cells, which did not receive monensin pretreatment, stained more faintly.  $\times 200$ .

hepatocytes, in which the same dose of HGF caused a 2.2-fold rise in [ $^3\text{H}$ ]thymidine uptake. The lack of a mitogenic response to HGF could not be attributed to the presence of other growth factors in serum-containing culture media; even when nonparenchymal liver cells were cultured in serum-depleted medium

(0.5% FCS), HGF failed to enhance DNA synthesis (data not shown). HGF also failed to exhibit any scatter factor activity on lipocytes or endothelial cells in primary culture. Spreading was prominent in both liver cell populations regardless of HGF treatment (Fig. 7, *a-d*). MDCK cells, by contrast, exhibited





**Figure 3.** Changes in HGF gene expression by liver cells in response to carbon tetrachloride. Graphs depict the relative abundance of HGF mRNA as a function of time after carbon tetrachloride in hepatocytes, endothelial cells, Kupffer cells, and lipocytes. HGF mRNA was quantitated in all cases by RNase protection. Initial (0 h) values for each cell type were arbitrarily set at 1.0, with the exception of hepatocytes, in which an HGF signal was undetectable. Note that the graphs only compare time intervals within each cell population; they do not compare the relative abundance of HGF mRNA in different cell

types (e.g., endothelial cells vs. lipocytes). Values represent mean  $\pm$  SEM for  $n \geq 3$ . \* $P < 0.05$  vs. 0 h, by Mann-Whitney  $U$  test.

typical scattering behavior in response to HGF (Fig. 7, *e* and *f*). In additional experiments, sinusoidal endothelial cells were plated on gel substrata in the presence or absence of HGF (data not shown). They migrated and formed capillary-like tube structures on these gels; but the rate of tube formation was not altered by HGF.

## Discussion

Previous studies have yielded conflicting results regarding the cellular origin of HGF in liver. Noji and colleagues (21), who used in situ hybridization to examine HGF gene expression in normal and regenerating liver, found abundant HGF tran-

**Table II. Quantitation of HGF in Liver Cell Homogenates and Cell Culture Medium\***

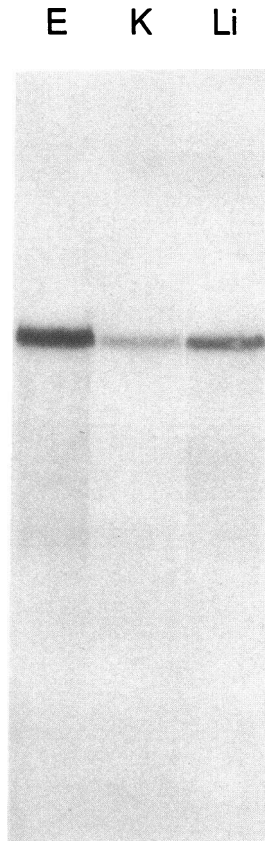
	Cell homogenate	Culture medium
	pg/ $\mu$ g DNA	pg/ $\mu$ g DNA per 24 h
Endothelial cells		
Normal	27.1 <sup>‡</sup>	654.2 $\pm$ 40.3
Vehicle-fed control, 6 h	22.0 $\pm$ 1.6	691.8 $\pm$ 127.0
CCl <sub>4</sub> , 6 h	30.7 $\pm$ 4.2	1484.7 $\pm$ 22.6 <sup>§</sup>
Lipocytes		
Normal	262.4 $\pm$ 21.8 <sup>  </sup>	3,541.5 $\pm$ 258.4 <sup>  </sup>
Vehicle-fed control, 6 h	253.2 <sup>‡</sup>	3,623.5 $\pm$ 491.5
CCl <sub>4</sub> , 6 h	293.3 $\pm$ 2.7 <sup>  </sup>	3,283.5 $\pm$ 42.5 <sup>  </sup>

\* Values represent mean  $\pm$  SEM.

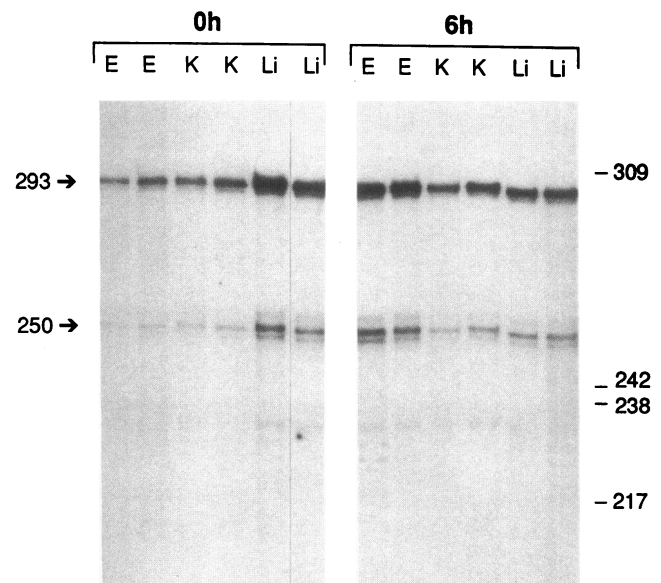
<sup>‡</sup>  $n = 1$ .

<sup>§</sup>  $P < 0.001$  vs normal and vehicle-fed control, by Student's  $t$  test.

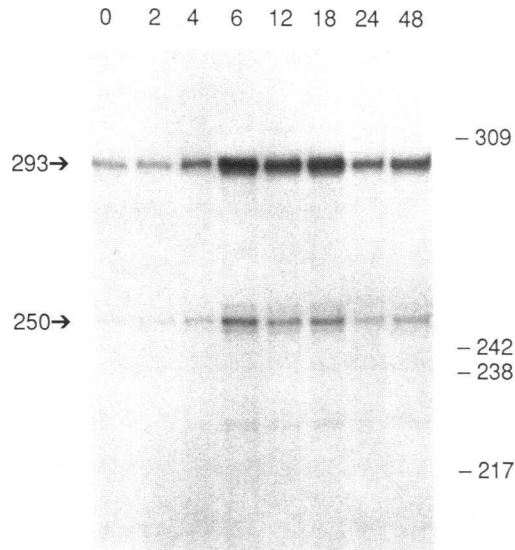
<sup>||</sup>  $P < 0.001$  vs endothelial cells, by Student's  $t$  test.



**Figure 4.** Northern analysis of HGF mRNA in liver cells. HGF mRNA was measured in endothelial cells, Kupffer cells, and lipocytes at 6 h after carbon tetrachloride, as described in Methods. 20  $\mu$ g of total RNA were used for hybridization; exposure time was 24 h. In all three cell types, HGF mRNA appeared as a single band of  $\sim 6$  kb. HGF transcripts in normal liver cells were identical in size to those in regenerating liver (data not shown).



**Figure 5.** HGF gene expression in three nonparenchymal cell populations at 0 and 6 h after CCl<sub>4</sub> measured by RNase protection. Autoradiogram illustrates HGF mRNA in endothelial cells (*E*), Kupffer cells (*K*), and lipocytes (*Li*) in normal liver (0 h) and at the peak of CCl<sub>4</sub>-induced HGF mRNA alteration (6 h). Molecular size markers are shown at the right in basepairs. Duplicate lanes represent cells from two different animals. Two HGF signals are present: one at 293 bp, corresponding to the full-length cRNA probe, and a second at 250 bp, representing a 15-bp deletion variant of HGF mRNA. The signal for the variant mRNA appears at 250 bp because the deleted sequence is positioned 29–43 nucleotides downstream from the 5' end of the cRNA. No signal is obtained when the HGF probe is hybridized with yeast transfer RNA as a negative control (not shown).



**Figure 6.** Parallel modulation of two HGF mRNA transcripts by  $\text{CCl}_4$ . The autoradiogram illustrates HGF mRNA in sinusoidal endothelial cells, in normal liver (0) and at various intervals (hours) after carbon tetrachloride. HGF mRNA was quantitated by RNase protection, as in Fig. 5. Molecular size markers (bp) are shown at the right. 5  $\mu\text{g}$  of total RNA were used for hybridization; exposure time was 20 h.

scripts in cells lining the hepatic sinusoid and judged them to be endothelial and Kupffer cells. Wolf et al. (23) similarly found that anti-HGF antibody highlighted cells with a sinusoidal distribution, but concluded that Kupffer cells were the primary source of HGF immunoreactivity. Both groups relied solely on morphologic criteria to identify HGF-positive cells; in so doing they may have overlooked lipocytes, which can be difficult to distinguish from endothelial and Kupffer cells without specific histochemical markers. The first investigators to implicate lipocytes as a source of HGF mRNA were Schirmacher and colleagues (24). Working with purified liver cell isolates, they demonstrated abundant amounts of HGF mRNA in lipocytes. In direct contrast with the previous studies, they failed to detect HGF transcripts in either sinusoidal endothelial cell or Kupffer cell isolates by Northern analysis;

**Table III.** DNA Synthesis in Liver Cell Cultures Incubated with HGF\*

Cells	n	$[^3\text{H}]$ -Thymidine incorporation	
		cpm/ $\mu\text{g}$ DNA $\dagger$	
		(-) HGF	(+) HGF (50 ng/ml)
Endothelial cells	4	22,023 $\pm$ 3,912	18,001 $\pm$ 5,497
Lipocytes	4	1,387 $\pm$ 162	1,403 $\pm$ 376
Hepatocytes	2	1,744 $\pm$ 203	3,757 $\pm$ 328

\* Cells were isolated from normal rat liver and plated in primary culture in medium 199 containing 5% FCS. HGF was added 16 h after plating, and removed after an additional 48 h.  $[^3\text{H}]$ thymidine labeling was performed as described in Methods.

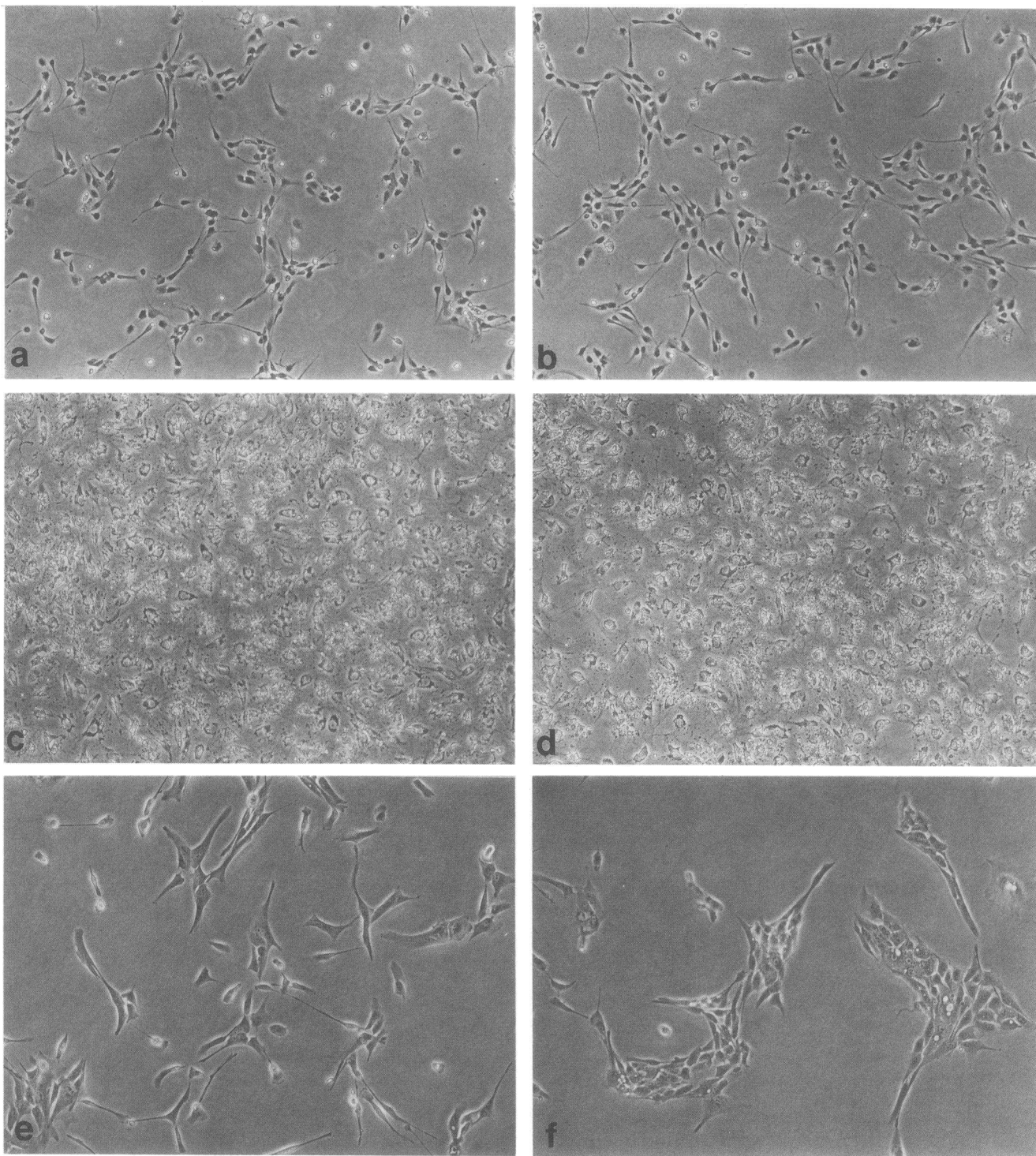
$\dagger$  Values represent mean $\pm$ SEM.

and concluded that lipocytes were the only source of HGF mRNA in normal liver. In the present study, we identify HGF and its mRNA in all three of the above cell populations in normal liver: endothelial cells, Kupffer cells, and lipocytes. Our ability to detect HGF in endothelial and Kupffer cell isolates is attributable to the sensitive solution hybridization and immunologic methods used. Lipocytes represent the principal source of HGF in normal liver, exhibiting high levels of HGF mRNA in vivo and secreting HGF constitutively in primary culture. Endothelial cells and Kupffer cells also produce HGF, albeit in much smaller amounts than lipocytes.

Within hours of a toxic insult to the liver, HGF gene expression and synthesis rise dramatically and selectively in endothelial cells. No change is apparent in either Kupffer cells or lipocytes. HGF production by endothelial cells increases well in advance of DNA synthesis, suggesting a contributory role in hepatic regeneration. The reason for such an exclusive change is uncertain, but is consistent with the hypothesis that endothelial cells in general represent inducible sources of HGF. Endothelial cells from both kidney (17) and lung (18) have been reported to exhibit increases in HGF gene expression during hepatic regeneration. Moreover, the response in lung was similar whether regeneration was induced by  $\text{CCl}_4$  or partial hepatectomy. It has been proposed that HGF production by endothelial cells is regulated by a soluble factor released during liver injury and distributed to extrahepatic organs via the circulation. One candidate polypeptide named "injurin" has recently been purified from the plasma of animals with acute liver injury (38). Injurin induces HGF gene expression by fibroblasts; it remains to be determined whether the compound also affects endothelial cells.

These experiments, which are the first to quantitate secretion of HGF by liver cells, demonstrate that HGF production (Table II) parallels HGF gene expression (Figs. 1 and 3). Lipocytes from normal liver contain ten times the intracellular HGF of endothelial cells (264.4 vs 27.1 pg/ $\mu\text{g}$  DNA,  $P < 0.001$ ), and secrete five times more HGF in culture (3,541.5 vs 654.2 pg/ $\mu\text{g}$  DNA per 24 h,  $P < 0.001$ ). 6 h after  $\text{CCl}_4$ , HGF secretion by endothelial cells increases substantially (1,484.7 vs. 654.2 pg/ $\mu\text{g}$  DNA per 24 h,  $P < 0.001$ ), whereas HGF secretion by lipocytes remains stable. Without a specific bioassay, we cannot be certain that liver-derived HGF is biologically active; indirect evidence, however, suggests that this is the case. The two mRNAs encoding HGF in liver cells predict peptides of 723 and 728 amino acids. Similar transcripts, when expressed by others in vitro (36), have yielded biologically active HGF. Small deletions or mutations in the HGF molecule can destroy its mitogenic activity (39, 40); the only naturally occurring variants of HGF that lack biologic activity, however, are encoded by substantially truncated mRNAs (26).

The amount of HGF secreted by endothelial cells and lipocytes from regenerating liver is sufficient to promote DNA synthesis by hepatocytes (4). On the basis of the current experiments, however, we cannot draw a firm conclusion regarding the significance of liver-derived HGF in relation to that which reaches the liver through the plasma. Because HGF binds avidly to heparin, circulating hormone may become sequestered in heparan sulfate-rich extracellular matrices (41). If sequestration in turn limits the bioavailability of plasma HGF, then local sources of the growth factor could assume primary importance. Studies examining the mechanism of HGF respon-



**Figure 7.** Absence of scattering behavior by endothelial cells and lipocytes in response to HGF. Endothelial cells (*a* and *b*) and lipocytes (*c* and *d*) from normal rat liver were plated in primary culture in medium 199 containing 5% FCS. MDCK cells (*e* and *f*), used as positive controls for scatter factor activity, were plated in subculture under the same conditions. Cells in *a*, *c*, and *e* were treated with 50 ng/ml recombinant human HGF; untreated controls are illustrated in *b*, *d*, and *f*. Endothelial cells and lipocytes exhibited similar morphology regardless of HGF treatment (*a*–*d*). Spreading was marked in both cell populations, even after 48 h of exposure to the growth factor. HGF-treated MDCK cells, by contrast, exhibited marked scattering after 24 h of incubation.

siveness suggest that an important control point may be activation of HGF receptors in injured organs (42). The relative importance of paracrine and endocrine sources of HGF remains to be determined.

The significance of upregulation of HGF in endothelial cells is speculative but may involve autocrine effects. Morimoto and colleagues (9) have shown that HGF promotes growth and migration of microvascular endothelial cells. In the

current experiments, HGF at a concentration of 50 ng/ml did not affect either endothelial cells or lipocytes in primary culture. However, these studies are limited in that they monitored only morphology and DNA synthesis in the presence and absence of HGF. It is possible that HGF receptors, if present on nonparenchymal cells in vivo, are rapidly downregulated in primary culture. Alternatively, HGF may exert effects on endothelial cells and lipocytes different from those examined here. Other growth factors elicit a wide variety of cellular responses, from extracellular matrix synthesis to angiogenesis (43, 44). Further experiments must be performed to determine whether nonparenchymal cells, which produce HGF, are also targets of HGF activity.

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