Increased insulin-like growth factor binding protein-4 expression after partial hepatectomy in the rat

ILARIA DEMORI, SARA BALOCCHO, ADRIANA VOCI, AND EMILIA FUGASSA
Istituto di Fisiologia Generale, Università degli Studi di Genova, I-16132 Genoa, Italy

THE INSULIN-LIKE GROWTH FACTORS (IGF-I and -II) are mitogenic peptides that promote the growth and differentiation of tissues (13). The availability of IGFs to interact with their specific receptors and trigger biological responses is regulated by the levels of high-affinity binding proteins (IGFBPs), a family of bifunctional adaptor proteins that specifically bind the IGFs and interact with the cell surface, extracellular matrix, or other protein components. The IGFBPs regulate cell growth by modulation of the bioactivity of IGFs and by mechanisms independent of their effects on IGF availability. The abundance of IGFBPs is regulated by hormonal, nutritional, and developmental factors that act at the gene expression or protein synthesis level as well as through specific proteases. This complex mechanism may, in turn, regulate IGF activity (19, 26).

Although the liver is the main site of biosynthesis for IGF-I and some IGFBPs, the role played by the IGF-IGFBP system in the regulation of hepatic growth is not obvious. Liver regeneration after partial hepatectomy (PHx) has been used as a model in experimental animals to study the response of the hepatic IGF-IGFBP system to internal changes and to investigate the possible role of IGFs and IGFBPs in the regeneration process. In fact, it has been demonstrated that, unlike normal rat hepatocytes, cells isolated from regenerating rat liver and proliferating HepG2 cells have high levels of IGF binding sites (4). Nonparenchymal cells such as Kupffer, Ito, and endothelial cells possess the type I IGF receptor (3, 37), and recent evidence supports a role for IGF-I in regulating the proliferation of nonparenchymal cells within the liver (1, 15, 16, 30). A role for the IGF-II receptor in liver regeneration has been suggested as well, because its levels appear to be increased in hepatocytes isolated from hepatotomized rats, which are more sensitive to IGF-II for the stimulation of DNA synthesis (31, 32). However, a rise in IGF-II production by the regenerating liver after PHx has not been observed (31), and several reports demonstrate a decrease in circulating IGF-I levels in PHx animals (28, 31, 36).

Concerning IGFBPs, it is well established that the IGFBP-1 gene is one of the most highly expressed liver-specific immediate-early genes, because it is rapidly and dramatically induced in rat liver soon after PHx (14, 23). Less is known about the effect of PHx on the other IGFBPs of hepatic origin. Ghahary et al. (14) observed a decline in hepatic IGFBP-3 mRNA and serum IGFBP-3 levels both in hepatotomized and sham-operated animals, which is likely caused by changes in nutrient intake and reduced pituitary growth hormone secretion. No data are available as yet about the effect of PHx on IGFBP-4, the second most abundant IGFBP in adult rat serum after IGFBP-3. IGFBP-4 is synthesized mainly in the liver and exhibits a high affinity for IGF-I and -II that is nearly equal to that of IGFBP-3 (9). In this work we investigated the expression of hepatic IGFBP in rats subjected to two-thirds hepatectomy and demonstrated an increase in IGFBP-4 expression during liver regeneration.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (250-300 g) were housed under conditions of controlled temperature and light. The animals were anesthetized by intraperitoneal injection of Ketalar (Parke-Davis, Barcelona, Spain) at the dose of 15 mg/100 g body wt and subjected to PHx or sham hepatectomy (SHx) between 800 and 1200. For PHx, the left lateral and median lobes (~2/3 of total liver mass) were ligated and excised through a median incision (17). This tissue was used...
as the zero time control. SHx animals underwent surgery and manipulation of the liver but no tissue resection. After surgery, the rats were warmed under a heat lamp until they regained consciousness and were allowed free access to water. Food was provided daily at 1800. To control for the animals’ nutritional status, in additional experiments SHx rats were fed the average intake of PHx animals, but no significant differences were seen in respect to freely feeding animals.

Rats were killed by cervical dislocation 3, 6, 9, 12, 24, 48, 72, and 96 h after surgery. Blood was collected and allowed to clot, and serum was stored at −80°C. Serum triiodothyronine (T₃) and thyroxine (T₄) were evaluated by an enhanced chemiluminescence (ECL) enzyme immunoassay (Diagnostic Products Corporation, Los Angeles, CA). The detection limit of the assay was 0.35 ng/ml for T₃ and 4 ng/ml for T₄.

Immediately after death, both liver and kidneys were rapidly dissected, quickly frozen in liquid nitrogen, and stored at −80°C until used. Kidneys from age-matched rats that did not undergo surgery were used as controls.

RNA isolation. Total RNA was prepared using acid guanidium thiocyanate-phenol-chloroform extraction (7). RNA was quantified by measuring absorbance at 260 nm. Samples were stored at −80°C until used.

CDNA probes. The rat IGFBP-1, -2, -3 and -4 CDNA probes were kindly provided by Dr. S. Shimasaki (Department of Cell Biology, Scripps Research Institute, La Jolla, CA). The rat 18S rDNA probe (BamHI-I-Hind III fragment; Ref. 5) was generously provided by Dr. I. G. Wool (Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL). The probes were labeled to high specific radioactivity by multiprime DNA labeling systems (Amersham International, Amersham, UK) using [3²P]dCTP (Du Pont NEN, Bad Homburg, Germany; specific radioactivity 3,000 Ci/mmol).

Northern blot analysis. Total RNA (20 µg) was subjected to electrophoresis on 1% agarose gel containing 2.2 M formaldehyde in 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM disodium EDTA. RNA was stained with ethidium bromide to check the integrity of the samples and to ensure equal loading in each lane. After transfer to nylon membrane by capillary blotting, prehybridization was performed for 2 h at 42°C in a solution containing 5× SSPE (1× SSPE = 0.15 M NaCl-0.01 M NaH₂PO₄-1 mM EDTA), 50% formamide, 5× Denhardt’s solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA (29). Hybridization was carried out for 24 h at 42°C in fresh buffer containing the [3²P]-labeled probe. After hybridization, the filters were washed sequentially in 2× SSPE-0.1% SDS, 1× SSPE-0.1% SDS, and 0.1× SSPE-0.1% SDS at 42°C, with each wash lasting for 20 min. Filters were then exposed to Amersham Hyperfilm-MP films at −80°C using intensifying screens.

To ensure even loading, the membranes were stripped and rehybridized with the rat 18S rDNA probe. The signal intensity of the autoradiograms was quantified densitometrically using the Scion Image PC software package. Arbitrary densitometric units were normalized to 18S and expressed as a percentage of the control values.

Measurement of serum IGF-I. Serum IGF-I was measured by RIA (Medgenix Diagnostics, Fleurs, Belgium) after acid-ethanol extraction according to Daughaday et al. (10). Tissue extraction for IGFBP’s. Liver tissue was homogenized in 20 mM Tris-2% Triton X-100 buffer (pH 7.4), and IGFBP’s were extracted following the method of Flyvbjerg et al. (12). Aliquots of extracts were stored at −80°C. Protein content of the extracts was measured using the biocinchonic acid protein assay (Pierce Chemical, Rockford, IL).

Ligand blotting and immunoblotting. For ligand blotting, sodium [125I]iodide (carrier free) was purchased from American.
PHx. The results are depicted in Fig. 1. The immediate-early increase in IGFBP-1 mRNA abundance during liver regeneration is in total agreement with data reported by others (14, 23). Hybridization of liver RNA with IGFBP-3 cDNA revealed a faint signal that was not significantly affected by PHx. In contrast, hepatic IGFBP-4 expression was markedly increased within 6 h, peaked at 9–12 h, and began to decrease at 24 h after PHx. As shown in Fig. 2, no significant increase in the abundance of hepatic IGFBP-4 mRNA was observed after SHx. IGFBP-2 transcript was not detected in the liver of intact rats and remained undetectable after PHx (data not shown).

Effect of PHx on renal IGFBP-4 mRNA levels. PHx also influenced kidney IGFBP-4 mRNA expression similarly to that observed in regenerating liver. As shown in Fig. 3, renal IGFBP-4 mRNA levels increased within 3 h, peaked at ~9 h, and returned to control values at 24 h after PHx. No significant change in renal IGFBP-4 mRNA expression was seen in the SHx rats (data not shown).

Effect of PHx on serum IGF-I and IGFBP levels. Serum IGF-I levels after PHx are shown in Table 2. Our data are in line with previous results by others reporting a decrease in serum IGF-I levels and activity after PHx (28, 31, 35, 36). In our experiments, a decline in circulating IGF-I was evident from 12 h after liver resection. Serum IGF-I levels were ~60% of normal at 24 h after surgery and returned to control values by 72 h after PHx.

The effect of PHx on circulating IGFBPs was studied by ligand blot analysis of sera collected from PHx rats. Autoradiograms revealed the presence of three different IGFBPs with molecular masses of ~41–45, 30, and 24 kDa, which were immunologically identified by antisera against rat IGFBP-3, -1, and -4, respectively (Fig. 4).

Figure 5 shows the time course of serum IGFBPs after PHx. The abundance of IGFBP-3 was decreased within 9–12 h after PHx and returned to control levels thereafter. A similar pattern was observed for the 30-kDa IGFBP-1. In contrast, as quantified in Fig. 5B, serum IGFBP-4 levels of PHx rats began to increase 12 h after surgery and remained high to the end of the observation period.

Effect of PHx on liver IGFBP levels. Figure 6 is a representative autoradiogram of ligand blot performed on liver extracts. IGFBPs with molecular masses similar to those observed in serum were detected. A depletion of liver IGFBP content was observed within 24–48 h after PHx, followed by a progressive return to control levels.
DISCUSSION

In this work, the expression of IGFBPs in the regenerating liver was investigated. Classic reports demonstrated that IGFBP-1 is an immediate-early gene, whose expression is rapidly and dramatically increased after 70% PHx (14, 23). In our experiments this pattern was confirmed. However, serum IGFBP-1 levels appeared to be decreased during the first day of liver regeneration, returning to control values thereafter. Similarly, a decline in IGFBP-3 serum levels was observed within 24 h after PHx, without significant differences in the hepatic mRNA abundance. A rapid decrease in circulating IGFBP levels also was reported by Phillips et al. (25), who proposed that this was caused by a rapid clearance of the binding proteins from blood once the main site of production was partially removed by two-thirds hepatectomy. Moreover, as observed by Ghahary et al. (14), a decrease in circulating IGFBP-3 during liver regeneration may be caused by a drop in nutrient intake and growth hormone secretion.

Our original observation concerns an enhanced expression of IGFBP-4 during liver regeneration. The hepatic IGFBP-4 mRNA abundance was increased about fourfold over control values 9–12 h after PHx and returned to basal levels 24–48 h after surgery. Consistent with what was observed at the mRNA level, serum IGFBP-4 concentration rose 12–24 h after PHx and remained high thereafter.

Although a reduction in food intake occurs in rats after PHx (28, 35), the enhancement in IGFBP-4 expression during liver regeneration was not caused by changes in nutritional status, because it did not occur in food-restricted SHx rats. Moreover, fasting was reported to reduce rather than increase IGFBP-4 mRNA levels in the liver (6). Finally, the changes in IGFBP-4 expression were limited to the first 24 h after PHx, a time at which nutritional inputs should not have great influence.

Interestingly, IGFBP-4 transcript levels also were elevated in the kidney of PHx rats. This is in line with the notion that the kidney is among the organs that are mostly affected by PHx; in fact, compensatory renal hypertrophy during liver regeneration in rats has been described (24), and elevated IGFBP-1 mRNA levels have been seen in both liver and kidney of PHx rats (14).

The increase in both hepatic and renal IGFBP-4 expression would suggest the involvement of some local and/or systemic factors induced by PHx and responsible for the enhanced IGFBP-4 expression during liver regeneration. Recently, we (11) demonstrated a stimula-

**Table 2. Serum IGF-I levels in rats killed at various times after PHx**

<table>
<thead>
<tr>
<th>Hours After PHx</th>
<th>Serum IGF-I, ng/ml</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>796 ± 26</td>
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<tr>
<td>3</td>
<td>810 ± 84</td>
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<tr>
<td>6</td>
<td>667 ± 14</td>
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<tr>
<td>9</td>
<td>624 ± 23</td>
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<tr>
<td>12</td>
<td>535 ± 24*</td>
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<tr>
<td>24</td>
<td>510 ± 16†</td>
</tr>
<tr>
<td>48</td>
<td>539 ± 17*</td>
</tr>
<tr>
<td>72</td>
<td>671 ± 39</td>
</tr>
<tr>
<td>96</td>
<td>730 ± 70</td>
</tr>
</tbody>
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*Insulin-like growth factor (IGF)-I values are means ± SE; n = 3 rats. *P < 0.05; †P < 0.01.
tion by thyroid hormone, which is able to increase IGFBP-4 production by rat hepatocytes, both in vitro and in vivo. However, in the case of liver regeneration after PHx, an involvement of thyroid hormone in enhancing IGFBP-4 production should be ruled out, because we observed a decline in T₃ and T₄ serum levels within 24–48 h after liver resection, confirming data reported by others (20, 21).

What is the physiological significance of the rise in IGFBP-4 production after hepatectomy? So far, IGFBP-4 has been described only as an inhibitor of the mitogenic actions of IGF-I on several cell types (22, 27, 34). It is known, and also documented in this paper, that serum IGF-I levels and activity are decreased after PHx (28, 31, 35, 36). The classic actions of circulating IGFBP-4 in this context would be to bind IGF-I, prolong its half-life, and affect its activity by preventing or delaying its binding to the receptors. Because high levels of

![Image](http://ajpgi.physiology.org/)

**Fig. 5. Effect of PHx on serum IGFBP abundance.**

A: representative autoradiogram of ligand blots showing 41- to 45-kDa IGFBP-3, 30-kDa IGFBP-1, and 24-kDa IGFBP-4. Positions of molecular mass standards are indicated. B: densitometric quantification of serum IGFBP-4 levels. Results are expressed as percentage of control. Values are means ± SE (n = 3 rats). **P < 0.01; ***P < 0.001.

IGF-I and -II receptors are present on hepatocyte membranes during liver regeneration (4, 31, 32). IGF-I-mediated effects on the hepatocyte itself cannot be excluded. Paracrine effects are also possible, because recent evidence suggests an important role for IGF-I in regulating the proliferation of nonparenchymal liver cells (particularly Ito cells) that possess the type I IGF receptor (1, 15, 16, 30). The depletion of liver IGFBP-4 protein content after PHx, as judged by ligand blot analysis of cell proteins, appears to be in line with an enhanced action of IGF-I on liver cells during regeneration. The finding that IGFBP-4 does not accumulate in the liver, despite the increase in mRNA, could indicate its prompt release and/or cellular catabolism. In fact, it is well established that an important site of regulation for IGFBP-4 is its proteolysis, resulting in fragments that do not bind IGF. Within the liver, the soluble lysosomal aspartic protease cathepsin D might cleave IGFBP-4 and be involved in the clearance of IGFBPs or IGFs (8).

Finally, the possibility exists that the IGFBPs exert IGF-independent effects. This has been validated for IGFBP-1, -3, and -5, which can bind the cell surface or the extracellular matrix (19). Although such interactions have not been documented for IGFBP-4, inhibitory action of the protein through an as yet unknown IGF-independent process has been suggested (33).

In conclusion, our finding of an increase in liver and kidney IGFBP-4 production in PHx rats, resulting in elevated serum IGFBP-4 levels, adds proof of the involvement of the IGF-IGFBP system in liver regeneration. The regulation of IGFBP expression could be part of a mechanism to modulate local and peripheral IGF activity after liver resection.

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