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Heparin-binding growth factor type 1 (acidic fibroblast growth factor): A potential biphasic autocrine and paracrine regulator of hepatocyte regeneration

(cell proliferation/transforming growth factor β)

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ABSTRACT **Heparin-binding growth factor type 1 (HBGF-1; sometimes termed acidic fibroblast growth factor) is potentially an important factor in liver regeneration. HBGF-1 alone (half-maximal effect at 60 pM) stimulated hepatocyte DNA synthesis and bound to a high-affinity receptor (K_d = 62 pM; 5000 per cell). Epidermal growth factor (EGF) neutralized or masked the mitogenic effect of HBGF-1 concurrent with appearance of low-affinity HBGF-1 binding sites. HBGF-1 reduced the inhibitory effect of transforming growth factor type β (TGF-β) on the EGF stimulus. Nanomolar levels of HBGF-1 decreased the EGF stimulus. An increase in hepatic HBGF-1 gene expression after partial hepatectomy precedes increases in expression of the EGF homolog, TGF-α, and nonparenchymal-cell-derived TGF-β in the regenerating liver. Expression of HBGF-1 mRNA occurs in both hepatocytes and nonparenchymal cells and persists for 7 days in liver tissue after partial hepatectomy. HBGF-1 acting through a high-affinity receptor is a candidate for the early autocrine stimulus that drives hepatocyte DNA synthesis prior to or concurrent with the EGF/TGF-α stimulus. It may allow hepatocyte proliferation to proceed in the presence of low levels of TGF-β. An EGF/TGF-α-dependent change in HBGF-1 receptor phenotype and increasing levels of nonparenchymal-cell-derived HBGF-1 and TGF-β may serve to limit hepatocyte proliferation.**

Adult hepatocytes rarely divide except in response to injury or xenobiotics. After partial hepatectomy, DNA synthesis in hepatocytes begins about 14 hr after the operation and peaks at 24 hr (1, 2). The liver remnant doubles after 36 hr. By 1 week 90% of the liver mass is restored, and hepatocytes return to quiescence. Liver cell DNA synthesis, cell division, and return to quiescence is thought to be controlled by growth-stimulatory and growth-inhibitory hormones of autocrine and paracrine origin (3–5). Isolated hepatocyte DNA synthesis is stimulated by several factors including insulin-like growth factors and epidermal growth factor (EGF) or homologs (6, 7) and is inhibited by transforming growth factor type β (TGF-β) (8–12). Hepatic TGF-β mRNA levels increase after partial hepatectomy in the nonparenchymal cells of liver and thus may act in a paracrine mode to modulate hepatocyte division (3–5). Heparin-binding growth factors (HBGF) are autocrine regulators of differentiated hepatoma cells in culture, and the activity is mediated by a receptor of apparent molecular mass of 100–150 kDa (13). Fetal human hepatocytes and normal adult rat liver membranes also exhibit specific binding sites for HBGF (13). These developments suggest a role of HBGF polypeptides and the HBGF receptor in liver cells. In this report, we examine the mitogenic activity, receptor properties, and the expression of HBGF in isolated liver cells and regenerating liver.

MATERIALS AND METHODS

Materials. Bovine brain HBGF-1 and EGF were from Upstate Biotechnology (Lake Placid, NY). Medium MCDB 107 was a gift of S. Yamada (Kyokuto Pharmaceutical, Tokyo). Insulin, transferrin, ethanolamine, bovine serum albumin, oleic acid, and soybean trypsin inhibitor were from Sigma. Type I/III collagen (Vitrogen) was from Collagen Corp. Collagenase was from Wako Pure Chemicals (Osaka, Japan). Porcine platelet-derived TGF-β1 was from R & D Systems (Minneapolis).

Hepatocyte Cultures. Liver of adult male F344 rats (200–250 g) were perfused through the portal vein with Hepes-buffered saline (pH 7.0) containing 5 mM EGTA and then were perfused for 10–15 min with the same solution containing 0.05% (wt/vol) collagenase, 1 mM calcium chloride, and soybean trypsin inhibitor (10 μg/ml). The perfused liver was excised and dispersed in Hepes-buffered saline. The cell suspension was filtered through gauze, and hepatocytes were collected by centrifugation (four times) at 50 × *g* for 1 min. Hepatocytes were plated at 1×10^5 cells in 35-mm plastic Petri dishes coated with type I/III collagen containing 3 ml of nutrient medium MCDB 107 (14) and 2% (vol/vol) fetal bovine serum. After 2 hr, the medium was removed and replaced with serum-free medium containing bovine serum albumin (1 mg/ml), oleic acid (4 μg/ml), dithiothreitol (50 μM), ethanolamine (5 μg/ml), and insulin (1 μg/ml). HBGF-1, EGF, and TGF-β were added as indicated in the text. At least 90% of cells in the experimental cultures were judged to be morphologic hepatocytes.

DNA synthesis was determined by addition of [*methyl*-^{3}H]-thymidine (1 μCi/ml; 80.9 Ci/mmol; 1 Ci = 37 GBq) after 48 hr of culture, and incorporation into acid-insoluble macromolecules was determined 24 hr later. Assays contained from 50,000 to 100,000 cells per dish.

^{125}I-labeled HBGF-1 (^{125}I-HBGF-1) binding to specific receptor sites and covalent affinity cross-linking to hepatocytes was performed as described (13).

Partial Hepatectomy, mRNA Analysis, and Immunoassays. Partial hepatectomy (69%) was performed by removal of the median and left lateral lobes (15) of livers from rats under ether/oxygen anesthesia. For sham-operated rats, the liver was manipulated but not removed. Rats were maintained in

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Abbreviations: HBGF-1 and HBGF-2, heparin-binding growth factor types 1 and 2; EGF, epidermal growth factor; TGF-α and TGF-β, transforming growth factor types α and β.

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temperature-controlled rooms in a 12-hr light/dark cycle, operations were performed within 2 hr of the same time each day, and sham-operated animals were paired in the same cages with partial hepatectomized animals.

Livers were removed at the indicated times after the operation, and the poly(A)$^+$ RNA was isolated and analyzed by Northern blot hybridization with nick-translated ^{32}P-labeled cDNA for HBGF-1 (4.8×10^8 cpm/μg) and HBGF-2 (4.5×10^8 cpm/μg) (13, 16). Hybridization *in situ* was performed as described (17). Hepatocytes were cultured on glass slides and fixed with 4% paraformaldehyde. Strand-specific ^{32}P-labeled RNA probes were prepared with T7 polymerase from HBGF-1 cDNA cloned in the antisense and sense orientations in the PTZ-19R plasmid (Pharmacia). For immunoassay, a polyclonal antiserum was raised in rabbits against amino acids 21–154 of HBGF-1 from bovine brain (18, 19). Tissue extracts were prepared and partially purified by heparin-agarose affinity chromatography (18), dotted (dot blots) on Immobilon membranes (Millipore), reacted with rabbit anti-HBGF-1, and then visualized by reaction with goat anti-rabbit IgG coupled to alkaline phosphatase. Samples were also analyzed by 15% SDS/PAGE, transferred electrophoretically to Immobilon membranes, and then reacted with the same antibody (Western blots). HBGF-like activity in liver tissue extracts and hepatocyte culture media was assayed by using human umbilical vein endothelial cell proliferation (20).

RESULTS AND DISCUSSION

Mitogenic Activity and Receptor Binding of HBGF-1 on Hepatocytes. To characterize the activity of HBGF on adult rat liver parenchymal cells, hepatocytes were isolated from perfused adult rat liver. Purified bovine brain-derived HBGF-1 (also called acidic fibroblast growth factor and endothelial cell growth factor) (18, 19, 21) stimulated hepatocyte DNA synthesis 3- to 5-fold, with a half-maximal effect at about 60 pM (Fig. 1 *A* and *B*). Autoradiographic analysis *in situ*

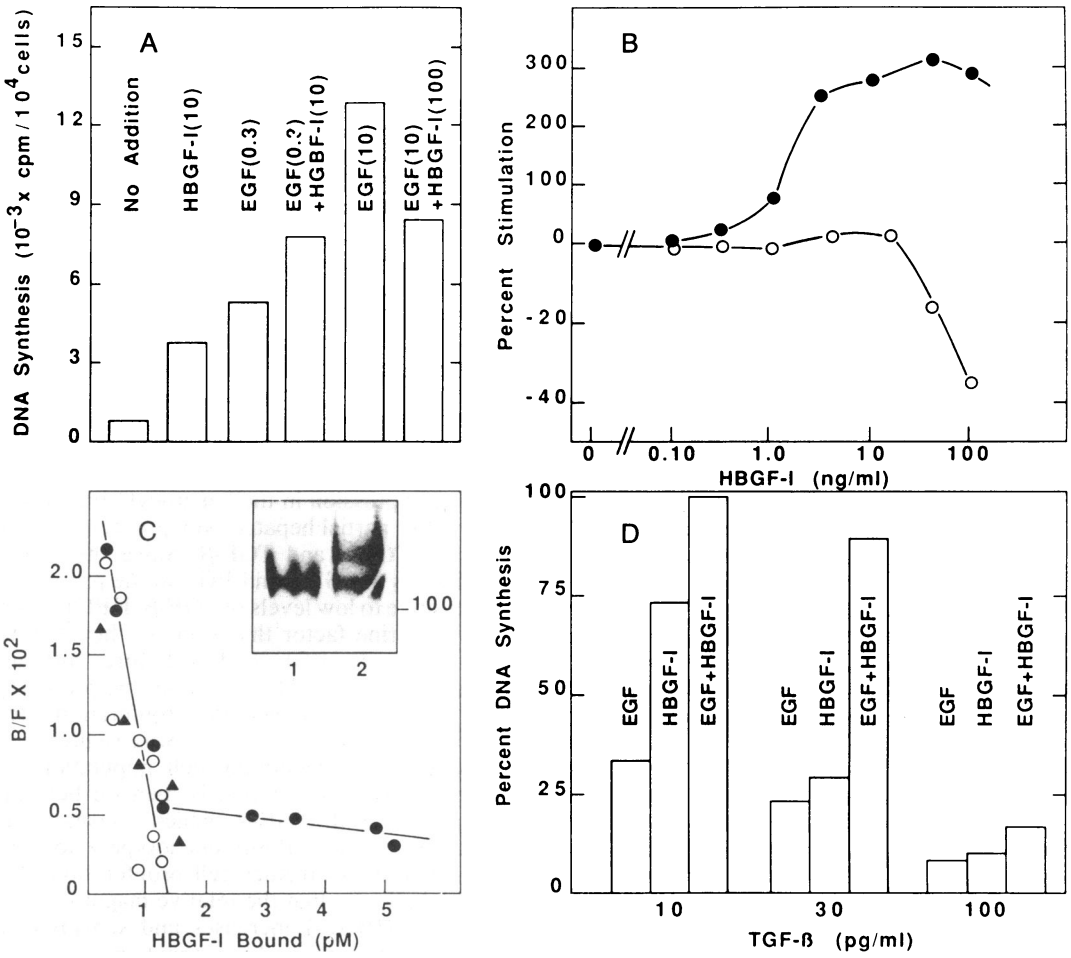

FIG. 1. Mitogenic effects and receptor binding of HBGF-1 on hepatocytes. Hepatocytes were prepared from adult rat liver and allowed to attach to culture dishes for 2 hr. HBGF-1, EGF, and TGF-β were then added as indicated, and DNA synthesis was determined after 48 hr of culture. Data are the mean of duplicate or triplicate assays from single representative experiments. (*A*) Mitogenic effect of HBGF-1 and EGF. HBGF-1 and EGF were added at the indicated concentrations (in ng/ml). (*B*) Effects of HBGF-1 alone (●) or in the presence of EGF (○) (10 ng/ml). For HBGF-1 alone, the percent stimulation was based on the baseline incorporation of [^{3}H]thymidine (1340 cpm per 10^4 cells). The percent stimulation of [^{3}H]thymidine incorporation in the presence of EGF was based on the incorporation in the absence of HBGF-1 and the presence of EGF at 10 ng/ml (13,530 cpm per 10^4 cells). (*C*) Scatchard plot of ^{125}I-HBGF-1 binding. Specific HBGF-1 receptor binding to cultured hepatocytes was determined after 2 hr (○) and 48 hr (▲) in the absence of added factors and after 48 hr in the presence of only EGF (10 ng/ml) (●). Binding assays (0.25 ml) contained 50,000 cells. HBGF-1 bound to nonreceptor, heparin-like sites (13) was constant in both conditions. (*Inset*) Bound ^{125}I-HBGF-1 was cross-linked to hepatocytes, and the resulting extracts were analyzed by SDS/PAGE and autoradiography (13). Hepatocytes were cultured for 2 hr (lane 1) in absence of EGF and HBGF-1 and for 48 hr (lane 2) in the presence of only EGF (10 ng/ml). Parallel assays containing 100-fold excess unlabeled HBGF-1 exhibited no radioactive bands. A 100-kDa marker is indicated. (*D*) Effect of TGF-β on HBGF-1 and EGF-stimulated DNA synthesis. The indicated amounts of TGF-β were added to hepatocyte cultures containing HBGF-1 and EGF at 10 ng/ml as indicated. Data are presented as a percent of DNA synthesis determined in the absence of TGF-β.

indicated that about 30% and 80% of the hepatocytes incorporated nuclear [^{3}H]thymidine in the presence of HBGF-1 and EGF, respectively. The maximal effect of EGF (3 ng/ml) was 3 to 5 times that of HBGF-1, and effect of HBGF-1 up to 600 pM was additive with that of suboptimal doses of EGF (Fig. 1*A*). HBGF-1 had no stimulatory effect in the presence of saturating levels of EGF. Nanomolar levels of HBGF-1 reduced the maximum EGF stimulus (Fig. 1 *A* and *B*).

Specific HBGF-1 binding sites on the isolated hepatocytes were characterized by Scatchard analysis and affinity cross-linking (Fig. 1*C*). After 2 and 48 hr of culture in the absence of HBGF-1 and EGF, the hepatocytes exhibited only a high-affinity class of HBGF-1 receptors (K_d = 62 pM; 5000 per cell). The presence of EGF for 48 hr resulted in a curvilinear Scatchard plot, indicating the appearance of a low-affinity class of HBGF-1 receptor sites. A single ^{125}I-labeled complex of apparent molecular mass of 122 kDa was apparent at 2 hr in the absence of HBGF-1 and EGF (Fig. 1*C Inset*, lane 1). A second 157-kDa ^{125}I-labeled complex appeared after 48 hr in the presence of EGF (Fig. 1*C Inset*, lane 2). Thus, the mitogenic activity of HBGF-1 is likely mediated by a high-affinity receptor with an apparent mass of 105 kDa. EGF induces the appearance of a low-affinity class of receptor whose occupancy by nanomolar levels of HBGF-1 may counteract the mitogenic effect of HBGF-1.

The effect of EGF on DNA synthesis and the properties of the HBGF-1 receptor in normal hepatocytes differs from the hepatoma cell line HepG2 (13). HepG2 cell DNA synthesis is not stimulated by EGF. HepG2 cells exhibit an EGF-independent biphasic mitogenic response to HBGF-1, an EGF-independent curvilinear Scatchard plot for HBGF-1 binding, and a broad band of ^{125}I-HBGF-I binding sites from 100 to 150 kDa that cannot be resolved into two distinct bands (13). Whether the hepatocytic 140- and 105-kDa polypeptides that link to ^{125}I-HBGF-1 are binding sites of different affinity, are the product of the same or different genes, and mediate the different mitogenic effects of HBGF on hepatocytes awaits isolation, chemical characterization, and reconstitution of the two receptor subtypes.

HBGF-1 Reduces Inhibition of Hepatocyte DNA Synthesis by TGF-β. TGF-β inhibits EGF-stimulated hepatocyte DNA synthesis *in vitro*, is activated after liver injury, and depresses the regenerative response of liver *in vivo* (3–5, 8–12). However, the medium of cultured hepatocytes contains endogenous stimulator(s) of hepatocyte DNA synthesis and factors that partially overcome the inhibitory effect of TGF-β (8). Previously we showed that the HBGF-stimulated growth of rat prostate tumor epithelial cells is refractory to inhibition by TGF-β, while TGF-β is an inhibitor of the same cells stimulated by EGF (22). Fig. 1*D* shows that TGF-β is a much less effective inhibitor of HBGF-1-stimulated hepatocyte DNA synthesis than of EGF-stimulated hepatocyte DNA synthesis. More remarkably, the presence of HBGF-1 resulted in nearly complete abrogation of the inhibitory effect of low levels of TGF-β (<30 pg/ml) on EGF-stimulated DNA synthesis (Fig. 1*D*). Thus HBGF-1 or a homolog is a candidate for the hepatocyte-derived factor that supports hepatocyte DNA synthesis despite the presence of low levels of TGF-β.

HBGF-1 mRNA, Antigen, and Activity Increases in Liver Remnants After Partial Hepatectomy. To determine whether HBGF genes are activated during liver regeneration, we analyzed poly(A)$^+$ RNA from partially hepatectomized livers by Northern blots with cDNA probes for HBGF-1 (21) and HBGF-2 (also called basic fibroblast growth factor) (23). Transcripts for HBGF-2 were undetectable. The major HBGF-1 transcript [4.8 kilobases (kb)] had increased by 4 hr to 80% of the peak at 24 hr (Fig. 2*A*), remained steady for 4 days (data not shown), declined significantly on day 5, and declined to normal levels by day 7 (Fig. 2*B*). HBGF-1 transcripts were evident in both primary liver cell suspensions enriched in parenchymal cells (hepatocytes) and nonparenchymal (bile duct epithelial, endothelial, and Kupffer) cells from the regenerating livers (Fig. 2*C*). As reported (3, 4), TGF-β mRNA, which appears at 4 hr, but peaks 3 days after partial hepatectomy, was only evident in the nonparenchymal-cell-enriched fraction (Fig. 2*C*). Northern (Fig. 2*C*) and *in situ* hybridization analysis (Fig. 2 *D* and *E*) of HBGF-1 mRNA from primary hepatocyte cell cultures from resting and regenerating liver (data not shown) confirmed the expression of HBGF-1 transcripts in hepatocytes.

Extracts of regenerating liver tissue 48 hr after partial hepatectomy exhibited 1000 times more total HBGF-like mitogenic activity per gram of original tissue than normal liver. Immunoblot analysis revealed the presence of antigens in the regenerating livers that migrated at the same position as bovine brain acetylAla-HBGF-1 (18 kDa) and amino acids 21–154 of HBGF-1 (16 kDa) (Fig. 2*F*). No antigen could be detected in extracts of normal liver. The medium of isolated hepatocytes after 3 and 48 hr of culture also exhibited HBGF-like activity on endothelial cells (20) and competed with specific ^{125}I-HBGF-1 binding to HepG2 cells (13). The same medium also exhibited the two antigen bands on immunoblots shown for regenerating liver extracts in Fig. 2*F*.

HBGF-1 as an Autocrine and Paracrine Regulator of Liver Regeneration. Increasing evidence suggests that EGF/TGF-α constitutes an autocrine stimulus of hepatocyte DNA synthesis during liver regeneration and TGF-β constitutes an opposing paracrine influence to limit the EGF/TGF-α stimulus (3–5, 8–12, 26). An increase in TGF-α transcripts is detectable at about 8 hr and peaks at 24 hr in liver tissue after partial hepatectomy, and TGF-α is expressed in the parenchymal cells (ref. 26; unpublished results). TGF-β transcripts are detectable at 4 hr, but do not peak until 3 days after the operation and appear to be expressed only in nonparenchymal cells (3–5). The results in this report suggest that the HBGF receptor and HBGF-1 in particular may also be important in the temporal control of liver regeneration by autocrine and paracrine mechanisms. The increase of HBGF-1 gene expression in the hepatocyte fraction of regenerating liver after partial hepatectomy precedes increases in expression of TGF-α and TGF-β. Since the mitogenic stimulus elicited by HBGF-1 and EGF in the presence of HBGF-1 is insensitive to low levels of TGF-β, HBGF-1 is a candidate for the autocrine factor that supports hepatocyte proliferation together with EGF/TGF-α before sufficient paracrine-derived TGF-β builds up to limit the proliferative response. We propose that hepatocyte proliferation after partial hepatectomy unfolds in a linear stepwise sequence where HBGF-1 expands the regenerating cell population by means of a high-affinity receptor that is then further expanded by response to EGF/TGF-α, which causes a shift in HBGF receptor to a low-affinity phenotype whose occupancy may be inhibitory to further cell proliferation. Preliminary evidence indicates that the relative magnitude of the mitogenic effect of HBGF-1 increases and sensitivity to TGF-β decreases in the cultured hepatocyte population if the effect of endogenous TGF-α is reduced by dilution or inhibition of EGF/TGF-α action at the receptor level. The population that is mitogenically responsive to HBGF-1 may be less sensitive to TGF-β. Whether the dose-dependent sensitivity to TGF-β reflects TGF-β receptor phenotype remains to be determined (8). This model is consistent with the temporal and dose-dependent inhibitory effects of TGF-β administered to livers of partially hepatectomized animals *in vivo* (11). Paradoxically, this model predicts that it is the expansion of the hepatocyte population by EGF/TGF-α that eventually limits long-term growth of cultured hepatocytes. It will be interesting to determine whether a pure proliferative population of HBGF-1-responsive cells exhibiting only the high-affinity

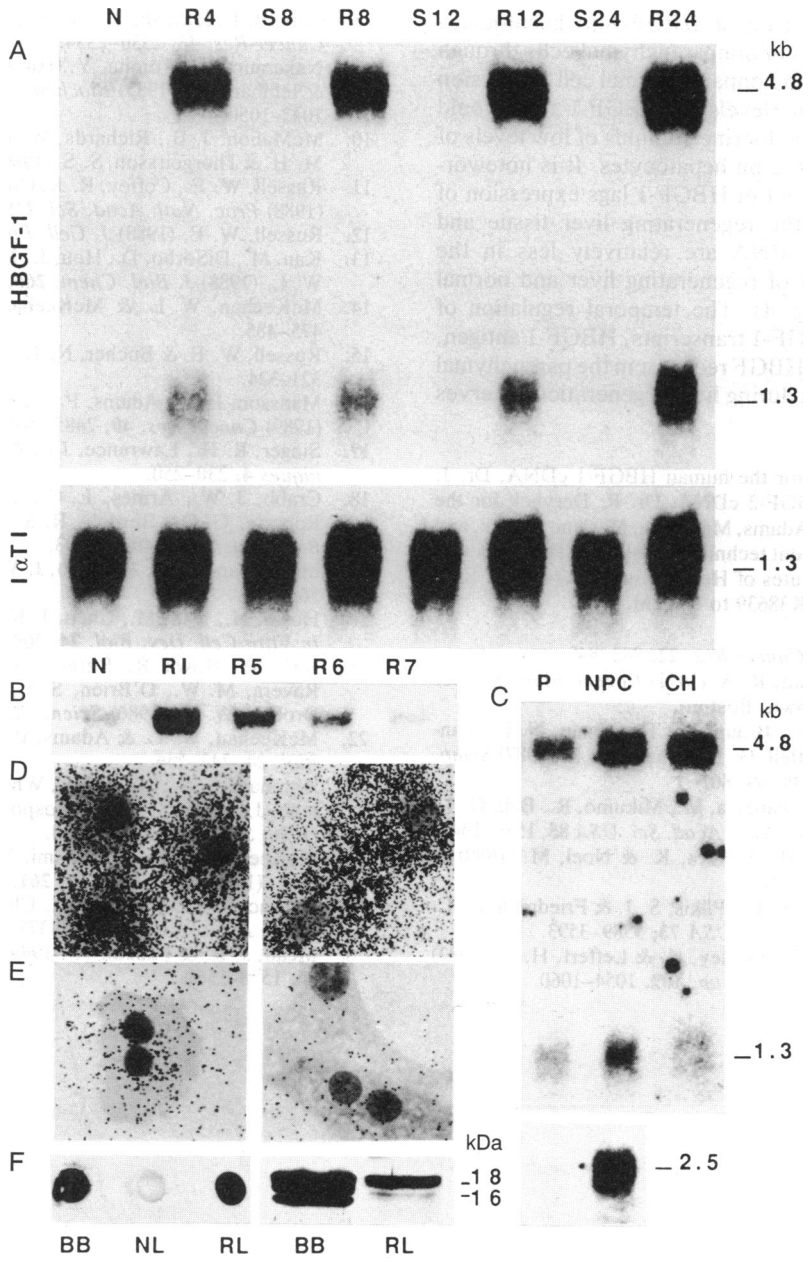

FIG. 2. Expression of HBGF-1 in regenerating liver and isolated hepatocytes. (*A*) HBGF-1 mRNA in regenerating liver. (*Upper*) Poly(A)$^+$ RNA (10 μg) was isolated from liver tissue and analyzed by Northern blot procedures with cDNA probes for HBGF-1 and HBGF-2. Blots were exposed for 3 days. (*Lower*) The same blots were then hybridized with cDNA (3×10^8 cpm/μg) for human inter-α trypsin inhibitor (IαTI), a liver secretory protein (13, 24), and exposed for 3 hr. Only the 1.3-kb region of the blot probed with IαTI cDNA is shown. N, normal liver; R, regenerating liver after partial hepatectomy; S, sham-operated. Numbers indicate hours after operation. (*B*) HBGF-1 mRNA levels in regenerating liver (R) at the indicated days after partial hepatectomy. N, normal liver. (*C*) HBGF-1 and TGF-β mRNA in parenchymal and nonparenchymal cell fractions. P and NPC indicate primary liver cell suspensions enriched in parenchymal and nonparenchymal cells, respectively, from collagenase-perfused livers 48 hr after partial hepatectomy. The P fraction was the fraction used for primary hepatocyte cultures (Fig. 1). The NPC fraction was prepared from the undissociated tissue remaining after collagenase dissociation, which was further treated with 0.1% collagenase/pronase for 20 min at 37°C (5). The P fraction is virtually free of nonparenchymal cells; however, the NPC fraction contains 5–10% hepatocytes. Poly(A)$^+$ RNA (10 μg) from the P and NPC fractions was first probed with cDNA for HBGF-1 (2×10^8 cpm/μg) and exposed for 3 days. The same two lanes were boiled for 3 min in water, exposed for 3 days to ensure deprobing, and then probed with cDNA (3×10^8 cpm/μg) for murine TGF-β1 (25) and exposed for 3 days. TGF-β cDNA hybridized to the indicated 2.5-kb band in the lane containing poly(A)$^+$ RNA from NPC. CH indicates poly(A)$^+$ RNA (5 μg) from hepatocytes from normal livers after 24 hr of culture. RNA from CH was probed with HBGF-1 cDNA (5×10^8 cpm/μg) and exposed for 7 days. (*D* and *E*) HBGF-1 mRNA in hepatocytes *in situ*. Isolated hepatocytes were cultured for 2 hr (*Left*) and 24 hr (*Right*). Fields were probed with HBGF-1 RNA cloned in the antisense direction (cRNA) (*D*) or with sense-strand RNA for HBGF-1 (*E*). (*F*) Expression of HBGF-like antigen in regenerating liver. (*Left*) Dot blots. (*Right*) Western blots. Regenerating liver extracts contain twice the amount of total heparin-binding proteins per wet weight tissue than does normal resting liver. BB, bovine brain (0.9 μg); NL, normal liver (9 μg); RL, regenerating liver (10 μg).

HBGF receptor can be maintained by blocking endogenous TGF-α action.

Expression of HBGF-1 mRNA in regenerating liver tissue is sustained through the 7-day regenerative phase of the liver. It remains to be determined if HBGF-1 expression in the hepatocyte fraction is transient and the sustained expression reflects expression in the nonparenchymal cells whose peak of DNA synthesis lags hepatocytes by 24 hr (1). Conceivably,

an early, transient expression of HBGF-1 in hepatocytes contributes to proliferation of nonparenchymal cells through a paracrine mechanism, and nonparenchymal cell expression of HBGF-1 contributes to levels of HBGF-1 that would eventually counteract the autocrine stimulus of low levels of hepatocyte-derived HBGF-1 on hepatocytes. It is noteworthy that the 1.3-kb transcript of HBGF-1 lags expression of the 4.8-kb transcript in the regenerating liver tissue and amounts of the 1.3-kb mRNA are relatively less in the parenchymal cell fraction of regenerating liver and normal cultured hepatocytes (Fig. 1). The temporal regulation of expression of the two HBGF-1 transcripts, HBGF-1 antigen, HBGF-1 activity, and the HBGF receptor in the parenchymal and nonparenchymal cells during liver regeneration deserves detailed examination.

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