Insulin-Like Growth Factors in Patients With Liver Cysts

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Insulin-like growth factors (IGFs) play an important role in cell growth and differentiation, and the liver is the main source of IGFs and IGF-binding proteins (IGFBPs) that appear in the circulation. The effect of liver cysts on the circulating IGF system was studied in this work. Serum concentrations of IGF-I and -II were measured by radioimmunoassay, IGFBP patterns were characterised by ligand-affinity and immunoblotting, and a lectin-binding assay was used to investigate the glyco component of IGFBP-3 complexes. IGF-I and -II concentrations in patients with cysts were significantly lower compared to those in healthy individuals ($P < 0.0001$ and $P < 0.01$, respectively), and the decrease was related to age but not sex. The overall mean concentrations of IGF-I and -II were

not significantly different whether the cysts were caused by *Echinococcus granulosus*, cross-reactive pathologies, or some other factor. IGFBP profiles correlated with the amount of IGF present: patients with lower IGF-I concentrations expressed decreased IGFBP-3 and elevated IGFBP-2 levels. Increased IGFBP-3 proteolytic activity in the patients' blood was not detected by immunoblotting. In the lectin-binding assay, IGFBP-3 complexes in the circulation of patients demonstrated reactivity similar to that in healthy persons, suggesting that the overall structure of the saccharide moieties of the IGFBP-3 complexes was not significantly altered due to liver cyst formation. *J. Clin. Lab. Anal.* 18:299–304, 2004. © 2004 Wiley-Liss, Inc.

Key words: IGF-I; IGF-II; IGFBPs; cystic lesions; ligand blotting; lectins

INTRODUCTION

The insulin-like growth factor (IGF) system plays an important metabolic role in cell growth and differentiation. It includes the peptides IGF-I and -II, and their binding proteins (IGFBP-1–6), receptors, and specific proteases. The liver is the major source of IGFs and IGFBPs that appear in the blood circulation. The synthesis of IGFs and IGFBP-3 is growth-hormone-dependent (1). IGFs form binary complexes with all IGFBPs, and ternary complexes with IGFBP-3 and -5. In plasma the ternary complex with IGFBP-3 and acid labile subunit (ALS) predominates and serves as a reservoir of IGF molecules. Thus, IGFBP-3 is the most abundant IGFBP in the circulation (about 3 mg/L), appearing in two glycosylation forms of 40 and 44 kD. IGFBP-1 (31 kD phosphoprotein) and IGFBP-2 (34 kD simple protein) are normally present in significantly lower concentrations (100- and 10-fold lower than IGFBP-3), partly because the binary complexes with IGF can leave the circulation through capillary walls. They have mainly inhibitory effects on IGF actions (2,3). Plasma concentrations of IGFBP-1 typically rise during fasting and after acute hypoglycemia, while IGFBP-2 provides an intermediate level of regulation

of IGF availability, especially under conditions in which there is insufficient IGFBP-3. Serum levels of IGFBP-2 increase when an organism is severely ill.

Hepatic injury is often accompanied by complex metabolic changes. In the progressive state of liver cirrhosis, the levels of both IGFs and IGFBP-3 decrease in the circulation, while concentrations of IGFBP-1 and -2 increase and then decrease (4). The intention of this work was to study the effect of cystic lesions of the liver on the circulating IGF system. In critically ill patients, the protease activity in the circulation may be increased (5); therefore, we analysed the proteolytic activity that could result in IGFBP-3 fragmentation. Changes in the concentration of specific molecules due to hepatic damage may be accompanied by small changes in structure resulting from the altered cellular milieu. The

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condition may be associated with functional changes in certain variables, such as binding constants. This type of alteration was previously found in the structure of the glyco component of IGFBP-3 in patients with liver cirrhosis (6). In this study we also investigated whether structural alteration of IGFBP-3 was present in patients with liver cysts, and examined interactions between IGFBP-3 and lectins.

MATERIALS AND METHODS

Serum samples were obtained from healthy adults ($n=81$, 36 men and 45 women, 20–75 years old, BMI=18.3–27.6 kg/m²) and patients with liver cysts diagnosed by ultrasonography ($n=143$, 51 men and 92 women, 25–76 years old, BMI=16.3–29.4 kg/m², habitual nutritional status, and with no malnutrition due to loss of appetite, vomiting, diarrhea, or malabsorption). All patients who were suspected of having echinococcosis had been sent for laboratory examination by their physicians, and consented to the use of their sera samples in this experiment.

We performed serodiagnoses (i.e., determined the presence of antiparasite antibodies) using a commercial indirect immunofluorescence assay (IFA; INEP, Zemun, Serbia) with FITC-labelled secondary antibodies (7). Samples with an antibody titre of 1:40 were considered seropositive. The patients were classified into three categories: 1) patients with anti-*Echinococcus granulosus* antibodies (I, $n=48$); 2) patients with cysts that shared antigenic determinants with *E. granulosus* protoscoleces (8,9) (i.e., with antibodies that cross-reacted with *E. granulosus*) (II, $n=45$); and 3) patients with cystic lesions of other origin (III, $n=50$).

Concentrations of IGF-I and -II in sera were measured by radioimmunoassay (RIA-IGF-I and RIA-IGF-II; INEP, Zemun, Serbia) with ¹²⁵I-labelled ligands (10,11). The assays were standardised against WHO reference materials (IGF-I: WHO 87/518; and IGF-II: WHO 96/538, NIBSC, Herts, UK). IGFBP patterns in sera were characterised by ligand-affinity and immunoblotting. SDS-PAGE was performed according to Hossenlopp et al. (12) with certain modifications, as previously described (6). Proteins were transferred to a nitrocellulose membrane, followed by autoradiography with ¹²⁵I-IGF-I or ¹²⁵I-anti-IGFBP-3 polyclonal antibodies (DSL; Webster, USA).

A solid-phase lectin-binding assay was performed in polystyrene test tubes (Spektar, Čačak, Serbia) coated with 1 µg/100 µL of lectin per tube, in 0.05 M PBS pH 7.4, overnight at 4°C (13). The lectins concanavalin A (Con A), wheat germ agglutinin (WGA), phytohemagglutinin (PHA), lentil lectin (LCA), and tomato lectin (LEA) were used. Nonspecific binding was inhibited by

1% casein in PBS. The serum samples (100 µL) were incubated with immobilized lectins for 2 hr at 4°C. After the samples were washed with 0.1% Tween-20, ¹²⁵I-IGF-I (5,000 cpm/100 µL) was added, and they were left overnight at 4°C. Bound radioactivity was measured in a gamma-counter. Specific binding was obtained as the difference between total and nonspecific binding (tubes coated with casein).

The concentration of serum bilirubin and the activities of transaminases (AST and ALT), γ -glutamyl transferase (GGT), and alkaline phosphatase (ALP) were determined with the use of commercial kits (RANDOX, Crumlin, UK). The reference values for these hepatic markers are 5–24 µmol/L for bilirubin, ≤ 40 U/L for AST, ≤ 37 U/L for ALT, 11–50 U/L for GGT, and 98–297 U/L for ALP.

Data Analysis

The numerical data are expressed as the mean (X) and standard deviation (SD). Differences between the groups were analysed with the use of a nonparametric Mann-Whitney U-test. The relative intensity of autoradiographic bands was estimated with the Glyco Band Scan computer program (version 5, 1998).

RESULTS

The patients that participated in this study were analysed as a group against the reference population, and as three subgroups that were compared (Fig. 1). The patients were subdivided into three groups according to the reaction of their sera towards *E. granulosus* protoscoleces. The subjects in group I were designated positive by the indirect immunofluorescent assay, while the patients in group III were determined to be negative. In group II, intensive fluorescent staining was localized only on the protoscolex tegument, while the antigen-antibody interaction of the protoscolex parenchyma was completely absent.

The overall mean concentrations of IGF-I and -II in the healthy population were 23.1 (SD=7.99) and 72.0 (SD=14.41) nmol/L, respectively (Table 1), and the values found were positively correlated ($P<0.001$), as reported earlier (14). Serum levels of IGF-I decreased with increasing age at similar slopes in both men ($P<0.001$) and women ($P=0.004$). There was an equivalent fall in IGF-II concentrations with age in women ($P<0.001$), but the age-related decrease in men was small and not statistically significant ($P>0.05$) (14). The overall mean concentrations of IGF-I and -II in patients with liver cysts were 14.4 (SD 6.53) and 66.0 (SD 21.05) nmol/L, respectively, and they were significantly decreased ($P<0.0001$ for IGF-I, and $P<0.001$ for IGF-II). IGF-I levels decreased with age in both

Fig. 1. Distribution of serum IGF-I and -II values in healthy adults and patients with liver cysts.

male and female patients ($P=0.005$ and $P=0.02$, respectively), and the sex-related differences were not significant. The concentrations of IGF-II were found to be significantly lower with increasing age only in men with cysts ($P=0.004$). When three groups of patients were compared with each other, it was found that the overall mean values of IGF-I and -II were similar and not significantly different (Table 1). The relatively greater decrease in IGF-I led to a significant increase in the ratio IGF-II/IGF-I ($P<0.03$). This effect was most marked in the patients in group II, in which the IGF-II/IGF-I ratio was higher compared to the other two groups ($P=0.01$). There was no correlation between the antibody titer and IGF-I and -II concentrations in group I individuals. Bilirubin concentration in the

patients' sera ranged from the reference interval up to 52 $\mu\text{mol/L}$, while AST, ALT, GGT, and ALP activities were either normal or increased (≤ 430 U/L, 1,200 U/L, 77 U/L, and 580 U/L, respectively). Although higher levels of hepatic markers were often found in patients with lower IGF concentrations, a direct correlation was not established.

We analysed a number of serum samples by ligand-affinity blotting to determine how liver cysts affected the expression of IGFBPs. Representative results are shown in Fig. 2. Ligand-affinity blotting was validated with the use of protein standards. The major IGFBPs from healthy persons had molecular masses of 40 and 44 kD, corresponding to the well-known IGFBP-3 doublet, as well as 34 kD (a weak band) that agreed with the mass

TABLE 1. Circulating IGF-I and IGF-II concentrations in healthy adults and patients with liver cysts

Liver cyst	IGF-I (nmol/L) X (SD)	IGF-II (nmol/L) X (SD)	IGF-II/IGF-I X (SD)
None, n = 81	23.1 (7.99)	72.0 (14.41)	3.4 (1.19)
Male, 20–39 years	28.4 (8.70)	75.0 (14.46)	
Male, 40–75 years	22.6 (6.79)	70.7 (12.18)	
Female, 20–39 years	29.1 (7.60)	83.0 (14.80)	
Female, 40–74 years	20.2 (6.99)	65.1 (12.09)	
All patients, n = 143	14.4 (6.53)	66.0 (21.05)	5.2 (2.23)
Male, 31–39 years	17.3 (4.89)	73.9 (16.35)	
Male, 40–76 years	13.2 (4.03)	57.3 (18.74)	
Female, 25–39 years	16.8 (4.06)	71.4 (18.88)	
Female, 40–72 years	13.7 (5.57)	65.4 (15.33)	
Group I, n = 48	15.7 (7.20)	64.2 (24.01)	4.6 (1.99)
Group II, n = 45	13.4 (7.28)	66.5 (21.23)	6.1 (2.54)
Group III, n = 50	15.3 (5.03)	67.3 (19.00)	5.0 (2.47)

Fig. 2. Ligand blot of the IGF-binding proteins in sera (lane 1: healthy person; lanes 2–10: patients with liver cysts). The molecular mass markers were ovalbumin (45 kD), carboanhydrase (36 kD), and glycerophosphate anhydrase (29 kD).

of IGFBP-2 (lane 1). Other IGFBPs are normally present in sera in much lower concentrations, and they could not be visualised. Sera from patients with liver cysts demonstrated an increased amount of IGFBP-2 (lanes 2–10) and most frequently a decreased level of IGFBP-3 (lanes 2–4 and 7–10). Since we had previously measured the serum level of IGF-I and -II in all of the samples used for the ligand-affinity blotting assay, we postulated that a correlation could exist. The reduction of IGF-I levels was much more significant (Table 1); therefore, we discuss only IGF-I concentrations in relation to the IGFBP profiles. The IGF-I concentration in the serum sample of healthy subject (corresponding to lane 1) was 28.2 nmol/L. In lanes 2–4, 8, and 9 (containing a large reduction of IGFBP-3) the concentration of IGF-I was 7.2, 9.9, 7.7, 10.5, and 8.6 nmol/L, respectively. In lanes 7 and 10, the intensity of IGFBP-3 was reduced compared to the control serum (lane 1), but to a lesser extent than in samples 2–4, 8, and 9. Accordingly, the level of IGF-I in these samples was higher (17.7 and 13.7 nmol/L) but still reduced compared to control serum. Sera in lanes 5 and 6 showed an IGFBP profile similar to that in the control serum, and the concentration of IGF-I in these samples was closer to that measured in the control sample (21.2 and 22.6 nmol/L). The presence of a 31 kD band was

not detected in any serum. In the immunoblotting assay, autoradiograms indicated the presence of native IGFBP-3 (40–44 kD), and there were no proteolysed fragments that could be recognised by polyclonal anti-IGFBP-3 antibodies (data not shown).

Randomly chosen sera from healthy persons ($n = 20$) and patients with liver cysts, which autoradiographically showed the presence of intact IGFBP-3 ($n = 20$), were analysed by a lectin-binding assay. The results of this assay should be regarded as an overall lectin-binding affinity of IGFBP-3 complexes. Although ternary complexes (IGF/IGFBP-3/ALS) predominate in the circulation, a certain amount of binary complexes (IGF/IGFBP-3) may be present as well. IGFBP-2 molecules cannot react with lectins, because IGFBP-2 is a simple protein. We chose the samples according to the IGFBP-3 band intensity (evaluated by densitometric band scan), which had to be similar to that of the control serum so that we could select a representative group in which the amount of IGFBP-3 and the reactivity towards ligand ^{125}I -IGF-I were not significant variables. All applied lectins reacted with IGFBP-3 complexes. Measurements made in radioactivity units indicated the amount of bound ^{125}I -IGF-I, which in turn reflected the quantity of IGFBP-3 complexes captured by the immobilized lectins. To determine the relative

reactivity, we assigned the greatest amount of lectin-bound ^{125}I -IGF-I/IGFBP-3 complexes from healthy individuals a value of 1.0. IGFBP-3 complexes reacted with the lectins in the following order of affinity: LEA (1.0 ± 0.21) = Con A (1.0 ± 0.24) > LCA (0.9 ± 0.30) > WGA (0.5 ± 0.32) = PHA (0.5 ± 0.30). On average, the intensities of the above interactions with patient sera were lower: LEA (0.9 ± 0.22) = Con A (0.9 ± 0.29) > LCA (0.8 ± 0.30) > WGA (0.5 ± 0.31) > PHA (0.4 ± 0.32). However, the differences between healthy persons and patients were not statistically significant.

DISCUSSION

Pathophysiological cellular changes and physical obstruction due to increasing mass of a cyst may compromise liver function. Cysts may be of different origins, which affects hepatic metabolism in various ways and to different degrees. In some cases, local tissue damage can be compensated for, while in others extensive systemic insufficiencies are observed. Liver cysts in the adult can be classified as developmental, neoplastic, inflammatory, hydatid, or miscellaneous (15). The patients involved in this study were divided into three groups according to the immunochemical reaction of their sera towards *E. granulosus* protoscolices. Group I comprised samples that were typically seropositive, confirming that *E. granulosus* was responsible for the cyst formation. Sera in group II exhibited specific immunofluorescent staining only on the protoscolex tegument, thus they recognised antigens that are common for *E. granulosus* and other cross-reactive pathologies. Reports of serological tests for hydatid giving false-positive results in various malignant diseases appear regularly, suggesting antigenic similarity between *E. granulosus* and neoplasms. A cross-reactivity was shown in cases of noncestode parasitic infections and malignancies (8). Rashed et al. (16) demonstrated that sera from tumor patients recognised 27 kD, 21 kD, 16 kD, and 13 kD protein bands from hydatid cyst fluid, while cross-reaction was also found with 200 kD, 175 kD, 62 kD, 52 kD, and 40 kD proteins in cases with anti-Schistosoma antibodies. The third group of patients included subjects that did not react to *E. granulosus* antigens.

IGFs are recognised as hepatic markers for evaluating liver damage due to cirrhosis (4,17). The current results demonstrate that there is also a significant decrease in circulating IGF-I and -II concentrations in patients with liver cysts. The decline was greater for IGF-I, and hence the IGF-II/IGF-I ratio increased significantly. This condition was not found in liver cirrhosis, which implies that different control mechanisms (e.g., acute phase reaction mediated by cytokines) may have been in-

involved. In contrast to IGF-I, where values were within or below the reference interval, there were several samples with IGF-II concentrations above the upper reference limit. Since hepatocellular carcinomas are frequently associated with IGF-II production (18), this experimental finding may suggest a direction of cyst progression. Determining pro-IGF-II values may be helpful for completing these data. Although the fall in IGF-I concentrations with age was significant for both male and female patients, no sex-related differences were found. Van den Berghe et al. (19) reported that during a protracted critical illness, men have lower concentrations of IGF-I than women, which implies that gender is an important factor. The patients involved in this study demonstrated alterations of the IGF system that apparently resulted from hepatic damage/obstruction, and no secondary reaction involving sex hormones (pituitary and/or gonadal). Since sex hormones were not measured in this work, the offered explanation awaits further evaluation.

IGFBP-3 and -2 were detected in patients' sera, and there was a correlation with IGFs. It is known that IGFs and IGFBPs are synthesized in different cells in the liver and are regulated by different hormones, growth factors, and cytokines. Hepatocytes synthesize IGF-I and -II, and IGFBP-1, -2, and -4, while IGFBP-3 and -4 are produced in Kupffer and endothelial cells (1,20). Hazel et al. (21) reported that IGF-I and IGFBP-1 and -2 are differentially expressed in the liver acinus (i.e., hepatocytes have specific expression patterns that depend on their position within the liver acinus). Unfortunately, we did not have complete information about the cysts, and their characteristics, number, and location may have affected the metabolic outcome (22). This could explain the variety of findings concerning the IGF system.

The absence of a IGFBP-1 protein band on the autoradiograms indicates that severe feeding difficulties (malnutrition) are not a factor in the etiology preceding IGF/IGFBP alterations. The absence of IGFBP-3 fragments on the immunoblots suggests that proteolytic degradation in the circulation is probably not a crucial event in the regulation of IGFBP-3 concentration in patients with cysts. However, the significance of this alteration (23) remains to be determined by quantification of IGFBP-3 protease activity in sera. In the lectin-binding assay, IGFBP-3 complexes from both groups of subjects (healthy controls and patients with liver cysts) demonstrated similar reactivity towards lectins. This suggests that the overall structure of the saccharide moieties of the IGFBP-3 complexes was not significantly altered, and that physiological interactions that depend on carbohydrate recognition (e.g., cell surface binding) most likely are unaffected.

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