A Possible Role for Insulin-like Growth Factor-binding Protein-3 Autocrine/Paracrine Loops in Controlling Hepatocellular Carcinoma Cell Proliferation

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Abstract
Hepatocellular carcinoma (HCC) is a common malignancy, but treatment outcomes have generally remained poor. Specific factors important for the pathogenesis of HCC are incompletely understood. Insulin-like growth factors (IGFs) are potent autocrine and paracrine mitogens for liver cancer cell proliferation, and their bioactivity is reduced by IGF-binding protein 3 (IGFBP-3). In the present study, we report that IGFBP-3 protein levels were either undetectable (28.5%) or low (71.5%) in human HCC samples examined compared with matched non-neoplastic liver tissue by Western blotting. IGFBP-3 was localized to nontumor liver cells by immunohistochemistry with greater immunointensity than neoplastic liver cells. Levels of type I receptor (IGF-IR) were found to be low in ~39% of human HCC samples examined compared with matched nontumor tissues. IGF-II was overexpressed in 32%, whereas IGF-I expression was decreased in 100% of HCC tissues. IGF-II was overexpressed in 32%, whereas IGF-I expression was decreased in 100% of HCC tissues. In vitro studies revealed that IGF-I and IGF-II induced HepG2 cell proliferation in a dose-dependent manner. Treatment of HepG2 cells with either human recombinant IGFBP-3 (hrIGFBP-3) or IGF-II antibody led to a significant reduction in cell proliferation. Cotreating these cells with hrIGFBP-3 significantly attenuated the mitogenic activity of IGF-I. IGF-I-induced phosphorylation of IGF-IR β subunit, IRS-1, mitogen-activated protein kinase, Elk-1, and Akt-1 as well as phosphatidylinositol 3′-kinase activity was significantly attenuated when hepG2 cells were pretreated with hrIGFBP-3. Our data indicate that loss of autocrine/paracrine IGFBP-3 loops may lead to HCC tumor growth and suggest that modulating production of the IGFs, IGFBP-3, and IGF-IR may represent a novel approach in the treatment of HCC.

Introduction
HCC is one of the most common malignancies worldwide, but treatment outcomes for HCC have remained generally poor. The majority of patients with HCC have inoperable disease with very poor prognosis (1). Survival in patients with curative resection carried out at dedicated centers is between 35–50% at 5 years and much lower elsewhere (2, 3). Long-term survival is uncommon because of the frequent presence of recurrence, metastasis, or the development of new primaries (4, 5). There is also currently no accepted adjuvant or palliative treatment modalities that have been conclusively shown to prolong survival in HCC (6).

Recent research on IGF-I and IGF-II have shown these to be potent mitogens for human hepatoma cells (7), and both IGF-I mRNA (8) and IGF-IRS (7, 8) have been detected in human hepatoma cell lines. In vitro studies on human HCC cell lines HuH-7 and HepG2 have demonstrated that these cells secrete IGF-II and that the inhibition of IGF-II expression in these cells led to a reduction in cell proliferation (8). IGF-I and IGF-II mRNA have also been detected in human hepatoma cell lines. In vitro studies on human HCC cell lines HuH-7 and HepG2 have demonstrated that these cells secrete IGF-II and that the inhibition of IGF-II expression in these cells led to a reduction in cell proliferation (8). IGF-I and IGF-II mRNA have also been detected in vivo in HCC, but the levels compared with matched nontumorous adjacent hepatic tissue were lower than in HCC for IGF-I mRNA (9) and higher in HCC for IGF-II mRNA (9–11). Furthermore, IGF-II fetal transcripts (5.6 and 4.5 kb) were found in HCC (9), whereas normal IGF-II transcripts were regressed (10). Coexpression of IGF-II protein and Ki-67 antigen in tumor cells were observed suggesting that IGF-II acts as an autocrine or paracrine growth factors in these tumors (10). Overexpression of IGF-II in transgenic mice has been shown to increase serum IGF-II and HCC (12). These observations suggest an important role for IGF-II in hepatocarcinogenesis.

Both IGF-I and IGF-II bind with high affinity to specific IGFBPs, which modulate their bioactivity. At least six IGFBPs have been described (reviewed in Refs. 13, 14). Expression of genes encoding the various IGFBPs has been observed in many tissues and is subject to intricate physiological regulation (reviewed in Refs. 13, 14). IGFBP-3 is the most abun-

Received 11/1/01; revised 12/27/01; accepted 1/7/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by grants from National Medical Research Council of Singapore (NMRC/0541/2001) and SingHealth Cluster Research Fund (EX 008/2001; to H. H.).
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3 The abbreviations used are: HCC, hepatocellular carcinoma; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; IGFBP, insulin-like growth factor-binding protein; hrIGFBP-3, human recombinant IGFBP-3; PI3K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; IRS-1, insulin receptor substrate 1; Akt, serine/threonine protein kinase; GH, growth hormone; PSF, phenol-red serum-free; SFM, serum-free medium.
IGFBP in the circulation, where it forms a Mr 150,000 complex with an acid-labile subunit and IGF-I or IGF-II (14). The IGFBP-3 gene is expressed in many tissues, and IGFBP-3 has affinities for IGFs that are either equal to or stronger than those of the IGF receptors and, therefore, inhibit the IGFs by sequestration in the extracellular compartment (reviewed in Refs. 13, 14). Recent evidence also demonstrates that IGFBP-3 has growth-inhibitory activity that is independent of its IGF binding properties (reviewed in Refs. 14).

Thus, existing evidence suggests that a better understanding of IGFBP-3 may allow modulation of its activities and the potential for therapeutic applications in the control of HCC.

**Results**

In the adult human, the liver is the main source of circulating IGF-I and IGFBP-3 (13, 14). Because local expression of IGF-I is important in autocrine and paracrine stimulation, and the IGFBP-3 appears to decrease the mitogenic activity of free IGF-I, presumably by competing with type I IGF receptors for the ligand, the IGFBP-3 levels in the HCC samples were determined. Total tissue lysates from both HCC cancers and adjacent morphologically normal liver cells were subjected to Western blot analysis. The resulting blots were incubated with antihuman IGFBP-3 (B, D, and F) and α-tubulin (A, C, and E) antibodies. Representative samples are shown. All normal adjacent liver tissues (N) had high levels of IGFBP-3, whereas IGFBP-3 protein was either undetected or absent from HCC tumors (T). Serum IGFBP-3 served as a positive control.

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**Table 1** Expression of IGFs, IGFBP-3, and IGF-IR in normal adjacent liver tissues and HCC tumors

<table>
<thead>
<tr>
<th>Genes examined</th>
<th>Normal adjacent liver tissues (n = 28)</th>
<th>HCC tumors (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intense staining of IGF-I</td>
<td>+++ (100%)</td>
<td>18/28 (64%)</td>
</tr>
<tr>
<td>Intense staining of IGF-II</td>
<td>+++ (100%)</td>
<td>19/28 (68%)</td>
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<tr>
<td>Expression levels of IGFBP-3 levels: (compared with normal and adjacent tissue of the same patient)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>28/28 (100%)</td>
<td>0/28 (0%)</td>
</tr>
<tr>
<td>Low</td>
<td>0/28 (0%)</td>
<td>8/28 (28.5%)</td>
</tr>
<tr>
<td>Undetectable</td>
<td>0/28 (0%)</td>
<td>20/28 (71.5%)</td>
</tr>
<tr>
<td>IGF-IR expression levels: (compared with normal and adjacent tissue of the same patient)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>28/28 (100%)</td>
<td>17/28 (61%)</td>
</tr>
<tr>
<td>Low</td>
<td>0/28 (0%)</td>
<td>11/28 (39%)</td>
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</table>
To confirm the above observation, immunohistochemical staining of HCC and normal adjacent liver tissues was performed using antihuman IGFBP-3 antibody, which recognized both intact and fragmented IGFBP-3 protein. IGFBP-3 protein was clearly localized to almost all of the cells in the adjacent normal tissue (Fig. 2A). However, this signal was absent (Fig. 2B) in HCC, indicating that IGFBP-3 expression was either inactivated or decreased in these neoplastic cells.

To determine whether HCC also expressed IGF-I and IGF-II, HCC and adjacent normal liver tissues were stained with anti-IGF-I and anti-IGF-II antibodies, respectively. Although normal adjacent tissues stained positively for IGF-I (Fig. 3A), the expression was significantly decreased in HCC (Fig. 3B). Approximately 64% (18 of 28) and 36% (10 of 28) of HCC samples examined had moderate and low IGF-I expression, respectively (Table 1), which is consistent with a previous report (9). IGF-II staining (Fig. 3C and D), in contrast, was approximately equal (68%; 19 of 28) or more intense (32%; 9 of 28) in HCC compared with normal liver tissue (Table 1). These observations are in agreement with previous reports demonstrating overexpression of IGF-II in HCC (9–11). Our data suggests that IGF autocrine and paracrine loops exist in HCC.

Because IGFs stimulate growth responses in liver cells by binding to the IGF-IR, IGF-IR levels in HCC and normal adjacent tissue samples were examined. Approximately 39% of HCC tumors had slightly lower IGF-IR levels than non-neoplastic adjacent liver tissues (Fig. 4; Table 1). When normalized for α-tubulin, IGF-IR expression was not significantly different between normal tissues and HCC (P < 0.01).

To test the hypothesis that IGF-I and IGF-II play a role in mediating tumor cell growth, HepG2 cells were treated with various concentrations of human recombinant IGF-I or IGF-II for 48 h. Fig. 5A shows dose-dependent growth stimulation by IGF-I. Similar effects were observed when HepG2 cells were treated with IGF-II (data not shown). Four-fold increase in cell number was observed at the dose of 25 ng/ml IGF-I (P < 0.01).

Because IGFBP-3 protein was undetectable or lost in the majority of HCC samples (Fig. 1) and IGFBP-3 has been shown to inhibit cancer cells in an IGF-independent pathway (14), we attempted to demonstrate the antiproliferative action of IGFBP-3 on HepG2 cells. Approximately 40% and 52% inhibition of basal proliferation was obtained via 250 ng/ml and 500 ng/ml of hrIGFBP-3, respectively (P < 0.01; Fig. 5B). Concentrations of hrIGFBP-3 up to 1 μg/ml did not inhibit additional proliferation.

To determine whether the proliferative action of IGF-I can be attenuated in the presence of IGFBP-3, HepG2 cells were treated with hrIGFBP-3 in the presence and absence of IGF-I for 48 h. As shown in Fig. 5C, treatment of HepG2 with 25 ng/ml IGF-I for 48 h resulted in a 2.8-fold increase in cell number (P < 0.01). IGF-induced HepG2 proliferation was significantly attenuated (P < 0.01) in the presence of 250 ng/ml IGFBP-3 and completely abolished at the concentra-

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Fig. 2. Immunostaining of normal liver (A) and HCC tumors (B) for IGFBP-3. Normal adjacent liver tissue and HCC tumors were stained with antihuman IGFBP-3 as described in “Material and Methods.” Adjacent normal liver tissue, showing intense expression of normal cells for IGFBP-3, whereas very low staining signal was observed for the morphologically disorganized HCC cells. Representative staining are shown. (original magnification ×400).

Fig. 3. Immunostaining of normal liver and HCC tumors for IGF-I and IGF-II. Normal adjacent liver tissue (A and C) and HCC tumors (B and D) were stained with antihuman IGF-I (A and B) or antihuman IGF-II (C and D) as described in “Material and Methods.” Representative samples are shown. Normal adjacent liver tissue shows intense staining for IGF-I, whereas low staining signals are seen for HCC cells. HCC tumors show more intense staining for IGF-II than normal adjacent tissue. (original magnification ×400).
tion of 500 ng/ml. This result suggests that IGFBP-3 attenuated IGF-I-induced HepG2 proliferation by reducing IGF-I bioavailability.

To test the hypothesis that autocrine production of IGF-II by liver cancer cells plays a role in mediating liver cancer cell growth, HepG2 cells, which has been shown to secrete IGF-II (8), were treated with anti-IGF-II, IGF-II, or with both combined for 48 h. Fig. 5D shows a 3-fold induction of basal proliferation by 25 ng/ml IGF-II (P < 0.01). This induction was significantly attenuated by IGF-II antibody (P < 0.01), whereas preimmune serum was not significantly affected. IGF-II antibody alone caused a 35% reduction in cell number. These results suggest that the rapid proliferation of liver cancer cells in vivo and in vitro may at least, in part, be a consequence of autocrine stimulation mediated by IGF-II expression.

Because MAPK and PI3k are important for the effect of IGFs on growth and apoptosis, the mechanisms involved...
in the attenuation of IGF-I by IGFBP-3 on proliferation of human HepG2 cancer cells were investigated. HepG2 cells were preincubated with 250 ng/ml hrIGFBP-3 for 24 h, or pretreated with 250 ng/ml hrIGFBP-3 for 24 h, washed, and stimulated with 25 ng/ml IGF-I. Lysates from these cells were immunoprecipitated with either anti-IGF-IR (B and C) or IRS-1 (D and E) antibodies as described in “Materials and Methods.” After SDS-PAGE, blots were immunoblotted with antibodies to IGF-IR (B), IRS-1 (D), and phosphotyrosine clone 4G10 (C and E). To detect total MAPK, phospho MAPK, and phospho Elk-1, cell lysates were subject to Western blotting as described in “Materials and Methods.” Blots were blotted with anti-α tubulin (A), anti-MAPK (F), phospho p44/42 MAPK (Thr202/Tyr204; G), and phospho Elk-1 (Ser383; H) antibodies. The figure is representative of three independent experiments.

**Discussion**

Very little information is available on the expression of IGFBP-3 in HCC. In the present study, we show that IGFBP-3 protein was lost or underexpressed in the majority of HCC. IGFBP-3 was intensely expressed in corresponding paired samples of normal liver cells. Because Kupffer cells are the main source of IGFBP-3 production in human liver (15), it is likely that these IGFBP-3-expressing cells are Kupffer cells. Because no low molecular weight IGFBP-3 bands were detected in Western blot analysis (data not shown), it is unlikely that proteolysis of IGFBP-3 is the cause of absent IGFBP-3 expression in HCC. The absence of IGFBP-3 in HCC was reinforced by immunohistochemical analysis showing that the normal hepatic cells expressed high levels of IGFBP-3 protein whereas HCC did not. The ability to detect low amount of IGFBP-3 in certain cancerous tissues by Western
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...tumor growth suggesting the possible involvement of IGFBP-3 in controlling HCC cell proliferation.

Because endogenous IGF-I and IGF-II are produced by both normal liver cells and HCC cells, the decrease or loss of IGFBP-3 production by HCC would allow more free IGFs to act in an autocrine or paracrine fashion to enhance tumor cell growth. Although expressions of other IGF-binding proteins in HCC such as IGFBP-1 and IGFBP-2 (data not shown) were also investigated, only IGFBP-3 levels were associated with HCC growth suggesting the possible involvement of IGFBP-3 in controlling HCC cell proliferation.

The observations that HCC tumors express less IGF-I than control liver, whereas IGF-II expression is higher in a high proportion of HCC tumors, are consistent with previous reports that showed IGF-I mRNA levels were lower in HCC as compared with nontumorous hepatic tissue from an adjacent area (9), whereas IGF-II mRNA was higher in a high proportion of HCC tumors, are consistent with previous reports. At the moment, the molecular mechanisms responsible for reduction in IGF-I and reactivation of IGF-II in HCC remain to be determined. It has been reported that hepatic IGF-I and IGF-IR expression is regulated by GH (13, 14) and estrogen (16). It is possible that the reduction in IGF-I and IGF-IR expression in HCC observed in the present study may be because of the loss of receptors for GH or estrogens. Experiments are under way to investigate this possibility.

Currently, the molecular mechanisms by which the IGFBP-3 expression is lost or reduced in HCC are not known. Several reports have suggested that IGFBP-3 expression is regulated by GH (reviewed in Refs. 13, 14), antiestrogen (17), transforming growth factor β (18), and retinoic acid (19). In HCC, receptors of these compounds may be lost or inactivated, leading to IGFBP-3 gene inactivation. It is possible that the presence of IGFBP-3 expression in HCC is a consequence of genetic alterations such as deletion, mutation, or inappropriate hypermethylation as described in a number of tumor suppressor genes (20, 21) and cyclin-dependent kinase inhibitor genes (22, 23), or chronic hepatitis and cirrhosis (24, 25). We are currently investigating the mechanisms responsible for silencing the IGFBP-3 gene and the relationship between hepatitis and cirrhosis with the expression of IGFBP-3 in HCC.

In our experimental system, we also observed that IGFBP-3 inhibited proliferation of HepG2 cells and could be an important factor in a negative control system regulating human cancer cell growth in vivo. Because the gene encoding IGF-II is expressed in HepG2 (8), the observed inhibitory action of IGFBP-3 likely involves the reduction of bioavailability of endogenous IGF-II for cell surface receptor binding. It is also possible that IGFBP-3 inhibits HepG2 by direct growth inhibitory signal transduction pathways (26, 27). However, these mechanisms are not mutually exclusive and both may be relevant in vivo.

Human liver cancer cell growth is inhibited by exogenous IGFBP-3. In addition, IGFBP-3 is a potent IGF-I antagonist. These properties suggest a possible therapeutic application for IGFBP-3 in the control of liver cancer cell proliferation. Also, the presence of IGFBP-3 in HCC specimens may possibly be a better marker for tumor stage, diagnosis, and prognosis than currently used histological parameters. Experiments are under way to analyze the association between levels of serum IGFBP-3 and tumor stage, metastasis, and other markers such as serum α-feto protein in patients with HCC.

Although many treatment modalities have been attempted in HCC, there is currently no proven practical therapy besides surgery, and attempts at both adjuvant therapy and therapy for inoperable HCC remains experimental (6). Because HCC is capable of endogenous production of both IGF-I and IGF-II (8–11), and hepatoma cells are very responsive to stimulation by these growth factors, agents that are capable of modulating endocrine, paracrine, and autocrine production of the IGFs and their receptors are likely to be useful in treatment of HCC. The present findings offer both a potential diagnostic parameter and as well as a potential novel strategy for HCC cancer endocrine therapy.

Materials and Methods

Reagents and Antibodies. MEM and FCS were obtained from Life Technologies, Inc., Grand Island, NY. hrIGFBP-3 was from Celltrix Laboratories, Richmond, VA. Human recombinant IGF-I and IGF-II were from GroPep, Adelaide, Australia. Rabbit anti-phospho Akt (Ser473), mouse anti-phospho p44/42 MAPK (Thr202/Tyr204), rabbit anti-Akt, and mouse anti-MAPK antibodies were purchased from New England Biolabs Inc., Beverly, MA. Rabbit anti-phospho Elk-1 (Ser383), rabbit antihuman IGF-I, antihuman IGF-IR, mouse anti-α-tubulin, and protein A-agarose were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Rabbit antihuman IGFBP-3, rabbit anti-IRS-1 and mouse antiphosphotyrosine 4G10 were from Upstate Biotechnology, Lake Placid, NY. Rabbit antihuman IGFBP-2 were from Austral Biologicals, San Ramon, CA. All of the antibodies were used at an indicated final concentration. Horseradish peroxidase-conjugated donkey antimouse or antirabbit secondary antibodies were from Pierce, Rockford, IL. Chemiluminescent detection system was from Amersham, Pharmacia Biotech.
Arlington Heights, IL. L-phosphatidyl inositol-4 monophosphate was from Sigma Chemical Co., St. Louis, MO.

Collection of Human HCC and Adjacent Nontumor Liver Specimens. Tissue samples were obtained intraoperatively from tumors and adjacent nontumor livers during liver resection for HCC in 28 patients at the Singapore General Hospital. The samples were snap frozen in liquid nitrogen and stored at −80°C until analysis. A similar set of samples was fixed in 10% formalin and paraffin embedded. The diagnosis of HCC was confirmed histologically in all of the cases. Prior written informed consent was obtained from all of the patients, and the study received ethics board approval at both institutions.

Immunolocalization of IGFBP-3. Formalin- and paraffin-embedded sections were used for IGFBP-3, IGF-II, and IGF-1 immunolocalization. This was performed using rabbit antihuman IGFBP-3 (1:500 dilution), rabbit antihuman IGF-I (3 µg/ml), and rabbit antihuman IGF-II (3 µg/ml) antibodies as described (28). Nonspecific staining was evaluated for each specimen using either a similar concentration of IgG or by absorbing the primary with appropriate specific immunogen. The slides were evaluated, and intensity of the staining was scored. Specific staining was semiquantitated by assigning a score of 0 to +++ based on increasing green fluorescence intensity. The results shown in Table 1 represent the average score obtained from twice staining.

Cell Culture. Human hepatoma HepG2 cells were obtained from American Type Culture Collection and maintained as monolayer cultures in MEM supplemented with 10% FCS growth medium. For proliferation study, confluent cultures of HepG2 cells were trypsinized and plated at 2 × 10^5 cells in 24-well plates (Nunc, Nalgene Nunc International, Rochester, NY) with growth medium. After 48 h, the cell monolayers were rinsed twice with MEM PSF medium and additionally incubated in PSF medium for 24 h. After 24 h, cells were treated with 250 ng/ml hrIGFBP-3 in SFM for 24 h. Cells were rinsed once and then incubated with 25 ng/ml IGF-I for 10 min. After IGF-I stimulation, cells were harvested and lysed in lysis buffer as described above. To determine the effects of IGFBP-3 on IGF-I-induced MAPK, Elk-1, and Akt phosphorylation, Western blotting was performed using phospho Akt (Ser473; 1 µg/ml), phospho p44/42 MAPK (Thr202/Tyr204; 1 µg/ml) and phospho Elk-1 (Ser383; 1 µg/ml) antibodies. Levels of IGF-IR β subunit and IRS-1, and their phosphorylated forms were determined by immunoprecipitation of total cell lysates using anti-IGF-IR (2 µg/ml) and IRS-1 (4 µg/ml) antibodies, respectively, as described by the supplier. Immunoprecipitated proteins was blotted using rabbit antihuman IGF-IR (1 µg/ml), antihuman IRS-1 (1 µg/ml), and antiphosphoryrosine 4G10 antibodies (1 µg/ml).

To study IRS/Pi3k p85 interactions, cell lysates were precipitated with anti-IRS-1 antibody. Immunocomplexes were used to determine p85 interactions associated with IRS-1. To measure p85 activity, 500 µg of total protein was incubated with 4 µg of anti-IRS-1 antibody for 3 h. The antigen/antibody complex was precipitated with 50 µl of protein A-agarose and washed with kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerol phosphate, 2 mM DTT and 0.1 mM Na3VO4]. Immunoprecipitates were resuspended in 25 µl of kinase buffer containing 20 µg of l-phosphatidyl inositol-4-monophosphate and 5 µl of 100 mM MgCl2, and then adding 30 µCl of [32P]ATP in 2.5 µl of 0.88 mM ATP per kinase reaction. Kinase products were resolved using TLC with a CHCl3/methanol/H2O/NH4OH (40:48:10:5) solvent system. Quantitative analysis of [32P]-labeled inositol 1,3,4–5 phosphate levels was determined by scanning the bands densitometrically.

Statistical Analysis. For quantitation analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated and normalized the amount of α-tubulin. Differences in cell number and the levels of proteins under studied were analyzed by the Mann-Whitney U test.

References


