Role of Growth Hormone (GH) in Liver Regeneration

PATRICIA A. PENNISI, JOHN J. KOPCHICK, SNORRI THORGEIRSSON, DEREK LEYROITH, AND SHOSHANA YAKAR

Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases (P.A.P., D.L., S.Y.), and Laboratory of Experimental Carcinogenesis, Center of Cancer Research, National Cancer Institute (S.T.), National Institutes of Health, Bethesda, Maryland 20892-1758; and Edison Biotechnology Institute, Ohio University College of Osteopathic Medicine, Ohio University (J.J.K.), Athens, Ohio 45701

Liver regeneration is a fundamental mechanism by which the liver responds to injury. This process is regulated by endogenous growth factors and cytokines, and it involves proliferation of all mature cells that exist within the intact organ. To understand the role of the GH/IGF-I axis in liver regeneration, we performed partial hepatectomies in three groups of mice: GH antagonist (GHa) transgenic mice, in which the action of GH is blocked; liver IGF-I-deficient mice that lack IGF-I specifically in the liver and also lack the acid-labile subunit (ALS; LIĐ+ALS KO mice), in which IGF-I levels are very low and GH secretion is increased; and control mice. Interestingly, the survival rate of GH transgenic mice was dramatically reduced after partial hepatectomy (57%) compared with the survival rate of controls (100%) or LIĐ+ALS KO mice (88%). In control mice, the liver was completely regenerated after 4 d, whereas liver regeneration required 7 d in LIĐ+ALS KO mice. In contrast, in GHa mice, liver regeneration reached only 70% of the original liver mass after 4 d and did not improve thereafter. Strikingly, 36 and 48 h after hepatectomy, the livers of control and LIĐ+ALS KO mice, respectively, exhibited intense 5-bromo-2′-deoxyuridine (BrdU) staining, whereas BrdU staining was dramatically decreased in the livers of GHa-treated mice. These results suggest that GH plays a critical role in liver regeneration, although whether it acts directly or indirectly remains to be determined. (Endocrinology 145: 4748–4755, 2004)

It has been well established that the liver can regenerate after resection. Liver regeneration occurs via a complex process that includes multiple signals and sequences of events. These signals drive hepatocytes from the quiescent G0 state into the G1 phase of the cell cycle and then through the restriction points of G1 into the S phase, where commitment to division has been reached (1–3). Liver regeneration may be initiated by the activation of one or more cytokine receptors, whereas growth factors usually act later on the already primed hepatocytes (4, 5). TNFα and IL-6 are thought to play major roles in initiating the process of liver regeneration. Mice lacking TNF receptor type 1 (TNFR-1) exhibited severely impaired liver regeneration after partial hepatectomy (6). This was found to be due to a defect in DNA synthesis and could be corrected by treatment with IL-6 (6). Similarly, IL-6 knockout mice showed impaired liver regeneration characterized by liver necrosis, liver failure, and a blunted DNA synthetic response in hepatocytes (7). Hepatocyte growth factor and TGFα are potent hepatic mitogens in vitro that are highly expressed after hepatectomy (4, 8, 9). In addition, epidermal growth factor (EGF) is a primary mitogen for hepatocytes in culture, and its expression increases after liver resection (5, 10). After partial hepatectomy, the activation of cytokine and growth factor signaling pathways leads to the induction of transcription factor complexes such as nuclear factor-κB, c-Myc, signal transducer and activator of transcription-3 (STAT3), cAMP-responsive element modulator, and activating protein-1 (1, 4–9). Mice with conditional knockouts of either STAT3 (11) or c-jun (12), specifically in the liver, exhibited impaired DNA synthesis, but liver regeneration still occurred.

GH is a member of the cytokine superfamily of polypeptide regulators (13). The growth-promoting effects of GH can be direct in selected target tissues, such as liver, or indirectly, via its endocrine mediator IGF-I. GH is the primary regulator of IGF-I synthesis and secretion in hepatocytes; in turn, IGF-I regulates GH secretion through a classical negative feedback loop (14, 15). In the circulation, IGF-I is bound to specific IGF-binding proteins (IGFBPs). Approximately 70–80% of the circulating IGF-I is found within a large ternary complex composed of the acid-labile subunit (ALS) and IGFBP-3 (16). A smaller proportion (15–20%) circulates as a binary complex with other IGFBPs, and less than 5% of IGF-I circulates in the free form (16, 17).

Despite the fact that the liver is the main source of circulating IGF-I and IGFBPs, hepatocytes have not been considered to be a primary target for IGF-I, because they do not express the IGF-I receptor (18). However, nonparenchymal cells within the liver, such as Kupffer cells and hepatic stellate cells, do express the IGF-I receptor and respond to IGF-I stimulation (19, 20), suggesting that IGF-I can act in a paracrine fashion in the liver. Interestingly, IGFBP-1 was shown to be one of the most rapidly induced and highly expressed proteins in regenerating liver (21). Mice that lacked IGFBP-1 exhibited normal development, but showed abnormal liver regeneration.
regeneration after partial hepatectomy, characterized by liver necrosis and reduced and delayed hepatocyte DNA synthesis (22, 23).

Recent evidence shows that GH can regulate EGF receptor (EGFR) expression in the liver as well as suppressors of cytokine signaling (SOCS) and glycoprotein 130 gene expression (24). It has also been shown that GH can stimulate tyrosine phosphorylation of the EGFR in cultured hepatocytes (25–27), raising the possibility that cross-talk between the GH receptor (GHR) and EGFR could also be important in liver regeneration.

The aim of our study was to investigate the role of GH (in the presence of reduced IGF-I levels) in liver regeneration. To that end we used three different mouse models. The first group included liver IGF-I-deficient (LID) mice that also lack the ALS (LID + ALSKO mice). The LID + ALSKO mice have very low levels of circulating IGF-I and markedly elevated GH levels, which correlate with increased liver weight (28). The second group included GH antagonist transgenic mice (GHa mice), which have low levels of circulating IGF-I and no detectable GH activity (29). Due to their low levels of GH activity, these mice show growth retardation and reduced liver weight (30). The third group, control mice, had normal circulating GH and IGF-I levels.

Here we demonstrate that GHa mice, which lack GH activity, show a retarded response to hepatectomy and are not able to restore liver mass even 7 d after partial hepatectomy. Taken together, our results suggest that GH plays a major role in the process of liver regeneration.

Materials and Methods

Animal husbandry and genotyping

The generation and genotyping of LID + ALSKO mice (with a mixed genetic background of FVB/N, C57Black, and 129sv) (28, 31) and GHa mice (also FVB/N, C57Black, and 129sv background) (29) has been described previously. All procedures that were used were approved by the animal care and use committee of the NIDDK, NIH (Bethesda, MD). Genotyping and breeding of mice were carried out at the NIH animal facility as previously described (28, 29, 31).

Partial hepatectomy

Male mice (6–8 wk old) were anesthetized with an ip injection of sodium phenobarbital (40 mg/kg). The abdominal area of the mice was shaved, and incisions of 2 cm were made. A partial hepatectomy was performed, in which approximately 70% or 30% of the liver was removed. The left lateral and median lobes of the liver were ligated before removal to prevent bleeding, as described previously (32). These lobes were removed intact, without damaging the remaining lobes. The remaining lobes were gently returned to the abdominal cavity. The incision was closed in two layers. The muscle layer was closed with catgut, and the skin was closed with stainless steel wound clips. The wound clips were removed 7 d later. The body weights of the mice were determined before and after surgery and during the follow-up period. Surgeries were performed in all mice at the same time of the day (during the morning hours). Mice were killed at the indicated time points, and livers were removed for histological analysis and isolation of RNA and protein preparations. Surgically removed liver lobes and the regenerating livers were weighed, and the relative percentage of body weight and the percentage of the corresponding original liver mass were calculated. To obtain dry weight data, three small pieces taken randomly from the removed liver lobes or regenerating livers were weighed and exhaustively lyophilized. Dried specimens were weighed again, and the dry/wet weight ratio was calculated.

Solution hybridization/ribonuclease (RNase) protection assay (RPA)

Mice were injected with a solution of BrdU (Amersham Biosciences, Piscataway, NJ; 100 mg/kg body weight) and were killed 2 h later. The remaining livers and portions of the gut were prepared and fixed in 4% paraformaldehyde for 14–16 h. For detection of incorporated BrdU, 5-μm sections from liver and gut (as an internal control), were processed according to the manufacturer’s instructions (Amersham Biosciences). On adjacent sections from the same liver samples, apoptotic cells were detected by the TUNEL assay with the ApopTag Plus peroxidase kit, according to the manufacturer’s instructions (Intergen, Purchase, NY). Mammary gland at an involutionary stage was used as an internal positive control. Hepatocytes were counted (between 1500 and 2000) from three different animals per group at each time point, and the average percentages of TUNEL-positive cells were calculated.

Statistical analysis

The experimental data were analyzed by t test, χ² test, and one-way ANOVA with Tukey’s test for additional comparisons when appropriate. P < 0.05 was considered statistically significant. Results are expressed as the mean ± sem, except when indicated.

Results

Increased mortality in GHa mice after partial hepatectomy

Partial hepatectomies (removal of ~70% of the liver) were performed in control, LID + ALSKO, and GHa male mice. GHa mice, which have low levels of circulating IGF-I and blunted GH activity, showed a dramatic increase in mortality after partial hepatectomy compared with control mice (P < 0.05, GHa vs. control; Table 1). In contrast, the mortality of LID + ALSKO mice, which exhibit low levels of circulating IGF-I, but increased levels of GH, did not differ significantly from that of control mice [P = not significant (NS), LID + ALSKO vs. control; Table 1]. To rule out the possibility that the general stress of the surgery contributed to the increased mortality, we performed partial hepatectomies in which only approximately 30% of the liver mass was removed in control and GHa mice. All mice that underwent removal of only 30% of the liver mass were able to recover completely during the first 12 h after surgery, demonstrating that neither the surgical procedure nor the anesthetic is the cause of premature death. Accordingly, the number of mice that died during surgery was the same in the groups, suggesting that there were no differences in the response to anesthesia between the groups.
Defective liver regeneration in the absence of GH activity

The vast majority of deaths in the GHa group occurred during the first 48 h after hepatectomy (Table 1), with only 60% of the mice surviving until 72 h after the surgery. A much smaller percentage of LID + ALSKO mice died during the first 48 h, and no control mice died after surgery. These results suggest that GHa mice were less able to overcome the loss of liver mass after partial hepatectomy.

**Defective liver regeneration in the absence of GH activity**

We next determined whether the reason for the premature death of GHa mice was the lack of their ability to regenerate liver mass. To do this, mice were killed at different time points after partial hepatectomy over a period of 7–10 d, and total body weight was measured before death. At death, the remaining liver lobes were harvested, and wet and dry weights were measured. The dry/wet weight ratios were calculated (see Materials and Methods) before and after surgery. There were no significant differences in the ratios between the groups before or after hepatectomy (0.263 ± 0.039 (control) vs. 0.263 ± 0.056 (LID + ALSKO), P = NS; 0.263 ± 0.039 (control) vs. 0.267 ± 0.025 (GHa), P = NS; 0.263 ± 0.039 (control) vs. 0.276 ± 0.035 (C after surgery), P = NS; 0.267 ± 0.025 (GHa) vs. 0.227 ± 0.040 (GHa after surgery), P = NS). Thus, wet weights of lobes or regenerating livers were used for additional calculations. Because the three groups of animals started with different absolute and relative liver masses (28, 31, 33), the mass of the liver regenerated during the study was calculated as a percentage of the average total liver mass of the nonoperated mice. As shown in Fig. 1A, control mice completely regenerated their original liver mass (5.0% of total body weight) after 4 d. LID + ALSKO mice were able to restore their original liver mass after 7 d (6.1% of total body weight). In contrast, GHa mice recovered only 70% of their original liver mass (4.6% of total body weight) after 4 d, and the original liver mass was not reached until the end of the study (P < 0.05, control vs. GHa). Control and GHa mice did not exhibit any significant changes in total body weight over a course of 7 d (Fig. 1B). However, LID + ALSKO mice showed an initial decrease in body weight (24 h after hepatectomy) (P < 0.05, by one-way ANOVA; highest significant difference, 1.43, by Tukey’s test), with no further reductions during the study.

**GHR, IGF-IR, and IGF-I gene expression before and after hepatectomy**

Because the mRNA changes in GHR and IGF-I correlate with GH action in the liver (34), we measured GHR, IGF-I, and IGF-IR expression with specific probes using the RPA as described previously (33, 35). GHR and IGF-I mRNA levels were expressed as the ratio of mRNA levels at various time points over the levels before surgery (basal). As shown in Fig. 2B, all three groups of mice exhibited a significant decrease in GHR and IGF-I mRNA levels during the first 24 h after partial hepatectomy compared with basal levels (P < 0.05, basal vs. d 1 control, GHa, and LID + ALSKO for GHR mRNA). In control and LID + ALSKO mice, GHR levels returned to basal expression levels 4 d after surgery, whereas GHa mice required 7 d to recover the original levels of GHR expression.

A similar decrease in IGF-I mRNA levels (Fig. 2C) was

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**TABLE 1. Survival rate and timing of death after partial hepatectomy**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of mice</th>
<th>% Surviving after surgery</th>
<th>No. of mice that died during hepatectomy</th>
<th>No. of mice that died after surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>100</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>LID + ALSKO</td>
<td>36</td>
<td>88*</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>GHa</td>
<td>26</td>
<td>57*</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Mice were monitored for up to 7–10 d after surgery. The survival rate was calculated as the percentage of animals that were able to recover from the procedure and survive until the time of death.

*aP = NS, 29/33 (LID + ALSKO) vs. 22/22 (control), by χ² test.

*bP < 0.05, 13/23 (GHa) vs. 22/22 (control), by χ² test; P < 0.05, 13/23 (GHa) vs. 29/33 (LID + ALSKO), by χ² test.

**FIG. 1. Liver mass regeneration.** Mice were subjected to partial hepatectomy (Hpx) and were killed 1, 4, or 7 d later, as described in Materials and Methods. A, Regenerating livers were harvested, weighed, and processed as described. Regeneration of liver mass was calculated as the percentage of the average liver mass of mice that did not undergo surgery in control ( ), LID + ALSKO ( ), and GHa ( ) animals at the indicated times. **, P < 0.05, GHa vs. control. B, Total body weight of the mice before and after surgery. Significant differences in body weight between the groups were observed at the beginning and end of the study. *, P < 0.05, LID + ALSKO vs. control; **, P < 0.05, GHa vs. control; #, P < 0.05, LID + ALSKO vs. GHa. No changes in body weight were observed in control and GHa mice during the days following partial hepatectomy (P = NS, by one-way ANOVA). Statistically significant changes were observed in LID + ALSKO mice on d 1, with no further reductions during the study (P < 0.05, by one-way ANOVA; *, P < 0.05, d 0 vs. d1, d4, d 5, d 7). Results are expressed as the mean ± SEM of at least three mice per time point.
observed 1 d after partial hepatectomy in control mice (P = 0.05, basal vs. d 1 in control and GHa), and their IGF-I expression levels returned to normal 4 d after surgery. In contrast, GHa mice, which express low levels of IGF-I due to blunted GH activity (40–50% of control), did not regain their original levels of IGF-I mRNA at any time point during the follow-up period after partial hepatectomy. LID+ALSKO mice lack IGF-I expression in the liver, because exon 4 of the IGF-I gene is specifically deleted in the livers of these mice.

IGF-IR mRNA was not detectable in the livers of any of the mice before surgery or after partial hepatectomy at any time during the study (Fig. 2A).

**Necrosis without apoptosis in livers of GHa mice after partial hepatectomy**

Normal liver architecture was observed in control, LID+ALSKO, and GHa mice in the basal state (data not shown). One day after surgery, little or no necrotic areas were observed in control (Fig. 3A, left panel) or LID+ALSKO (middle panel) mice. However, necrotic areas were apparent in the livers of most of the GHa mice (right panel, areas indicated by arrows) 24 h after surgery. On d 4, no necrosis was detected in the livers of control, LID+ALSKO, or GHa mice (Fig. 3B, left, middle, and right panels, respectively).

A TUNEL assay was carried out to test the possibility that an increase in the apoptotic index could contribute to the lack of liver regeneration in GHa mice. There was no difference in the apoptotic index between the groups at any given time point (Fig. 4C), suggesting that the lack of regeneration in the liver of GHa mice was not caused by increased apoptosis.

**Reduced BrdU incorporation in the livers of GHa mice**

Because neither the extent of necrosis nor the low levels of apoptosis could explain the lack of complete liver regeneration in GHa mice, we decided to test whether there was a defect in the proliferative response of hepatocytes in GHa mice. Several studies have shown that after partial hepatectomy in mice, DNA synthesis remains very low until 30–32 h after surgery, when it starts to increase, and DNA synthesis reaches a maximum after 36–40 h (1, 36). BrdU incorporation was used to determine the number of cells in S phase at several time points after surgery in control, LID+ALSKO, and GHa mice. No BrdU staining was detected in the livers of any of the mice before surgery, which is consistent with
hepatocytes being in the quiescent stage (1). BrdU incorporation was similar in control and LID/H11001 ALSKO mice (P/H11005 NS), and the peak was reached after 36 h in control mice and after 48 h in LID/H11001 ALSKO mice (Fig. 4A, left and middle panels, respectively). In contrast, there was dramatically less BrdU incorporation in GHa mice. Furthermore, maximal levels of BrdU incorporation in GHa mice (at 48 h) were 15% and significantly lower (P < 0.03 vs. control) than the maximal levels of BrdU incorporation observed in control mice (66%, at 36 h; P < 0.005 vs. basal; Fig. 4A, right panel). Portions of the gut from each animal were processed as internal controls for the entire procedure. The quantitation and kinetics of BrdU incorporation are shown in Fig. 4B. Although maximal BrdU incorporation was delayed in LID+ALSKO mice compared with control mice, this maximum was followed by a sharp decrease, and both groups exhibited similar low levels of BrdU incorporation by 72 h. Like LID+ALSKO mice, GHa mice exhibited a maximum incorporation at 48 h. However, in GHa mice this low level of incorporation did not decrease, and was sustained at least until 72 h post surgery. Calculation of the area under the curve (see inset in Fig. 4B) was similar in control and LID+ALSKO mice [13.8 (control) and 13.6 (LID+ALSKO)], but was reduced approximately 25% in GHa mice [13.8 (control) vs. 10.5 (GHa)]. Thus, a low level of sustained cellular proliferation occurs in GHa mice at a time when neither control nor LID+ALSKO mice exhibit such activity, presumably because these mice have completed the process of liver regeneration. Taken together, the BrdU experiment indicates a prolonged G0/G1 or G1/S transition in the LID+ALSKO mice and essentially very low levels of DNA synthesis in GHa mice.

**Discussion**

The loss of functional liver mass by surgical resection or hepatocyte loss caused by viral or chemical injury triggers a rapid proliferative response in these liver cells, which are normally quiescent (1). In the present study we show that GHa mice, which lack GH activity and have reduced levels of IGF-I (40% of control levels) (33), have impaired liver regeneration and increased mortality. In contrast, LID/H11001 ALSKO mice, which also have reduced levels of IGF-I (15% of control levels) (28, 35), but have approximately 15-fold increased levels of GH, can successfully recover from partial hepatectomy and are able to restore the original liver mass during the first week after surgery. In both groups LID/H11001 ALSKO and GHa mice, the peak of BrdU staining is retarded and appears 12 h later than in controls, suggesting that serum IGF-I levels may play a role in the events immediately following hepatectomy and are able to restore the original liver mass during the first week after surgery. In both groups LID+ALSKO and GHa mice, the peak of BrdU staining is retarded and appears 12 h later than in controls, suggesting that serum IGF-I levels may play a role in the events immediately following hepatectomy. Previous studies have not been able to confirm the up-regulation of IGF-IR expression in the liver after partial hepatectomy (37). In the present study using RPA, IGF-IR mRNA before or after partial hepatectomy could not be detected in any of the groups. Although we cannot exclude the possibility that IGF-IR is expressed in other cell types in the liver, our results suggest that GH plays a main role in liver regeneration.
regeneration of these three models and that IGF-I might have a minor impact in this process.

As the liver is a vital organ, the inability to regenerate the liver mass would be expected to prevent recovery after surgery. Therefore, it was important to evaluate the mortality rates of the mice that underwent partial hepatectomy. During the first 48 h after surgery, 100% of control mice and 88% of LID mice died during the first 48 h after surgery. A similar temporal pattern and a high percentage of mortality (~50%) were reported in at least three other knockout animal models associated with impaired liver regeneration: IL-6+/− mice (7), TNFR-1+/− mice (6), and mice with a conditional liver-specific knockout of c-jun (c-jun−/− mice) (12). Remarkably, IL-6, TNF-α, and c-jun have all been identified as key regulatory factors in the proliferation and survival of hepatocytes during liver regeneration. TNFR-1+/− mice exhibited some distortion of the liver architecture and a decreased ratio of liver to body weight that persisted even 14 d after surgery (6). Both IL-6+/− mice and c-jun−/− mice showed large areas of necrosis 36–72 h after surgery, but in both cases the mice that survived reversed those lesions (7, 12). In the current study necrotic foci were observed only in GHa mice 24 h after surgery. These lesions were not seen at later time points; thus, it is possible that although liver regeneration is impaired in GHa mice, they are still able to resolve necrotic lesions.

In the rat after partial hepatectomy of 70%, the original liver mass is completely restored within 7–10 d (1, 9). Even though the original shape of the liver is not recovered, the cellular architecture ultimately appears normal. In the present study the original liver mass was restored 4 d after surgery in control mice. As expected, normal cellular architecture was achieved by the end of the study, indicating that in control mice, 7 d were sufficient to complete the process. LID+ALSKO mice recovered both their original liver mass and cellular architecture 7 d after surgery. LID+ALSKO mice have an increased liver weight compared with controls, most likely due to the excess GH in the circulation. It has been well documented that liver size is proportional to body size, and that signals from the body can both positively and negatively regulate liver mass until the appropriate size is reached (38). In addition, studies demonstrated that GH affects liver mass (39–41). Therefore, it is possible that the delay observed in reaching the original liver mass in LID+ALSKO mice is the consequence of the higher original mass that needed to be restored in these mice.

The gain in liver weight after partial hepatectomy exhibited by the three groups of mice in this study was mainly the
of hepatocytes is the earliest event of the regenerative process, the first all-
that have been shown to be regulated by GH are involved in GHR expression has been restored. Because most of the genes
in the subsequent different responses exhibited by the three groups. Therefore, if GH is having an effect on the ability of
this decrease in food intake during the first hours of recovery from surgery. Because LID+ALSKO mice lack IGF-I expression in the liver, no IGF-I mRNA was detected in the livers of these mice at any time.

The role of GH in liver regeneration is not yet fully defined. Our findings suggest that the effect of GH on liver regeneration is not mediated through IGF-I, because both LID+ALSKO and GHa mice exhibit very low levels of circulating IGF-I. GH may affect several signaling pathways involved in this regenerative process. It could exert its effect at the transcriptional level by regulating the expression of receptors or transcription factors or by modulating the activity of receptors other than the GHR. Additional work is needed to delineate the mechanisms by which GH regulates the process of liver regeneration.

Acknowledgments

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Address all correspondence and requests for reprints to: Dr. Shoshana Yakar, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Room 8D12, Building 10, National Institutes of Health, Bethesda, Maryland 20892-1738. E-mail: shoshanay@intra.niddk.nih.gov.

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