Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver

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Zimmermann, Ellen M., Lina Li, Eileen C. Hoyt, Jolanta B. Puciloswka, Steven Lichtman, and P. Kay Lund. Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G447–G457, 2000—The liver is a major source of circulating insulin-like growth factor I (IGF-I), and it also synthesizes several classes of IGF binding proteins (IGFBPs). Synthesis of IGF-I and IGFBPs is regulated by hormones, growth factors, and cytokines. They are nutritionally regulated and expressed in developmentally specific patterns. To gain insight into cellular regulatory mechanisms that determine hepatic synthesis of IGF-I and IGFBPs and to identify potential target cells for IGF-I within the liver, we studied the cellular sites of synthesis of IGF-I, IGF receptor, growth hormone (GH) receptor, and IGFBPs in freshly isolated rat hepatocytes, endothelial cells, and Kupffer cells. We also localized cellular sites of IGFBP synthesis by in situ hybridization histochemistry. Western ligand and immunoblot analyses were used to determine IGFBP secretion by isolated cells. Two IGF-I mRNA subtypes with different 5' ends (class 1 and class 2) were detected in all isolated liver cell preparations. Type 1 IGF receptor mRNA was detected in endothelial cells, indicating that these cells are a local target for IGF actions in liver. GH receptor was expressed in all cell preparations, consistent with GH regulation of IGF-I and IGFBP synthesis in multiple liver cell types. The IGFBPs expressed striking cell-specific expression. IGF-BP-1 was synthesized only in hepatocytes, and IGFBP-3 was expressed in Kupffer and endothelial cells. IGF-BP-4 was expressed at high levels in hepatocytes and at low levels in Kupffer and endothelial cells. Cell-specific expression of distinct IGFBPs in the liver provides the potential for cell-specific regulation of hepatic and endocrine actions of IGF-I.

INSULIN-LIKE GROWTH FACTOR I (IGF-I) is synthesized in most if not all tissues, but the liver appears to be a major source of circulating IGF-I (17, 19). IGF-I mRNA abundance is 40- to 100-fold higher in liver than in nonhepatic tissues (20). Rates of secretion of IGF-I from isolated, perfused rat liver are sufficient to account for the concentrations of IGF-I found within the circulation (31). Growth hormone (GH) is a major determinant of levels of circulating IGF-I and abundance of hepatic class 1 and class 2 IGF-I mRNAs (13, 14). Hepatic IGF-I is also regulated by nutritional status and modulated in certain pathological conditions, such as sepsis and cirrhosis (34, 40, 45). The liver has not been traditionally considered a major target tissue for IGF-I actions because hepatocyte cell membranes contain few type 1 IGF receptors and type 1 IGF receptor mRNA is not readily detected in poly(A)+ RNA prepared from whole rat liver (23, 26). Because hepatocytes represent the majority of cells within the liver, these observations do not exclude the possibility that type 1 IGF receptor is expressed in less abundant liver cell types. In the present study, we compared the levels of expression of type 1 IGF receptor mRNA in RNA extracted from whole adult rat liver and from freshly isolated hepatocytes, Kupffer cells, and endothelial cells prepared from adult liver. We also measured class 1 and class 2 IGF-I mRNAs and GH receptor mRNA expression in these cell populations to determine whether these cells represent potential target cells for IGF-I.

A family of circulating IGF binding proteins (IGFBPs) is important modulators of IGF-I actions (15). In adult mammalian species, including rats and humans, IGFBP-3 is the most abundant circulating IGF BP (15). IGF-I, IGFBP-3, and an acid labile subunit form a 150-kDa ternary complex that prolongs the plasma half-life of IGF-I and limits the amounts of free, biologically active IGF-I in circulation. IGF-I also circulates bound to the other IGFBPs, but their physiological significance is less well established. The liver is a known source of IGFBP-1 to -4 (1, 12, 30, 33). The IGFBPs may act to control transport of IGF-I from the circulation to specific tissues, to provide for cell- and tissue-specific localization of IGF-I, and to modulate the interaction between IGF-I and its receptor (15). IGF-I-independent actions of the IGFBPs are also emerging (27, 50). The IGFBPs are regulated by multiple factors and conditions, including IGF-I, GH, nutritional status, and diabetes.

Cell-specific hepatic expression of IGFBPs has been demonstrated previously, although the results differ between studies and techniques employed. For example, hepatocytes were shown to synthesize only IGFBP-1 in one study (1) and IGFBP-1, -2, and -4 in another (33). Cultured Kupffer cells were shown to
synthesize only IGFBP-3 in one study (1) and IGFBP-2 and -3 in another (33). Cultured hepatic stellate cells secreted IGFBP-3 and -4 in one study (32) and no IGFBPs in another (1). Endothelial cell expression of IGFBPs has been less well studied. Information from in situ hybridization also suggests expression of IGFBP-1 mRNA in parenchymal cells (1, 12) and IGFBP-3 mRNA in Kupffer cells (1). IGFBP-2 mRNA was detected in parenchymal cells and was significant only in rats fed a low-protein diet (12).

Cell-specific patterns of hepatic IGFBP expression are emerging from studies to date, but it is clear that technical factors can greatly affect IGFBP secretion and ultimately affect conclusions regarding IGFBP biology. For example, cultured cells have been shown to markedly change expression of IGFBPs with time in culture (32). IGFBP-3 mRNA from Kupffer cells and IGFBP-4 mRNA from hepatocytes were markedly increased at 7 days compared with cells cultured for 2 days. During the same time period, the IGF type 1 receptor expression markedly decreased. In the present study we analyzed expression of IGFBP-1 to -6 mRNAs and proteins in freshly isolated hepatocytes, Kupffer cells, and hepatic endothelial cells. IGFBP mRNA abundance in RNA from freshly isolated cell populations was compared with expression in whole liver, and in situ hybridization histochemistry was used to assess the cellular sites of expression of the different liver-derived IGFBPs in vivo. We present evidence for distinct cellular patterns of expression of IGFBP-1, -3, and -4 mRNAs in adult rat liver.

MATERIALS AND METHODS

Animals and tissue collection. Adult 200- to 250-g male Sprague-Dawley strain rats were purchased from Sasco (Omaha, NE). Rats were fed standard rat chow ad libitum. To obtain tissue for RNA extraction, in situ hybridization histochemistry, and cell fractionation, rats were anesthetized by intraperitoneal injection of 50 mg/kg ketamine (Parke Davis, Morris Plains, NJ) and 5 mg/kg xylazine (Mobay, Shawnee, KS). After complete anesthesia, rats were killed by decapitation and liver was collected for processing and analyses. All animal experiments were approved by the Instrumental Animal Care and Use Committee of the University of North Carolina at Chapel Hill and the University Committee on the Care and Use of Animals at the University of Michigan. Animal experiments were conducted in accordance with the standards of humane animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Hepatic cell fractionation. Isolated preparations of hepatocytes, Kupffer cells, and endothelial cells were prepared from isolated rat liver by a modification of the technique described by Knoop and Sleyster (Ref. 16; see also Ref. 18). Livers were perfused first with Gey's balanced salt solution (GBSS) and then with a calcium- and magnesium-free solution, followed by perfusion with GBSS containing 0.25% collagenase and Pronase. The liver was then raked with a steel comb to disperse cells. Cells were centrifuged three times at 50 g for 2 min to pellet hepatocytes, and the supernatant was removed. The supernatant was centrifuged for 5 min at 250 g to pellet the nonparenchymal cells. The pellet containing nonparenchymal cells was resuspended in 15 ml of GBSS, loaded onto a Beckman J 2/21ME centrifugal elutriator (Beckman Instruments; Palo Alto, CA), and centrifuged at 4°C in a Beckman J E-6B rotor at 2,500 rpm. Cells were elutriated at 10, 18, 21, 36, and 42 ml/min with GBSS buffer containing 1% albumin. The purified endothelial cell population was collected at a rate of 18 ml/min. The purified Kupffer cell population was collected at a rate of 36 ml/min. Hepatocytes, Kupffer cells, and endothelial cells were pelleted by centrifugation and then quick frozen in liquid nitrogen and stored at −80°C until RNA extraction. With these methods purity of Kupffer cells was estimated at 90–95% based on immunostaining with a Kupffer cell-specific monoclonal antibody (KU-1; generously provided by Dr. J. Reichner, Rhode Island Hospital; Providence, RI) (18). Histological evaluation indicates that contamination of endothelial cells with hepatocytes and Kupffer cells also was <5%. To confirm relative purity of the different cell populations albumin mRNA was used as a marker for hepatocytes. Interleukin-1β (IL-1β) is synthesized by both Kupffer and endothelial cells (11, 18), and IL-1β mRNA was used as a marker for nonparenchymal cells.

RNA extraction and analysis. Poly(A)+-enriched RNA was isolated from liver and isolated cells by the guanidine thiocyanate-cesium chloride and oligo(dT) cellulose chromatography methods previously described (51). Concentration of poly(A)+ RNA was determined by absorbance at 260 nm. RNA integrity was verified by electrophoresis of poly(A)+ RNAs on agarose gels containing ethidium bromide. Class 1 and class 2 IGFBPs were analyzed by RNase protection using methods previously described (51).

The IGF-I probe used for RNase protection was a Bgl 2 digest fragment of an IGF-I class 1a cDNA (8). The expected size of the class 1 protected fragment was 586 nt. Class 1 and class 2 mRNAs differ in their 5′-untranslated regions and signal peptide sequences. The Bgl 2 fragment extended 27 nt 5′ of the common region of class 1 and class 2 IGF-I cDNAs, and therefore the expected size of the class 2 protected fragment was 559 nt. The IGF-I receptor probe protected a 271-nt fragment of the IGF-I receptor (40). The protected fragment contained 15 nt of the 5′-untranslated region, the signal peptide, and 159 nt of the coding region of the α-subunit.

Other mRNAs were analyzed by Northern blot hybridization. For Northern blot hybridization, 10- to 20-µg aliquots of poly(A)+ RNAs were denatured, size fractionated on a 1% agarose gel, and then transferred to Genescreen (NEN Research Products; Boston, MA). Prehybridization and hybridization of blots were carried out at 60°C in a standard buffer (41) that contained 50% formamide (Fluka Chemical, Ronkonkoma, NY) and 2.5× saline-sodium citrate (SSC) (20× SSC = 3 M NaCl, 0.3 M Na citrate, pH 7.0). Hybridizations were carried out at 60°C using 1 × 106 counts/min per milliliter of radiolabeled RNA probe. Blots were washed successively in 1× SSC at 65°C for 1 h and 0.1× SSC for 30 min at 70°C. After being washed, blots were exposed to Kodak X-Omat AR film with intensifying screens at −80°C. The relative intensity of bands on the same gel was assessed by analysis of scanned autoradiograms (Silver Scanner II; La Ciel, Beaverton, OR; Adobe Photoshop version 3.0, Adobe Systems, Mountain View, CA) using image analysis software (NIH Image; National Institutes of Health, Bethesda, MD).

In situ hybridization. Liver was collected for in situ hybridization histochemistry to localize IGFBP mRNAs. Liver was frozen in isopentane to −40 to −50°C and then maintained at −80°C until the time of sectioning. In situ hybridization histochemistry was performed using methods previously described (51). Briefly, frozen sections of liver (10 µm thick) were fixed in 4% paraformaldehyde, washed, and then treated with proteinase K (1 mg/ml) for 10 min at 37°C. After acetylation...
and dehydration, sections were incubated with 50 µl of hybridization buffer per slide, containing 75% deionized formamide, 10% dextran sulfate, and 3% SSC, 50 mM Na-phosphate buffer, pH 7.4, for 48-24 h. Hybridization was performed at 55°C for 18-24 h. After hybridization, sections were incubated in RNase A (200 µg/ml; Sigma Chemical, St. Louis, MO) for 30 min at 37°C and then in decreasing concentrations of SSC at room temperature, followed by a 1-h incubation in 0.5× SSC at 55°C. Sections were then dehydrated, air-dried, and exposed to Kodak NTB-2 radiographic emulsion at 4°C. Slides were developed in Kodak D19 and Kodafix, stained with Mayer’s hematoxylin, and coverslipped. Sections were observed and photographed under light and dark field illumination (Olympus BH-2 microscope; Olympus, Lake Success, NY). Sections were hybridized with both antisense and sense RNA probes corresponding to each mRNA under test. The sense probes serve as a negative control for specificity of hybridization signals observed with antisense probes (see RESULTS). As an additional control, some sections were pretreated with RNase A (200 µg/ml; Sigma Chemical) before hybridization with the antisense probe. The RNase controls uniformly yielded negative results, which are defined as grain density over tissue that is indistinguishable from grain density in an area of slide containing no tissue (data not shown).

Probe synthesis. Antisense or sense RNA probes were derived from 1) rat IGF-I cDNA (8) subcloned into pGEM 3Z (Promega, Madison, WI), 2) rat type 1 IGF receptor cDNA (49) subcloned into pBluescript vector (Stratagene; Menasha, WI) and provided by D. C. Reith and C. T. Roberts (National Institutes of Health, Bethesda, MD), 3) rat GH receptor cDNA (22) subcloned into pBluescript (Stratagene) and provided by Dr. L. Mathews (University of Michigan, Ann Arbor, MI), 4) rat IGFBP-1, -2, -3, and -5 cDNAs prepared by PCR based on published sequences, subcloned into pBluescript (Stratagene) and provided by Dr. A. J. D’Ercole (University of North Carolina, Chapel Hill, NC), 5) rat IGFBP-4 and -6 cDNAs provided by Dr. A. J. D’Ercole (University of North Carolina, Chapel Hill, NC), and 6) rat IL-1β probe subcloned into pGEM 3 (Promega) and provided by Dr. Alan Shaw, Glaxo Institute of Molecular Biology in Geneva, Switzerland (now with Merck, Sharp, and Dohme, West Point, PA). Plasmids were linearized with appropriate restriction enzymes to allow preparation of sense or antisense RNA riboprobes. For RNAse protection or Northern blot hybridization, antisense RNA probes were labeled with [32P]UTP (800 Ci/mM; Amersham, Arlington Heights, IL) using T7, T3, or SP6 RNA polymerase (Promega) as previously described (41, 51). For in situ hybridization histochemistry, sense and antisense IGFBP riboprobes were labeled with [33P]UTP (>1,000 Ci/mM; Amersham).

Western ligand and immunoblot analysis. Freshly isolated cells were placed in serum-free DMEM and incubated for 24 h. The medium was collected by gentle centrifugation and concentrated 10 times using Centricon microconcentrators (Amicon, Beverly, MA). Twenty microliters of concentrated medium were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Ligand blots were performed by incubating membranes with 300,000 counts/min per milliliter of recombinant human IGF-I and IGF-II and visualized by autoradiography (44). Immunoblots were performed by incubating membranes in IGFBP-1 to -5 specific antibodies in a 1:1,000 dilution (kindly provided by D. R. Clemmons, Chapel Hill, NC), and bands were visualized using a Chemiluminescence Western Blotting Kit (Boehringer Mannheim). Human amniotic fluid and rat serum were used as positive controls.

RESULTS

Albumin and IL-1β mRNAs. The purity of the elutriated cell preparations was assessed by measuring albumin and IL-1β mRNA in the three hepatic cell preparations. As expected, abundant albumin mRNA was detected in the hepatocyte preparation (Fig. 1). Albumin mRNA was detected at low levels in Kupffer cells and was not detected in endothelial cells (Fig. 1). This indicates that minor contamination of the Kupffer cell preparation with hepatocytes, IL-1β mRNA was not detected in hepatocytes and was present, as expected, in both Kupffer and endothelial cells (Fig. 1). These data, routine histological evaluation, and the distinct patterns of IGFBP mRNA expression indicate little cross-contamination of different isolated liver cell preparations.

IGF-I, type 1 IGF receptor, and GH receptor expression. RNAse protection assays revealed that adult rat liver, isolated hepatocytes, Kupffer cells, and endothelial cells each express class 1 and class 2 IGF-I mRNAs (Fig. 2). In each case the abundance of class 1 mRNA was higher than that of class 2 mRNA, and there was no discernible difference in relative abundance of the two mRNAs across different liver-derived cells (ratio of class 1 to class 2: 1.4:1 in hepatocytes, 1.6:1 in Kupffer...
cells, and 1.6:1 in endothelial cells). Similar signal intensity of class 1 and class 2 IGF-I mRNAs in 2 µg of hepatocyte RNA analyzed vs. 15 µg of Kupffer or endothelial cell RNA (Fig. 2) indicates much greater levels of expression of IGF-I mRNAs in hepatocytes than in other liver cell types studied. Consistent with prior studies (51), only class 1 mRNA was detected in rat cecum used as a positive control (data not shown). Type 1 IGF-I receptor mRNA was not detected in poly(A)$^+$-enriched RNA extracted from whole liver, hepatocytes (even when greater amounts of RNA were analyzed), or Kupffer cells but was detected at low levels in RNA from the isolated hepatic endothelial cells (Fig. 2) and from rat cecum used as a positive control. Autoradiogram was exposed overnight at −80°C with intensifying screens.

GH receptor mRNA (4.3 kb) and a smaller mRNA of 1.3 kb encoding GH binding protein were detected at high levels in liver (data not shown) and hepatocytes (even when greater amounts of RNA were analyzed), or Kupffer cells but was detected at low levels in RNA from the isolated hepatic endothelial cells (Fig. 2) and from rat cecum used as a positive control.

IGFBP mRNA expression. Expression patterns of different IGFBPs in whole rat liver and isolated hepatocytes, Kupffer cells, and endothelial cells are shown in Fig. 4 and summarized in Table 1. The sizes of the transcripts identified by the IGFBP probes were consistent with published reports (reviewed in Ref. 38): 2.0 kb for IGFBP-1 (24), 1.8 kb for IGFBP-2 (21), 2.5 kb for IGFBP-3 (36), 2.6 kb for IGFBP-4 (25), 6.0 kb for IGFBP-5 (39), and 1.3 kb for IGFBP-6 (37). IGFBP-1 was expressed in poly(A)$^+$-enriched RNA from whole rat liver. In isolated hepatic cell preparations, IGFBP-1 mRNA was expressed primarily in the hepatocyte preparation (Fig. 4A) with trace expression in the Kupffer cell preparation. IGFBP-2 mRNA was barely detectable in poly(A)$^+$-enriched RNA from whole rat liver even on long exposures of autoradiograms (Fig. 4B). Interestingly, IGFBP-2 mRNA was expressed at relatively high levels in isolated hepatocytes. This finding has been observed consistently across different liver and isolated hepatocyte preparations. The reason for this is unknown; however, it may be caused by the isolation procedure (see DISCUSSION).

IGFBP-3 mRNA was expressed in whole rat liver and in Kupffer and endothelial cells but not in hepatocytes (Fig. 4C). IGFBP-4 mRNA was expressed in whole liver and in all elutriated cell preparations, with higher-level expression in hepatocytes than in Kupffer or endothelial cells (Fig. 4D). Trace amounts of 6-kb IGFBP-5 mRNA were detected in rat liver RNA and in RNA from isolated endothelial and Kupffer cells but not hepatocytes (Fig. 4E). Longer exposures revealed smaller bands between 3.5 and 1.8 kb that hybridized with the IGFBP-5 probe (Fig. 4E). The identity of these bands is not defined. Given the comigration with other IGFBP mRNAs and some limited homology of IGFBP-5 mRNA and other IGFBP mRNAs, it is possible that at
least some of these smaller bands reflect cross hybridization of the IGFBP-5 probe with other hepatic IGFBP mRNAs. The trace expression of IGFBP-5 mRNA in liver and negative in situ hybridization data (see Localization of IGFBP mRNAs) argues against significant expression of IGFBP-5 in adult rat hepatocytes; however, low-level expression by nonparenchymal cells may exist. There was no detectable expression of the 1.3-kb IGFBP-6 mRNA in rat liver or elutriated cells (Fig. 4 F).

Localization of IGFBP mRNAs. Different IGFBP mRNAs were localized in sections of liver by in situ hybridization histochemistry. The aim was to obtain an independent morphological evaluation of cellular sites of IGFBP expression that would complement Northern blot hybridization data on isolated liver-derived cells. In situ hybridization data also served as a control for the possibility that patterns of IGFBP expression could be influenced during the isolation of distinct liver cell types. The results of in situ hybridization studies for IGFBP-1, -3, and -4 are shown in Figs. 5, 6, and 7 and largely confirm Northern blot hybridization data. The IGFBP-1 antisense probe showed intense hybridization in hepatocellular regions of the liver lobule (Fig. 5). Labeled cells were identified as hepatocytes by their large size, staining characteristics with routine hema-
toxylin and eosin stain, and location within the liver lobule. The absence of detectable IGFBP-1 mRNA in portal triads or around the central vein is consistent with the lack of detectable IGFBP-1 mRNA in isolated hepatic endothelial cells (Fig. 5). IGFBP-1 mRNA was not detected in bile duct epithelium (Fig. 5). Because of the intimate anatomic association between hepatocytes and Kupffer cells within the lobule and the high-level expression of IGFBP-1 in hepatocytes, it was not possible to demonstrate IGFBP-1 mRNA expression in Kupffer cells by in situ hybridization.

Sites of localization of IGFBP-3 mRNA by in situ hybridization histochemistry were consistent with Northern blot hybridization data. IGFBP-3 was localized to small cells with spindle-shaped nuclei and scant cytoplasm distributed throughout the hepatic lobule (Fig. 6), characteristic of the histological appearance and localization of Kupffer cells. Intense hybridization signals for IGFBP-3 mRNA were localized to endothelial cells lining the portal vein and central hepatic vein (Fig. 6). Weaker hybridization signals were observed in cells within the wall of the hepatic artery and bile duct epithelium (data not shown). There was no detectable hybridization of IGFBP-3 antisense probe to hepatocytes.

IGFBP-4 mRNA was localized throughout the hepatic lobule (Fig. 7), consistent with Northern blot hybridization data indicating expression of IGFBP-4 in multiple liver cell types. IGFBP-4 mRNA was highly expressed in hepatocytes, and there was no change in the level of IGFBP-4 expression in hepatocytes in different regions of the hepatic lobule (e.g., those in zone 1 vs. zone 3). IGFBP-4 was also expressed in Kupffer cells within the hepatic parenchyma. In the portal triads, IGFBP-4 was expressed in cells lining the portal vein and hepatic artery but no IGFBP-4 was detected in the bile duct epithelium (Fig. 7). IGFBP-4 mRNA was detected in endothelial cells surrounding the central vein, although at much lower levels than seen for IGFBP-3 (Fig. 7).

Specificity of hybridization signals obtained with the IGFBP-1, -3, and -4 antisense probes was confirmed by the uniformly negative signals obtained with the corresponding sense probes (Figs. 4–6). In situ hybridization data are not presented for IGFBP-2, -5, and -6 because signals were not discernibly different from those for sections hybridized with the control sense probes.

Western ligand and immunoblot analysis. To further verify cell-specific synthesis of IGFBP expression we performed Western ligand blot and immunoblot analysis on conditioned medium from short-term (24 h) cultures of freshly isolated hepatocytes, Kupffer cells, and hepatic endothelial cells. Kupffer and endothelial cells demonstrated similar patterns of protein expression. Four bands were seen on Western ligand blot at ~40, 32, 28, and 24 kDa (Fig. 8A). The 40-, 32-, and 24-kDa proteins were consistent with the sizes for IGFBP-3, -2, and -4, respectively. A faint, unidentified band at ~28 kDa was observed on Western ligand blot in endothelial cells and Kupffer cells (Fig. 8A) and also observed on normal rat serum used as a positive control (not shown). The size of the faint band was consistent with the size of IGFBP-1 or -5; however, neither the IGFBP-1- nor the IGFBP-5-specific antibody identified this band on immunoblot analysis. The most abundant

Table 1. Summary of Northern blot data

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IGFBP, insulin-like growth factor binding protein. See Fig. 4 for additional data.
protein on Western ligand blot was the 32-kDa protein, which was identified by immunoblot as IGFBP-2 (Fig. 8B). A faint 22-kDa band and a 14-kDa band were observed on IGFBP-2 immunoblot. These bands are consistent with products of specific IGFBP-2 proteolytic activity reported in other cell systems (48). The 24-kDa band seen on Western ligand blot was identified by immunoblot as IGFBP-4 (Fig. 8C). Hepatocyte-conditioned medium was negative by Western ligand and immunoblot analysis despite 30 times concentration of the samples. These data demonstrate secretion of IGFBP protein from isolated cell preparations. Interestingly, hepatocytes do not secrete detectable quantities of IGFBPs under these isolation and culture conditions.

**DISCUSSION**

The present study establishes the cellular sites of expression of several components of the IGF system in adult rat liver, a predominant source of circulating IGF-I in adult rat and other mammals (17, 19). As expected, IGF-I mRNA was highly expressed in hepatocytes. IGFBP-3 mRNA was also demonstrated in Kupffer and endothelial cells. These findings indicate that IGF-I secreted from nonparenchymal cells may contribute to the circulating pool of IGF-I derived from liver, albeit at lower levels than IGF-I derived from hepatocytes. Kupffer and endothelial cells play a key role in immune recognition, cytokine production,
and immune cell recruitment within the liver (11). In other systems, IGF-I expression by macrophages and endothelial cells may participate in processes such as wound healing (28) and vascular repair after endothelial damage (43) and in the pathogenesis of pulmonary and intestinal fibrosis (29, 51). Our present findings raise the possibility that IGF-I derived from hepatic macrophages or endothelial cells may serve as a trophic factor during hepatic responses to infection, ischemia, tumor, or other inflammatory processes.

Expression of IGF-I in three distinct liver-derived cells indicates the potential for cell-specific regulation of IGF-I expression within liver. However, our data also provide some evidence for common transcriptional control mechanisms in these cells. In rat and other mammalian species, two distinct IGF-I mRNA types, termed class 1 and class 2 IGF-I mRNAs, arise via use of distinct transcription start sites within two different 5'-leader exons of the IGF-I gene (19). These mRNAs encode the same IGF-I sequence but differ in signal peptide coding sequence and 5'-untranslated region. Class 1 mRNAs are expressed in a wide range of tissues, whereas class 2 mRNAs are expressed almost exclusively in liver (19). Surprisingly, Kupffer cells and hepatic endothelial cells expressed a ratio of class 1 to class 2 IGF-I mRNA that was similar to that of hepatocytes and very different from most nonhepatic tissues or cells. Common liver-specific control mechanisms may therefore dictate activation of the class 2 IGF-I promoter in multiple liver cell types.

Our analyses of type 1 IGF receptor mRNA in different liver cell types indicate that in normal adult rats endothelial cells, but not hepatocytes or Kupffer cells, express type 1 IGF receptor. The liver traditionally has not been considered a major target for IGF-I actions because of the low-level expression of IGF-I receptor in most studies (23, 26). The finding of type 1 IGF receptor on endothelial cells is consistent with the hepatic endothelial cell as a target cell for autocrine or paracrine actions of IGF-I and not unexpected based on the finding of type 1 IGF receptor on nonhepatic vascular endothelial cells (43). In cultured human umbilical vein endothelial cells, IGF-I mediated many biological effects, including induction of adhesion molecule expression (5), nuclear factor-kB activation (5), and increased endothelial cell-monocyte adhesion (4). In addition, IGF-I stimulated corneal endothelial cell proliferation (10). The biological effects of IGF-I on endothelial cells have been implicated in the pathogenesis of atherosclerosis, in restenosis after angioplasty, and in retinal vascular disease.

Our study did not examine nonparenchymal cell populations, including hepatic stellate cells (Ito cells), natural killer cells, or lymphocytes. These populations are difficult to study in freshly isolated populations because quantities of cells are small. Stellate cells can be cultured, however, and cultured cells have been shown to be IGF-I responsive and express IGF-I type 1 receptor (32). Therefore, in normal adult rat liver, both endothelial and stellate cells express the IGF-I receptor and are likely targets for IGF-I actions. These observations encourage investigation of hepatic targets for IGF-I action in pathological states such as hepatitis and cirrhosis (3, 30).

GH is a primary regulator of IGF-I gene transcription and mRNA abundance in rat liver and in a number of nonhepatic tissues (13, 14, 19). Observations that Kupffer and endothelial cells, like hepatocytes, express GH receptor mRNA indicate that these cells have the capacity to respond to GH. GH receptor mRNA is expressed at much higher levels in hepatocytes than in Kupffer or endothelial cells, which may contribute to higher-level expression of IGF-I in hepatocytes. On the basis of in situ hybridization data, Chin et al. (9) concluded that GH receptor mRNA was expressed exclusively in hepatocytes and not in hepatic nonparenchymal cells. This contrasts with our findings in isolated cells. A possible explanation for this difference is that high-level expression of a specific mRNA in hepatocytes makes detection of the same mRNA in nonparenchymal cells difficult by in situ hybridization, because of the close anatomic localization of parenchymal and nonparenchymal cells. In our studies, the differential expression of hepatocyte and parenchymal cell marker mRNAs and differential expression of IGFBP mRNAs in different isolated cell types argue against cross-

Fig. 8. Detection of IGFBPs in elutriated cells by Western blot analysis. Elutriated endothelial cells (EC) and Kupffer cells (KC) were cultured in serum-free DMEM for 24 h. Medium was concentrated, and proteins were size separated by SDS-PAGE. A: Western ligand blots were performed using 125I-IGF-I and 125I-IGF-II. B and C: immunoblot analyses were performed using antibodies specific for IGFBP-2 and IGFBP-4, respectively. Bands were visualized using Chemiluminescence Western Blotting Kit (Boehringer Mannheim). Representative blots are shown.
contamination as a cause for our detection of GH receptor mRNA in nonparenchymal cells.

Northern blot hybridization data and in situ hybridization data demonstrated cell-specific expression of IGFBP-1, -3, and -4 in adult rat liver. Consistent with prior observations, hepatocytes were primary sources of IGFBP-1 (2, 46). In addition, our data show that hepatocytes were a major source of IGFBP-4, which is the second most abundant serum IGFBP in adult rats and other species (15). Expression of high levels of IGF-I, IGFBP-1, and IGFBP-4 in hepatocytes indicates that formation of IGF-I to IGFBP-1 and IGF-I to IGFBP-4 complexes before or after secretion from hepatocytes may serve to limit the amounts of free IGF-I secreted by the liver into circulation and thereby prevent insulin-like hypoglycemic actions of IGF-I. To our knowledge, expression of IGFBP-1 in Kupffer cells has not been reported previously. We cannot exclude the possibility that the expression of IGFBP-1 mRNA in the Kupffer cell preparation represents minor contamination of the Kupffer cell preparation with hepatocytes. Expression of IGFBP-1 by Kupffer cells raises the possibility of differential regulation of Kupffer cell-derived vs. hepatocyte-derived IGFBP-1. Because Kupffer cells are also a source of IGF-I, IGFBP-1 coreleased from Kupffer cells may modulate the actions of IGF-I during immune challenge or liver damage. This hypothesis awaits further study.

Our observations that IGFBP-3 mRNA is expressed primarily, if not exclusively, in hepatic nonparenchymal cells are in agreement with prior reports (42, 47). Our findings extend these observations by demonstrating that IGFBP-3 mRNA is expressed in both endothelial and Kupffer cells. Our observations differ from those of Chin et al. (9) who, using in situ hybridization, concluded that IGFBP-3 mRNA is expressed in hepatic endothelial cells and not in Kupffer cells. We believe that this difference reflects the difficulty in definitively distinguishing Kupffer and endothelial cells based solely on morphological data from in situ hybridization studies. Consistent with our findings, IGFBP-3 was demonstrated in conditioned medium from Kupffer cells and sinusoidal endothelial cells (33, 47).

Expression of IGFBP-3 in the two hepatic nonparenchymal cell populations raises some interesting issues regarding the regulation of IGFBP-3 expression. It has long been established that IGFBP-3 is a GH-dependent IGFBP (15, 47). IGFBP-3 mRNA expression is reduced in liver of hypophysectomized rats and increased after GH replacement. Studies suggest that GH regulation of IGFBP-3 is indirect and possibly mediated by IGF-I (7). In support of this, Villafuerte et al. (47) found that IGF-I, but not GH, increased IGFBP-3 mRNA and IGFBP-3 secretion into conditioned medium from nonparenchymal cells. Previously, it has been difficult to attribute GH regulation of hepatic IGFBP-3 expression solely to IGF-I given the paucity of type 1 IGF receptors or type 1 IGF receptor mRNA in rat liver. The present observation that hepatic endothelial cells, major sources of IGFBP-3 mRNA, express type 1 IGF receptor, suggests that these cells may be targets for IGF-I and could account for IGF-I-induced hepatic IGFBP-3 synthesis.

Our demonstration that GH receptor mRNA is expressed on Kupffer and endothelial cells, the major cellular sources of hepatic IGFBP-3 mRNA, suggests that GH effects on IGFBP-3 expression in these cells should not be entirely ruled out. It is difficult to maintain GH responsiveness of cells in culture, and this may contribute to lack of observed effects of GH in cultured Kupffer and endothelial cells (47).

Our studies demonstrate that IGFBP-4 mRNA is expressed in hepatic endothelial and Kupffer cells, as well as in hepatocytes. To our knowledge, the cellular sites of hepatic IGFBP-4 synthesis have not been described previously. High-level expression of IGFBP-4 mRNA in hepatocytes suggests that they are important sites of synthesis of circulating IGFBP-4. The finding of IGFBP-4 mRNA in hepatic endothelial cells is consistent with prior reports of IGFBP-4 expression in cultured endothelial cells from nonhepatic sites (6). Hepatic nonparenchymal cells may thus be important sites of synthesis and cell-specific regulation of hepatic and circulating IGFBP-4.

IGFBP-2 was the only binding protein for which analyses from isolated cells yielded discordant results with analyses of RNA extracted from whole rat liver or in situ hybridization analyses. Observations that levels of IGFBP-2 mRNA in adult rat liver assessed by Northern hybridization or in situ hybridization are very low are consistent with prior Northern hybridization data (35). Relatively high-level expression of IGFBP-2 mRNA in isolated hepatocytes as found here has not been reported previously, and in one study time-dependent increases in IGFBP-2 mRNAs were observed when hepatocytes were cultured in the absence of serum or hormonal supplements (35). Interestingly, our ligand and immunoblot studies demonstrated high-level expression of IGFBP-2 in Kupffer- and endothelial cell-conditioned media after 24 h, but no IGFBP-2 mRNA was detected in freshly isolated Kupffer or endothelial cells. It is unlikely that this represented contamination by hepatocytes because IGFBP-2 was not detected in hepatocyte medium. Patterns of IGFBP-2 remain most puzzling because IGFBP-2 mRNA is expressed in isolated hepatocytes but not in whole liver, and IGFBP-2 protein is highly secreted by Kupffer and endothelial cells despite the lack of IGFBP-2 mRNA in freshly isolated cells. One possibility is that IGFBP-2 is not expressed in intact liver but is quickly induced by isolation procedures or short-term cultures. This is consistent with prior observations that IGFBP-2 mRNA expression markedly increases with time in culture. Further studies on transcriptional and posttranscriptional regulation of IGFBP-2 may address this issue.

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