Modulation of Insulin-like Growth Factor-II/Mannose 6-Phosphate Receptors and Transforming Growth Factor-β1 during Liver Regeneration*

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Transforming growth factor-β1 (TGF-β1) is a potent mito-inhibiting substance that is thought to play an important function in regulating hepatocyte proliferation during liver regeneration. In this investigation, we have shown by immunohistochemistry that hepatocytes contain significant intracellular concentrations of TGF-β1 12 h after a two-thirds partial hepatectomy. This elevation in hepatocyte TGF-β1 concentration was initially confined to those cells that resided in the perportal region of the liver. The elevation of intracellular TGF-β1 was, however, transient, and within 36 h, the hepatocytes positive for TGF-β1 had changed in a wavelike fashion from the perportal to the pericentral region of the liver lobules. By 48 h, most hepatocytes no longer contained TGF-β1. Interestingly, this temporary increase in TGF-β1 always preceded the onset of hepatocyte replication by approximately 3–6 h. Since TGF-β1 mRNA has been shown to be absent from hepatocytes normally and throughout liver regeneration, these results imply that the increase in intracellular TGF-β1 resulted from an augmented uptake. We have further shown that the insulin-like growth factor-II/mannose 6-phosphate (IGF-II/Mann-6-P) receptors were up-regulated during liver regeneration and that the increased expression of this receptor co-localized in those hepatocytes containing elevated concentrations of TGF-β1. The latent TGF-β1 phosphomannosyl glycoprotein complex has been shown to bind to the IGF-II/Mann-6-P receptor. Therefore, our data are consistent with the hypothesis that this latent complex is internalized through the IGF-II/Mann-6-P receptor to the intracellular acidic prelysosomal/endosomal compartments where the mature TGF-β1 molecule could be activated by dissociation from the latent complex.

Following a two-thirds partial hepatectomy, the remaining tissue regenerates to the liver's original mass within 1–2 weeks (1, 2). Consequently, this is an excellent and often used model for investigating those factors involved in the initiation and termination of normal cell replication. Though most hepatocytes ultimately divide in response to a partial hepatectomy, cellular proliferation is not initiated at the same time throughout the liver lobule (1). Rather, hepatocyte DNA synthesis starts in the perportal region 15–18 h after a partial hepatectomy, and by 48 h, only the pericentral hepatocytes are actively proliferating. In contrast, the nonparenchymal cells proliferate later with maximal DNA synthesis occurring approximately 48 h after partial hepatectomy.

Though the kinetics and the intralobular distribution of hepatocyte proliferation during liver regeneration are well described, the molecular mechanisms involved in the control of liver cell growth are only now being elucidated (3-5). The expression of proto-oncogenes has been shown to be modulated during liver regeneration. Within 1–2 h after a partial hepatectomy, the steady state mRNA levels of c-fos, c-myc, and c-jun are significantly increased, whereas that of c-ras and c-raf maximize approximately 30 h later (6-8). Interestingly, the tumor suppressor gene, p53, is also up-regulated (9). This occurs, however, in the G1 phase of the cell cycle, suggesting that wild-type p53 plays an important function prior to the initiation of hepatocyte DNA synthesis.

Numerous hepatocyte growth-stimulating substances have been discovered that are involved in initiating this cascade of proto-oncogenes (3, 6-8) and other immediate early gene (4, 5) expression during liver regeneration (for review, see Ref. 2). These factors can be divided into two groups, complete mitogens and co-mitogens. Complete mitogenic agents are defined as those substances that by themselves stimulate primary cultures of hepatocytes to replicate in serum-free media. These include epidermal growth factor, TGF-α,1 fibroblast growth factor, hepatocyte growth factor, and hepatopoietin B. In contrast, other factors shown to play an important role in liver regeneration, such as the catecholamines, vasopressin, angiotensin II, insulin, and glucagon do not directly stimulate hepatocyte DNA synthesis in culture. They do, however, significantly enhance the proliferative effectiveness of the complete mitogenic agents and, therefore, act as co-mitogens.

Though hepatocyte replication is rapidly initiated following a partial hepatectomy, it is not an uncontrolled process since approximately 48 h later liver cell growth ceases. TGF-β1 is a potent inhibitor of cellular proliferation, including that of hepatocytes (for review, see Ref. 9). Picomolar concentrations of this growth factor inhibit hepatocyte proliferation in culture (10, 11), and TGF-β1 injected into partially hepatectomized rats significantly delays the onset of DNA synthesis (12). Thus, it has been suggested that TGF-β1 is involved in preventing uncontrolled hepatocyte proliferation following a two-thirds partial hepatectomy (3, 6). This postulate is further...
TGF-β1 is synthesized as a prepro molecule that is cleaved at a dibasic cleavage site (residues 278) by a protease having an acidic pH optimum (14). The pro region of the molecule (residues 30–278) is further glycosylated at three N-linked glycosylation sites, and two of the carbohydrate side chains are phosphorylated at mannose residues forming mannose 6-phosphate (15, 16). The dimeric form of the pro region binds noncovalently to the mature TGF-β1 homodimer (residues 279–390), inactivating its biological activity (17–20). In platelets and certain cell lines, an additional polypeptide molecule is cross-linked by disulfide bonds to the pro region (9, 21, 22). It is the latent complex that is secreted normally from cells. Thus, for mature TGF-β1 to regain its biological activity, it must first be dissociated from this protein complex. Therefore, the regulation not only of the synthesis of TGF-β1 but also its activation would be expected to play an important role in controlling liver regeneration. Conditions such as low or high pH, heat, urea, and proteases such as plasmin have been shown to activate TGF-β1 (9, 18, 23). It has been postulated, however, that the glycosylated pro domain of TGF-β1, in addition to being necessary for TGF-β1 secretion (14), may also play an important role in the activation of TGF-β1, since it binds to the IGF-II/Man-6-P receptor (16, 17, 24).

The structure of the IGF-II receptor and the calcium-independent mannose 6-phosphate receptor have been shown to be identical, and thus it binds two structurally unrelated ligands (25). Recently, the IGF-II/Man-6-P receptor has also been shown to be maternally imprinted and closely linked or identical with the Tme (T-associated maternal effect) gene (26). The receptor consists of a single polypeptide chain with a large extracellular domain that possesses distinct binding regions for IGF-II and phosphomannosyl residues. In contrast, the very small intracellular portion of the receptor lacks a tyrosine kinase domain but interacts and activates G12ζ, a GTP-binding protein (27). Though the IGF-II/Man-6-P receptor is thought to function mainly in the sorting of newly synthesized lysosomal enzymes and in the endocytosis of extracellular phosphorylated lysosomal enzymes (28), secreted growth factors such as prolifiner (29) and the latent complex of TGF-β1 (16, 24) have been shown to also bind to this receptor. By subsequently directing the TGF-β1 latent complex to intracellular acidic prelysosomal compartments, TGF-β1 may be activated. Alternatively, as recently demonstrated, the extracellular activation of TGF-β1 by plasmin may be facilitated by the binding of the TGF-β1 latent complex to the IGF-II/Man-6-P receptor (30).

In both normal and regenerative liver, TGF-β1 has been shown by Northern blot analysis (6, 31) and in situ hybridization (32, 33) to be produced solely by the nonparenchymal cells. The cellular distribution of the TGF-β1 protein during regenerative liver regeneration has not, however, been previously determined. With the use of antibodies specific for the mature TGF-β1 molecule, we now show that the periporal hepatocytes, which normally do not contain TGF-β1, have high intracellular levels of TGF-β1 approximately 12 h after a partial hepatectomy. This increased intracellular concentration of TGF-β1 is transient and occurs in the Gi phase of the cell cycle. In a wavelike fashion, the hepatocytes positive for TGF-β1 progress from the periporal to the pericentral regions of the liver lobule. Furthermore, a marked increase in the IGF-II/Man-6-P receptor co-localizes in those hepatocytes with elevated intracellular TGF-β1. These findings suggest that the increase in TGF-β1 observed during liver regeneration may result from enhanced hepatocyte uptake of the complete latent TGF-β1 phosphomannosyl glycoprotein complex by the IGF-II/Man-6-P receptor.

**MATERIALS AND METHODS**

**Animals**—Male Fischer 344 rats (Charles River Laboratories, Wilmington, MA) weighing 150–200 g were maintained in a temperature control room with a 12-h light-dark cycle. The animals were provided Purina 5010 rat chow and water ad libitum. All animals were two-thirds partially hepatectomized by the method of Higgins and Anderson (34) between 9:00 and 10:00 a.m. At various times thereafter the animals were killed, and the remaining lobes of the liver were removed. A portion of the liver liver was then fixed for immunohistochemistry, and the remainder was quick frozen in liquid nitrogen and stored at −75 °C until RNA isolation.

**Antibodies and cDNA Probes**—The three primary antibodies used for the immunolocalization of TGF-β1 were kindly provided by Dr. M. Sporn (National Cancer Institute, Bethesda). The LC1–30 and LC79–109 antibodies to mature TGF-β1 are rabbit polyclonal antibodies generated from synthetic peptides with the amino acid sequences of the N-terminal and C-terminal regions of mature TGF-β1, respectively (23). The polyclonal antibody made to a synthetic peptide with the amino acid sequence of the C-terminal portion of the proteolytically cleaved pro region of the prepro-TGF-β1 molecule (20, 35). The rabbit polyclonal IGF-II/Man-6-P receptor antibody (C-1 antiserum) was raised to purified rat IGF-II/Man-6-P receptor protein (36). A 320 bp pair of cDNA clone of the rat IGF-II/Man-6-P receptor (clone 4P6) was kindly provided by R. MacDonald (University of Nebraska, Omaha) (37).

**Immunohistochemical Techniques**—Liver tissues were fixed overnight in Omnifix (AN-CON Genetics, Inc., Melville, NY), paraffin-embedded, serial-sectioned (6 μm), and placed on glass slides for peroxidase immunohistochemical staining. The histological sections were deparaffinized in xylene for 10 min and placed in 100% ethanol for an additional 10 min. Endogenous peroxidase activity was eliminated by exposing the sections for 30 min to 0.3% H2O2 in methanol. The tissue sections were then rehydrated for 30 min in phosphate-buffered saline containing 0.1% bovine serum albumin. This was followed by a 30-min exposure at 37 °C to hyaluronidase (1 mg/ml), and nonspecific binding of the primary antibodies was blocked by exposing the sections for 60 min to phosphate-buffered saline containing 10% goat serum (Cedarlane Laboratories, Ltd., Hornby, Canada) and 5% milk diluted blocking solution (Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD). These processed tissue sections were then incubated overnight at 4 °C in a humidified chamber with the antibodies to TGF-β1 (10 μg/ml IgG) and the IGF-II/Man-6-P receptor (10 μg/ml C-1 antiserum); identical concentrations of rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as controls. The next day, the tissues were washed 3× 5 min with phosphate-buffered saline (0.1%, bovine serum albumin) and immunoperoxidase-stained according to the recommended procedures provided with the rabbit IgG Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA); diaminobenzidine was used as the substrate. All immunohistochemical studies were performed with two replicate sections from each animal, and two different animals were used at each time point following partial hepatectomy.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from liver tissue with the use of RNAzol (Cinna/Biotecx, Friendswood, TX) (38). The RNA concentration was determined from the OD at 260 nm. Total RNA (50 μg) was denatured in formamide (60%) and formaldehyde (17.5%) for 15 min at 55 °C. The samples were then chilled, and 1 μg of ethidium bromide was added. The RNA samples were fractionated by electrophoresis on horizontal denaturing formaldehyde (2.2%) agarose gels. The gels were photographed under UV light, and the RNA was transferred to nitrocellulose membranes (Schleicher & Schuell) by standard techniques (39). The filters were baked in a vacuum oven for 2 h at 80 °C. 32P-labeled DNA probes were labeled with [α-32P]dCTP (NEN Research Products, Boston, MA) by a mung bean translation (Promega Biotec, Madison, WI), according to the manufacturer’s recommended procedure (specific activity > 10 6 cpm/μg DNA). The nitrocellulose filter was prehybridized for 2 h at 42 °C in 25 ml of hybridization buffer composed of 5× SSPE (3.6 M NaCl, 0.2 M NaH2PO4, H2O, 20 mM sodium citrate [pH 7.7], 2× Denhardt’s reagent [5% Ficoll, 5% polyethylene glycol, 1% bovine serum albumin], 50% formamide, 0.1% SDS, and 6 mg of denatured salmon testes DNA (Sigma). After the prehybridization, the filters were hybridized with denatured cDNA probe at 42 °C.
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ization buffer was removed, the [α-32P]dCTP-labeled cDNA for the IGF-II/Man-6-P receptor (10⁶ dpm/ml) was added to 4 ml of the hybridization buffer and the filter incubated overnight (16 h) at 42 °C. The filter was then washed three times with 2 x SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) and 0.1% SDS for 30 min at 22 °C, followed by two washes with 0.1 x SSC and 0.1% SDS for 30 min at 50 °C. The filter was finally exposed to Kodak X-Omat film (Sigma) with intensifying screens for 1 week at -70 °C. Molecular weights were determined with the use of the 0.24-9.5-kilobase RNA Ladder (Bethesda Research Laboratories) stained with ethidium bromide and photographed under UV light.

RESULTS

Immunohistochemical staining of normal rat liver for TGF-β1 and IGF-II/Man-6-P receptor is shown in Fig. 1, A and B. The LC[1-30] antibody (Fig. 1A) demonstrated that hepatocytes in the liver are very slightly positive for TGF-β1 when compared with the IgG control (Fig. 1D). However, only the nonparenchymal periductular cells and the cells lining the sinusoids stained strongly positive with the pre[266-278] antibody that is specific for the pro region of the prepro-TGF-β1 molecule (Fig. 1C). This distribution of immunohistochemical staining is identical with the pattern of cells previously shown by in situ hybridization to express the TGF-β1 gene (32). Staining of normal liver for the IGF-II/Man-6-P receptor showed that it was present both intracellularly and in the extracellular membrane of hepatocytes. As previously shown, however, the hepatocytes in the pericentral region of the liver lobule had the greatest receptor concentration (Fig. 1B) (40). The nonparenchymal periductular cells also stained positive for the IGF-II/Man-6-P receptor.

In contrast to the low levels of mature TGF-β1 observed in normal liver with the LC[1-30] antibody, hepatocytes stained strongly positive with this antibody during liver regeneration. This increase in intracellular TGF-β1 was observed first in the periporal hepatocytes approximately 12 h after a two-thirds partial hepatectomy (Fig. 2A). Hepatocytes with high intracellular concentrations of mature TGF-β1 also contain the pro region of the prepro-TGF-β1 molecule since they stained positive with the pre[266-278] antibody (Fig. 2C). The intensity of staining for the pro region was, however, always less than that observed for the mature TGF-β1 molecule. This may result from the epitope that the pre[266-278] antibody recognizes being blocked upon binding to the IGF-II/Man-6-P receptor, whereas the mature TGF-β1 portion upon dissociation from the receptor complex is readily recognized by the LC[1-30] antibody. This intracellular increase in TGF-β1 was also found to be transient. By 30 h, the initially positive periporal hepatocytes no longer contained TGF-β1, whereas those in the midzonal region of the lobule were now positive (Fig. 3A). Fig. 3C shows a higher magnification of this midzonal region of a liver lobule. It is clear that hepatocytes in mitosis no longer contain TGF-β1, and we have shown previously that cells in the S phase of the cell cycle are also

**Fig. 1.** Immunohistochemical staining of control liver for TGF-β1 and the IGF-II/Man-6-P receptor. Mature TGF-β1 (A) and the IGF-II/Man-6-P receptors (B) were stained with the LC[1-30] antibody and C-1 antiserum, respectively. The pro region of the prepro-TGF-β1 molecule (C) was immunohistochemically localized with the pre[266-278] antibody. Nonimmune rabbit IgG and serum were used as negative controls. Both control sections were unstained, and only the rabbit IgG-stained section is shown (D). Portal spaces (P) and the central lobular region of the liver (CL) are marked. All sections were counterstained with hematoxylin. ×100.
negative for TGF-β1 (41). Finally, by 48 h, TGF-β1 had almost completely disappeared from hepatocytes, except in a few lobules where only the parenchymal cells adjacent to the terminal hepatic veins contained significant quantities of TGF-β1 (Fig. 4A). The LC[78–109] antibody that recognizes epitopes in the C-terminal portion of the mature TGF-β1 molecule produced a staining pattern identical with that seen with the LC[1–30] (results not shown). Thus, hepatocytes became transiently positive for TGF-β1 following a partial hepatectomy, and the lobular position of TGF-β1 positive hepatocytes changed, in a wavelike fashion, from the periporal to the pericentral region of the liver lobule in approximately 36 h. At all positions in the liver lobule, the hepatocytes replicated following the disappearance of the TGF-β1.

Further investigations demonstrated that the IGF-II/Man-6-P receptor was also increased in hepatocytes after a two-thirds partial hepatectomy. Northern blot analysis (Fig. 5) showed an increase in the steady state mRNA levels for the IGF-II/Man-6-P receptor that maximized between 12 and 24 h after a partial hepatectomy; by 48 h, it had almost returned to control levels. Equal loading of the samples was confirmed by photographing the ethidium bromide-stained RNA gel under UV light (data not shown). Of importance was the finding that the enhanced IGF-II/Man-6-P receptor expression (Figs. 2B, 3B, and 4B) co-localized both spatially and temporally in those hepatocytes that contained elevated levels of TGF-β1 (Figs. 2A, 3A, and 4A). These immunohistochemical results correlated well with the kinetics of IGF-II/Man-6-P receptor gene expression determined by Northern blot analysis. The spatial and temporal distribution of both the IGF-II/Man-6-P receptor and TGF-β1 in hepatocytes and their relationship to hepatocyte replication during liver regeneration are shown diagrammatically in Fig. 6.

**DISCUSSION**

In this report, we demonstrate for the first time that TGF-β1 is present transiently at high concentrations in hepatocytes, and that this temporary increase in TGF-β1 precedes hepatocyte replication. Interestingly, at later times (48 h) when TGF-β1 has been postulated to inhibit further hepatocyte proliferation (3, 6), the elevated concentration of TGF-β1 initially observed in hepatocytes has almost completely disappeared. A concomitant increase in the concentration of the IGF-II/Man-6-P receptor in those cells with elevated TGF-β1 suggests that the whole latent TGF-β1 phosphomannosyl glycoprotein complex may be endocytosed by the IGF-II/Man-6-P receptor and targeted to the acidic prelysosomal/endosomal compartments where the mature TGF-β1
molecule could be activated by dissociation from this complex. Further experimentation is required, however, to substantiate this postulate.

If the transient increase in intracellular TGF-β1 that we observed in hepatocytes during liver regeneration results from an increased uptake of the latent TGF-β1 complex through the IGF-II/Man-6-P receptor rather than to enhanced synthesis, we would expect that 1) as in normal adult hepatocytes, the TGF-β1 gene would remain untranscribed during liver regeneration and 2) either the rate of IGF-II/Man-6-P receptor internalization or the receptor concentration would be increased in those hepatocytes with elevated TGF-β1. Though fetal hepatocytes synthesize TGF-β1 (31), it has been shown to not be produced by adult hepatocytes (32). Furthermore, during liver regeneration, TGF-β1 gene transcription has been found to be induced only in the nonparenchymal cells, not in the hepatocytes (6, 31, 33); we have obtained similar results in this study (results not shown). Therefore, we postulated that the increased intracellular TGF-β1 observed in hepato-
cytes during liver regeneration resulted from augmented uptake through the IGF-II/Man-6-P receptor, not through increased synthesis.

IGF-II/Man-6-P receptor concentration and the steady state level of IGF-II/Man-6-P receptor mRNA have both been demonstrated to be significantly increased following a two-thirds partial hepatectomy (40, 42-44). We are the first, however, to demonstrate that the IGF-II/Man-6-P receptor expression is also modulated spatially in the liver lobule and that an elevation in hepatocyte IGF-II/Man-6-P receptors is highly correlated with an increased intracellular TGF-β1 concentration. The time at which the IGF-II/Man-6-P receptor up-regulation peaks has been reported to range between 24 and 48 h (43, 44). Neither of these studies investigated times earlier than 24 h, and our results demonstrate that the steady state mRNA level for IGF-II/Man-6-P receptor may actually peak somewhere between 12 and 24 h. The reason for the differences in the kinetics of the up-regulation of the IGF-II/Man-6-P receptor gene in these various studies is not known. Different rat strains were used in these experiments, and in this study and that of Burguera et al. (43), where male rats were used, the time of maximum gene expression was less than that observed by Scott et al. (44), who used female rats.

Regardless of the reason for these observed differences in the kinetics of IGF-II/Man-6-P receptor expression, it is clear that the IGF-II/Man-6-P receptor level is modulated both temporally and spatially during liver regeneration, suggesting that it plays a necessary role in this process.

Since the growth factor, IGF-II, is a ligand for the IGF-II/Man-6-P receptor, an increase in receptor number may serve to enhance the probability of hepatocyte replication. IGF-II has been proposed to act as a mitogen during fetal development, since its level is high in fetal and neonatal rats (45), and in mice containing an inactivated IGF-II gene, embryonic growth is retarded (46). High levels of IGF-II are also found in hepatocellular carcinomas of woodchucks infected with hepatitis virus, suggesting that it may be involved in liver tumor growth (47). The role of IGF-II in liver regeneration is, however, less clear, since the expression of IGF-II in the liver (6) and the plasma concentration (42) do not change significantly during liver regeneration. The results of Scott and Baxter (42), however, clearly show that IGF-II, though not a complete hepatocyte mitogen, is a potent co-mitogen. Furthermore, its ability to enhance epidermal growth factor-induced DNA synthesis is significantly augmented in hepatocytes from partially hepatectomized animals. This enhanced ability of IGF-II to act as a co-mitogen for regenerating hepatocytes may result from the increased number of IGF-II/Man-6-P receptors present in these cells. The IGF-II/Man-6-P receptor is, however, only transiently increased in hepatocytes (Fig. 6). Therefore, by binding IGF-II, the IGF-II/Man-6-P receptor may play an important role in controlling both the spatial and temporal distribution of hepatocyte proliferation. The initial signal to increase IGF-II/Man-6-P receptor synthesis during liver regeneration is unknown, but it must occur soon after a partial hepatectomy. A co-mitogenic substance, such as norepinephrine or hepatocyte growth factor, may be involved in this process. Alternatively, the latent TGF-β1 complex could be endocytosed after binding to the IGF-II/Man-6-P receptor and targeted to the acidic prelysosomal/endosomal compartments where, rather than being degraded in lysosomes, it is activated and subsequently released by a nondegradative retroendocytotic pathway (50). If this novel mechanism for activating TGF-β1 exists in hepatocytes, it predicts the intriguing possibility that activated TGF-β1 may be present transiently 3-6 h prior to the initiation of hepatocyte DNA synthesis.
Whether this putatively activated TGF-β1 would then be involved in regulating the local synthesis of growth factors and extracellular matrices (9, 23) or in maintaining hepatocytes at a restriction point in G1 (51) remains to be determined. This latter possibility is, however, particularly interesting, since the continuous exposure of cultured keratinocytes to mature TGF-β1 reversibly inhibits their growth, with the majority of the cells blocked in the G1 phase of the cell cycle (52). Furthermore, the addition of TGF-β1 in mid to late G1 prevents the phosphorylation of the tumor suppressor Rb gene product scheduled for this cell cycle stage (53) and arrests cells at this point in the cell cycle (54), possibly by blocking c-myc expression (55). If in regenerating hepatocytes the phosphorylation state of the Rb gene or other tumor suppressor genes like p53 are similarly cell cycle-regulated by TGF-β1, the function of a transient increase in the concentration of this growth inhibitor during late G1 would become more apparent. Though additional research will be required to discriminate between these various paradigms, these data suggest that the function of TGF-β1 in liver regeneration may be more complex than originally thought.

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