

# Insulin-like Growth Factor System Abnormalities in Hepatitis C–associated Osteosclerosis

## Potential Insights into Increasing Bone Mass in Adults

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### Abstract

Hepatitis C–associated osteosclerosis (HCAO) is a rare disorder characterized by a marked increase in bone mass during adult life. Despite the rarity of HCAO, understanding the mediator(s) of the skeletal disease is of great interest. The IGFs-I and -II have potent anabolic effects on bone, and alterations in the IGFs and/or IGF-binding proteins (IGFBPs) could be responsible for the increase in bone formation in this disorder. Thus, we assayed sera from seven cases of HCAO for IGF-I, IGF-II, IGF-IIIE (an IGF-II precursor), and IGFBPs. The distribution of the serum IGFs and IGFBPs between their ternary (~ 150 kD) and binary (~ 50 kD) complexes was also determined to assess IGF bioavailability.

HCAO patients had normal serum levels of IGF-I and -II, but had markedly elevated levels of IGF-IIIE. Of the IGFBPs, an increase in IGFBP-2 was unique to these patients and was not found in control hepatitis C or hepatitis B patients. IGF-I and -II in sera from patients with HCAO were carried, as in the case of sera from control subjects, bound to IGFBP-3 in the ~ 150-kD complex, which is retained in the circulation. However, IGF-IIIE was predominantly in the ~ 50-kD complex in association with IGFBP-2; this complex can cross the capillary barrier and access target tissues. In vitro, we found that IGF-II enhanced by over threefold IGFBP-2 binding to extracellular matrix produced by human osteoblasts and that in an extracellular matrix-rich environment, the IGF-II/IGFBP-2 complex was as effective as IGF-II alone in stimulating human osteoblast proliferation. Thus, IGFBP-2 may facilitate the targeting of IGFs, and in particular IGF-IIIE, to skeletal tissue in HCAO patients, with a subsequent stimulation by IGFs of osteoblast function. Our findings in HCAO suggest a possible means to increase bone mass in patients with osteoporosis. (*J. Clin. Invest.* 1998. 101:2165–2173.) Key words: dense bones • bone formation • osteoporosis • IGFBP-2 • IGF-IIIE peptide

### Introduction

Hepatitis C–associated osteosclerosis (HCAO)<sup>1</sup> is a recently described, rare syndrome characterized by a marked increase in skeletal mass in adults who are infected with the hepatitis C virus (1–8). Spine and hip bone mineral densities are elevated as much as twofold in these affected individuals, who represent the most dramatic example of acquired osteosclerosis in humans (3). Radiographs show dense bones in the appendicular and axial skeleton, with sparing of the calvarium and facial bones. Biochemical markers of bone formation are usually elevated, and transiliac bone biopsies generally show increased bone formation rates. Nevertheless, osseous tissue from these patients appears histologically to be of good quality with intact lamellar patterns, unlike the abnormal, rapidly remodeling woven bone found in patients with Paget bone disease (1–8). Although HCAO seems to be an extremely rare syndrome, an understanding of the mediator(s) of the increased skeletal mass is of great interest (9), because controlled stimulation of osteoblast function is sought after to treat metabolic bone diseases such as osteoporosis (1–5, 10).

To date, 10 cases of HCAO have been reported (1–8). However, it is apparent that only a small percentage of all patients infected with hepatitis C develop osteosclerosis, as skeletal radiographs of 107 randomly selected hepatitis C–infected patients failed to show dense bones (11). Thus, although it is uncertain whether hepatitis C is the causative agent of the skeletal disease (3, 11, 12), a plausible hypothesis is that either hepatitis C or another parenterally transmitted agent increases hepatic production of a growth factor(s) that stimulates osteoblast function (1, 10).

IGF-I and -II are important skeletal growth factors that are produced both by the liver and by osteoblasts and are thus potential candidates for mediating the increase in osteoblastic activity in HCAO (13, 14). IGF-I and -II are synthesized as precursor forms with carboxy-terminal E-domain extensions (15, 16). These prohormones are then processed, by removal of the E-domain, into mature ~ 7-kD peptides normally present in the circulation (17, 18). IGF-II prohormone (IGF-IIIE) appears to have biologic activity similar to IGF-II (19, 20), and increased circulating levels of ~ 10–20-kD IGF-IIIE produced by certain mesenchymal and epithelial tumors have been im-

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1. Abbreviations used in this paper: ALS, acid labile subunit; HCAO, hepatitis C–associated osteosclerosis; IGFBP, insulin-like growth factor binding protein; NICTH, nonislet cell tumor hypoglycemia; rh, recombinant human.

plicated in the pathogenesis of nonislet cell tumor hypoglycemia (NICTH; 21–24).

The activities of the IGFs are modulated both systemically and locally by a family of high affinity IGF-binding proteins (IGFBPs), six of which have been characterized to date (13). The IGFBPs are structurally homologous yet functionally distinct (13). They serve a number of important roles, including transport of IGFs in the circulation, delivery of IGFs to target tissues, and modulation of cell responses to IGFs (13). 95% of IGFs in the circulation are tightly bound to IGFBP-3 with an acid labile subunit (ALS) in a ternary complex of  $\sim 150$  kD. This complex is restricted to the vascular space and serves to limit bioavailability of IGFs to target tissues (25). Approximately 5% of IGFs circulated bind to IGFBP-1, -2, and -4 in  $\sim 50$ -kD binary complexes that can cross the capillary barrier and may be responsible for targeting IGFs to specific tissues (25).

Given the importance of the IGFs in skeletal growth and differentiation, we studied the IGF/IGFBP system in 7 of the 10 HCAO cases reported to date (1–5), in comparison to four control groups: healthy age-matched individuals, hepatitis C patients without osteosclerosis, hepatitis B patients, and patients with Paget bone disease. In HCAO, we find specific abnormalities in the IGF/IGFBP system that could increase bioavailability of IGFs to skeletal tissue, causing increased bone formation.

## Methods

**Patients and control groups.** After informed consent, overnight fasting serum samples were obtained from seven of the previously reported cases of HCAO (1–5). All patients had documented acquired osteosclerosis (assessed radiologically and histologically) and serological evidence of chronic hepatitis C infection, although none had symptomatic liver disease and the hepatitis C infection tended to be an incidental clinical finding. There were five men and two women aged 32–73 yr. Sera were kept either on dry ice or at  $-70^{\circ}\text{C}$  until analyzed. Control groups included normal healthy individuals ( $n = 9$ , age range 36–71 yr), hepatitis C patients without clinical or radiologic evidence of osteosclerosis ( $n = 9$ , age range 22–70 yr), hepatitis B patients (as a control for an unrelated liver disease;  $n = 6$ , age range 23–51 yr), and patients with Paget bone disease (as a control for increased bone formation unrelated to hepatitis C infection;  $n = 5$ , age range 54–71 yr).

**IGF/IGFBP measurements.** IGF-I and IGF-II were measured by specific RIA after separation from IGFBPs by G-50 acid chromatography as described previously (26). IGF-IIE was measured by a previously validated RIA (24) using an antibody generated against a synthetic 16-amino acid segment of the predicted IGF-II E-domain (E-II<sub>69–84</sub>). E-II<sub>69–84</sub> was used as radioligand and for the standard curve. This assay is specific for proteins with the E-II domain and does not detect the mature form of IGF-II. IGFBP-3 levels were measured by RIA as previously described using covalent [ $^{125}\text{I}$ ]IGF-II: IGFBP-3 as tracer (27). IGFBP-2 levels were assayed by RIA with a polyclonal anti-IGFBP-2 antibody (1:2,000 final dilution) provided by Werner Blum (Tubingen, Germany). Recombinant human (rh) IGFBP-2 purchased from Austral (San Ramon, CA) was used for the tracer and the standard curve. IGFBP-1 was measured by a two-site immunoradiometric assay and ALS by immunosorbent assay (Diagnostic Systems Laboratories, Webster, TX).

**Column chromatography.** Sephadex G-50 column chromatography in acid was performed as previously described (24). Briefly, patient or normal serum (1 ml) was fractionated on a 120-cm G-50 Sephadex column (Pharmacia, Piscataway, NJ) in 1% formic acid. 5-ml fractions were collected, lyophilized, and then redissolved in

RIA buffer. For Superdex 200 (S-200) chromatography, a 120-cm S-200 (Pharmacia) column was equilibrated in 0.1 M Tris/HCl with 0.15 M NaCl, pH 7.4 buffer, and 0.5 ml of each serum sample was fractionated at a flow rate of 0.5 mL/min. 2-ml fractions were collected, and the IGFs and IGFBPs assayed as described above. Columns were calibrated with aldolase (158 kD), ovalbumin (43 kD), myoglobin (19 kD), cytochrome *c* (12.4 kD), and IGF-I and -II ( $\sim 7$  kD).

**Western ligand blotting.** Western ligand blot analysis for IGFBPs was performed as previously described (28). Briefly, 1  $\mu\text{L}$  of serum or 50  $\mu\text{L}$  of extracellular matrix sample (see below) was separated by SDS-PAGE with a 7.5–15% linear gradient under nonreducing conditions. The separated proteins were electroblotted onto nitrocellulose filters, and the filters were then blocked, probed with [ $^{125}\text{I}$ ]IGF-II overnight at  $4^{\circ}\text{C}$ , and visualized by autoradiography according to the method of Hossenlopp et al. (29). Scanning densitometry and molecular size determinations were performed using an UltraScan XL laser densitometer and Gel-Scan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

**IGFBP-2 binding to extracellular matrix.** Binding assays were performed using extracellular matrix derived from normal adult human osteoblastic cells (30) or fetal human osteoblastic cells immortalized with a temperature-sensitive T antigen (31). Cells were grown to confluency in 24-well plates (Primera, Falcon Laboratories, Logan, UT). Monolayers were washed and the cells removed as described by Aria et al. (32). The extracellular matrix that remained on the plates was used for the binding studies. In one set of experiments, extracellular matrix was incubated overnight at  $4^{\circ}\text{C}$  with rhIGFBP-2 (500 ng/ml, kindly supplied by Sandoz Pharmaceuticals, Basel, Switzerland) without and with 400 ng/ml IGF-I, IGF-II, or insulin. Extracellular matrix proteins were extracted and IGFBP content determined by Western ligand blotting. In a second set of experiments, extracellular matrix-coated wells were incubated overnight at  $4^{\circ}\text{C}$  with [ $^{125}\text{I}$ ]IGFBP-2 (50,000 cpm; 5  $\mu\text{Ci}/\mu\text{g}$ ) without and with unlabeled IGF-I, IGF-II, or insulin. Extracellular matrix was extracted and counted in a gamma counter (ICN Micromedex Systems, Huntsville, AL). Nonspecific binding was defined as the amount of [ $^{125}\text{I}$ ]IGFBP-2 bound in the presence of 400 ng/ml unlabeled IGFBP-2. Nonspecific binding was subtracted from total binding to determine specific binding.

**Human osteoblast proliferation studies.** Proliferation studies using normal adult human osteoblasts were performed as described previously by our laboratory (33, 34). In brief, adult human osteoblastic cells were plated in 24-well plates and grown for either 1 d or for 10 d. Cells were washed and changed to serum-free medium without or with 10 nM of IGF-II, IGFBP-2, or the combination. [ $^3\text{H}$ ]Thymidine incorporation was measured at 22–26 h.

**Statistical analysis.** Overall group differences were assessed using ANOVA. When the ANOVA was statistically significant ( $P < 0.05$ ), individual comparisons were performed using nonpaired two-sided *t* tests. Data are presented as mean  $\pm$  SEM.

## Results

**Serum levels of IGFs/IGFBPs/ALS.** Table I presents serum levels of IGFs, IGFBPs, and ALS in the HCAO patients and the four control groups. Serum levels of IGF-I and -II were not altered in HCAO and were not significantly different among the groups. Immunoreactivity for the E-peptide domain of IGF-II was elevated  $\sim 2.6$ -fold in HCAO, into the range of values previously reported for patients with NICTH using this assay (24). However, IGF-II E-domain levels were also elevated in hepatitis C patients without osteosclerosis, but not in hepatitis B patients. Among the viral hepatitis patients, therefore, IGF-II E-domain elevations were specific for the two groups of hepatitis C patients. However, this abnormality was not unique to the hepatitis C patients, because IGF-II

Table I. Serum Levels of IGFs and IGFBPs in HCAO and Control Groups (Mean $\pm$ SEM)

	IGF-I	IGF-II	IGF-II E	IGFBP-1	IGFBP-2	IGFBP-3	ALS
	$\mu\text{g/liter}$	$\mu\text{g/liter}$	$\mu\text{g/liter}$	$\mu\text{g/liter}$	$\mu\text{g/liter}$	$\mu\text{g/liter}$	$\mu\text{g/ml}$
Normals ( $n = 9$ )	228 $\pm$ 27	657 $\pm$ 55	2.7 $\pm$ 0.1	30 $\pm$ 9	377 $\pm$ 44	2274 $\pm$ 195	20.1 $\pm$ 1.0
HCAO ( $n = 7$ )	190 $\pm$ 18	505 $\pm$ 60	7.1 $\pm$ 1.0*	52 $\pm$ 11	803 $\pm$ 159 <sup>‡</sup>	1354 $\pm$ 196 <sup>§</sup>	12.2 $\pm$ 2.7 <sup>§</sup>
Hepatitis C controls ( $n = 9$ )	207 $\pm$ 20	598 $\pm$ 25	5.4 $\pm$ 0.2*	31 $\pm$ 9	353 $\pm$ 61	1505 $\pm$ 121 <sup>§</sup>	10.9 $\pm$ 1.6*
Hepatitis B controls ( $n = 6$ )	195 $\pm$ 35	546 $\pm$ 142	2.3 $\pm$ 0.2	90 $\pm$ 54	320 $\pm$ 32	1595 $\pm$ 269 <sup>  </sup>	11.2 $\pm$ 3.1 <sup>§</sup>
Paget's disease ( $n = 5$ )	129 $\pm$ 6	669 $\pm$ 38	6.7 $\pm$ 0.2*	14 $\pm$ 4	291 $\pm$ 75	2364 $\pm$ 328	17.3 $\pm$ 3.1
ANOVA ( $P$ value)	0.13	0.42	< 0.001	0.21	0.001	0.004	0.01

\* $P < 0.001$  versus normals; <sup>‡</sup> $P < 0.02$  versus normals; <sup>§</sup> $P < 0.01$  versus normals; and <sup>||</sup> $P = 0.06$  versus normals.

E-domain levels were also elevated in patients with Paget bone disease.

The IGF-II E-domain immunoreactivity in the sera of the HCAO patients was further characterized using Sephadex G-50 acid chromatography. Data from two of these patients are shown in Fig. 1. The IGF-II E-domain immunoreactivity in the HCAO sera had a molecular mass of  $\sim 10$ – $20$  kD, with some size heterogeneity, as has previously been reported for IGF-II prohormone (20, 23, 35). There was no evidence for low molecular weight E-domain fragments in the sera of these patients. Similar molecular mass estimates of  $\sim 15$  kD for the IGF-II E-domain immunoreactivity were obtained by immunoblotting HCAO sera with IGF-II antibody (data not shown). Thus, the IGF-II E-domain immunoreactivity in sera of HCAO patients corresponded to the intact IGF-II prohormone, IGF-II-E.

Table I also presents immunoassayable levels of IGFBP-1, IGFBP-2, IGFBP-3, and ALS in the HCAO and control groups. IGFBP-1 levels were not significantly different among the groups. IGFBP-3 and ALS levels were lower in the hepatitis C and B patients compared to either the normal subjects or the patients with Paget bone disease. The unique abnormality in the HCAO patients, however, was a marked increase in IGFBP-2 levels (Table I). The immunoassay data were confirmed by Western ligand blot analysis of HCAO versus normal sera (Fig. 2), which also demonstrated the significant increase in IGFBP-2 in these patients at the expected molecular size for the intact protein on SDS-PAGE ( $\sim 34$  kD). The Western ligand blot data were independently verified by immunoprecipitating with IGFBP-2 antibody (data not shown). Immunoreactive IGFBP-2 in HCAO sera was confirmed to be intact protein by Biogel P-60 acid chromatography (data not

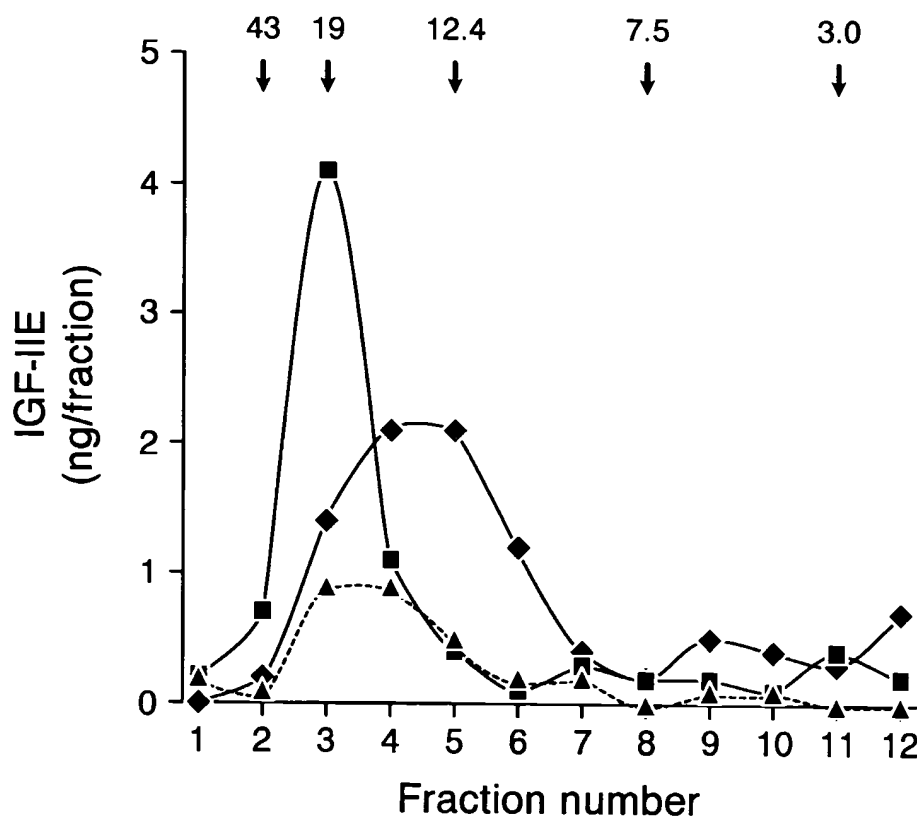


Figure 1. Sephadex G-50 chromatography at acid pH. Sera of two HCAO patients ( $\diamond$ ,  $\blacksquare$ ) and a pool of normal control serum ( $\blacktriangle$ ) were gel filtered on a Sephadex G-50 column in 1% formic acid. Fractions were assayed for IGF-II E-peptide by RIA. The arrows indicate the elution volume of proteins used as molecular size markers, with the numbers indicating the size in kD.

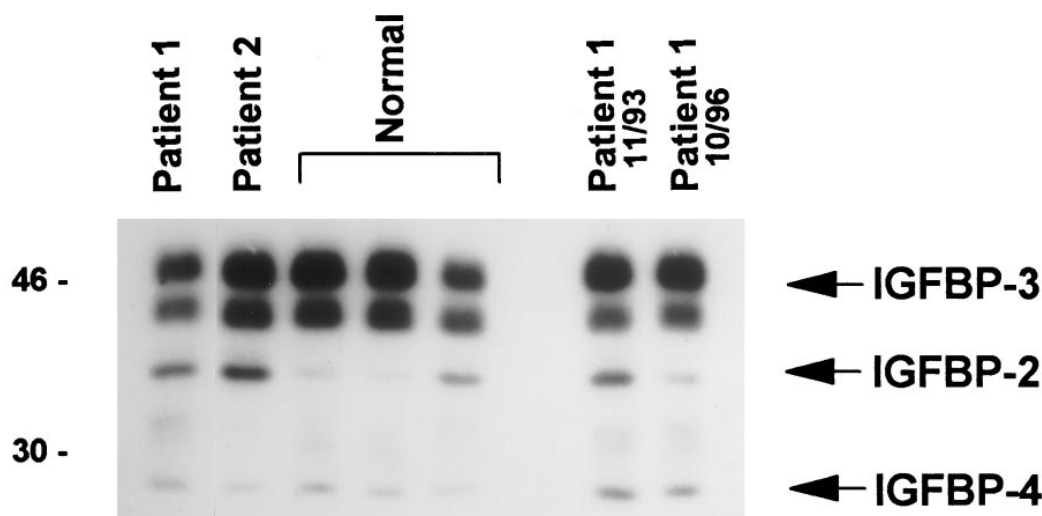


Figure 2. Western ligand blot of IGFBPs in serum from HCAO patients and normal controls. The arrows identify IGFBP-2, -3, and -4.

shown) as well as by nondenaturing S-200 chromatography (see Fig. 4). Whereas IGFBP-4 levels were not measured by immunoassay, Fig. 2 indicates that there was no apparent difference in IGFBP-4 levels between HCAO and normal subjects.

Fig. 2 also shows the decrease in serum IGFBP-2 levels between 1993 and 1996 in one of the HCAO patients who had clinical remission of bone pain over this time period (5). This remission was associated with a decrease in serum levels of liver enzymes (aspartate aminotransferase), as well as in markers of bone formation (alkaline phosphatase, osteocalcin). The biochemical changes were also associated with a marked decline in both serum IGFBP-2 and IGF-II to levels indistinguishable from the mean for the normal subjects (Table II). Treatment during this period consisted of subcutaneous calcitonin and bisphosphonates (intravenous pamidronate followed by oral etidronate) in an attempt to decrease bone turnover (5). Because increased bone turnover per se, as present in the Paget's patients, did not result in significant elevations in IGFBP-2 levels (Table I), the decline in IGFBP-2 levels over this time period was likely related to the course of the disease, perhaps due to remission of the liver disease, rather than to a nonspecific effect of a decrease in bone turnover.

**Distribution of serum IGFs and IGFBPs between 150- and 50-kD complexes.** In addition to measuring serum levels of the IGFs and IGFBPs, we also assessed the distribution of the

IGFs bound to IGFBPs in the ~150- and ~50-kD complexes in HCAO serum (Fig. 3). IGFBP-3 is the only binding protein that forms a 150-kD ternary complex with IGF and ALS (25). As shown in Fig. 3 A, most of the IGFBP-3 in the HCAO serum was found in the 150-kD complex, as is the case for normal serum. Essentially all of IGF-I in HCAO serum was associated with IGFBP-3 in the 150-kD complex, and this distribution of IGF-I did not differ between HCAO and normal serum (Fig. 3 B). Nearly all of the IGF-II in normal serum was also in the 150-kD fraction, and although most of the IGF-II in HCAO serum was in the 150-kD fraction, there was some increase in IGF-II in the 50-kD fraction in HCAO compared to normal serum (Fig. 3 C).

All of the IGFBP-2 in the HCAO patients', as well as normal sera, was in the 50-kD fraction. However, the absolute amount of IGFBP-2 was significantly increased in the patient sera (Fig. 4 A). There was no evidence by S-200 chromatography of low molecular fragments of IGFBP-2 in HCAO sera. Moreover, as shown in Fig. 4 B, the majority of IGF-II in the patient and control serum was also found in the 50-kD complex, presumably in association with IGFBP-2, which is the principal component of this complex. In HCAO serum, there was some evidence for IGF-II in the 150-kD complex as well.

**In vitro studies on IGFBP-2 interactions with the extracellular matrix of human osteoblasts.** The above in vivo data indicated not only that HCAO patients had an increase in both intact IGF-II and IGFBP-2, but also that the two proteins circulated together in the 50-kD IGF/IGFBP complex. To determine if IGFBP-2 could facilitate the transport of IGF-II and/or IGF-II to bone matrix in these patients, we assessed whether the IGF/IGFBP-2 complex could bind to human bone matrix in vitro. As shown in Fig. 5, rhIGFBP-2 (500 ng/ml) was incubated overnight at 4°C with human osteoblast-derived extracellular matrix without and with 400 ng/ml of IGF-I, IGF-II, or insulin. The extracellular matrix was washed and rhIGFBP-2 binding determined by Western ligand blot of solubilized matrix. Extracellular matrix incubated without IGFs showed minimal binding of rhIGFBP-2 (lane a). However, in the presence of IGF-I or IGF-II, the binding of rhIGFBP-2 to extracellular matrix was significantly enhanced (lanes e and f). By densitometry, rhIGFBP-2 binding to the osteoblast extracellular matrix

Table II. Changes Over Time in Serum Aspartate Aminotransferase (AST), Alkaline Phosphatase, Osteocalcin, IGF-II, and IGFBP-2 Levels in a Patient with HCAO

	November 1993	October 1996
AST, U/liter*	68	34
Alkaline phosphatase, U/liter <sup>‡</sup>	3187	248
Osteocalcin, µg/liter <sup>§</sup>	219	60
IGF-II, µg/liter	7.2	1.6
IGFBP-2, µg/liter	862	372

\*Reference range, 12–31 U/liter; <sup>‡</sup>Reference range, 98–257 U/liter; and

<sup>§</sup>Reference range, 8.8–29.7 µg/liter.

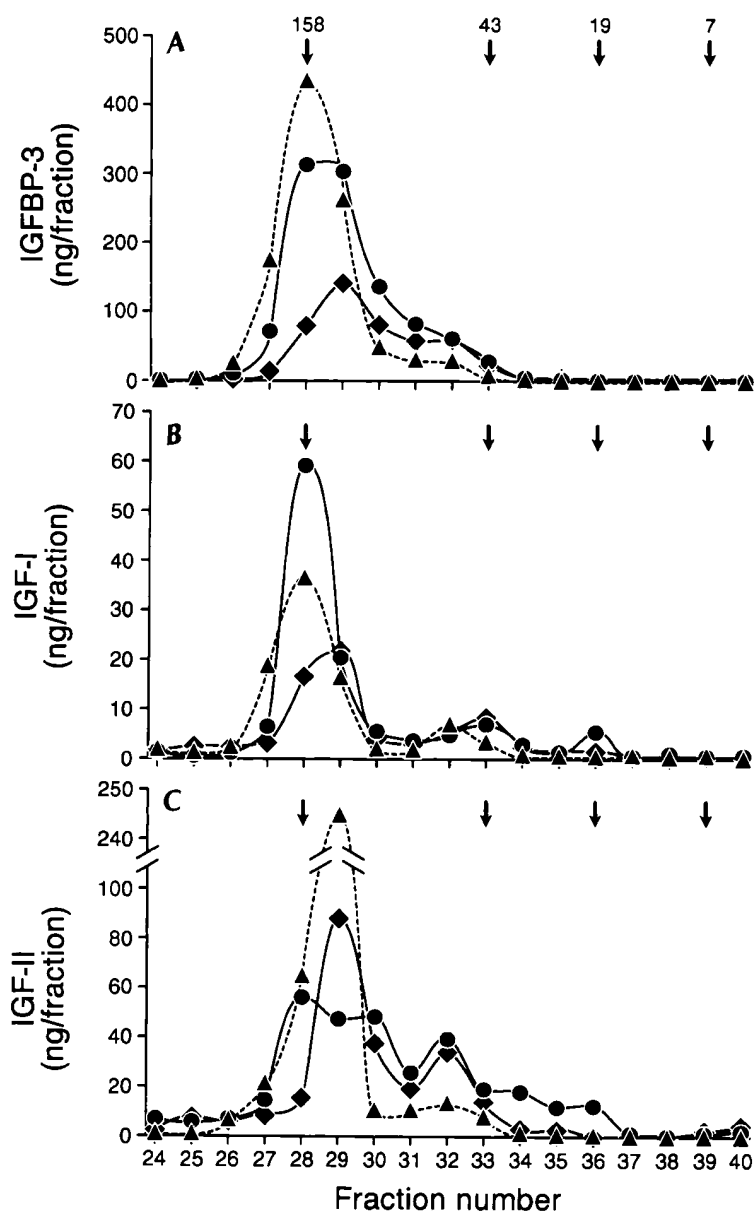


Figure 3. S-200 column chromatography at neutral pH. Sera from two HCAO patients (◆ and ●) and a control subject (▲) were filtered on a S-200 column. Fractions were assayed for IGFBP-3 (A), IGF-I (B), and IGF-II (C). The arrows indicate the elution volume of proteins used as molecular size markers, with the numbers indicating the size in kD.

was increased twofold in the presence of IGF-I, whereas rhIGFBP-2 binding was increased over threefold in the presence of IGF-II. Insulin, which is structurally related to the IGFs but does not bind IGFBPs, did not enhance rhIGFBP-2 binding to the osteoblast extracellular matrix (lane g). In the absence of exogenous rhIGFBP-2, there was little or no endogenous IGFBP present in the osteoblast extracellular matrix (lanes b–d). These findings were confirmed in another set of experiments that measured specific  $^{125}\text{I}$ -rhIGFBP-2 binding to the extracellular matrix produced by human osteoblasts. As shown in Table III, unlabeled IGF-I increased specific  $^{125}\text{I}$ -rhIGFBP-2 binding to the osteoblast extracellular matrix by approximately twofold, whereas the same concentration of IGF-II increased  $^{125}\text{I}$ -rhIGFBP-2 binding by over fourfold. The converse experiment yielded similar findings, i.e.,  $^{125}\text{I}$ -IGF-II alone did not bind extracellular matrix ( $0.05 \pm 0.05\%$ ), whereas in the presence of unlabeled rhIGFBP-2,  $^{125}\text{I}$ -IGF-II specific binding was  $2.86 \pm 0.10\%$  ( $P < 0.001$ ).

*Studies assessing IGFBP-2 effects on IGF-II stimulation of osteoblast proliferation.* The above studies indicated that IGFBP-2 could facilitate the transport of IGF-II and/or IGF-IIe to bone matrix in HCAO patients. To test whether the IGF-II/IGFBP-2 complex could stimulate human osteoblast proliferation and to assess the potential role of extracellular matrix in modulating this effect, we compared IGF-II versus IGF-II + IGFBP-2 stimulation of human osteoblast proliferation either 1 or 10 d after plating. These cells have been shown to progressively produce extracellular matrix in vitro and, in fact, form mineralized nodules with continuous culture under appropriate conditions (30). Thus, the 1-d cultures represent a relatively extracellular matrix-poor environment, whereas the 10-d cultures represent a relatively extracellular matrix-rich environment. Under both conditions, IGF-II markedly stimulated osteoblast proliferation: the percent [ $^3\text{H}$ ]thymidine incorporation increased after stimulation with IGF-II from  $0.12 \pm 0.003$  to  $0.74 \pm 0.086\%$  in the 1-d cultures

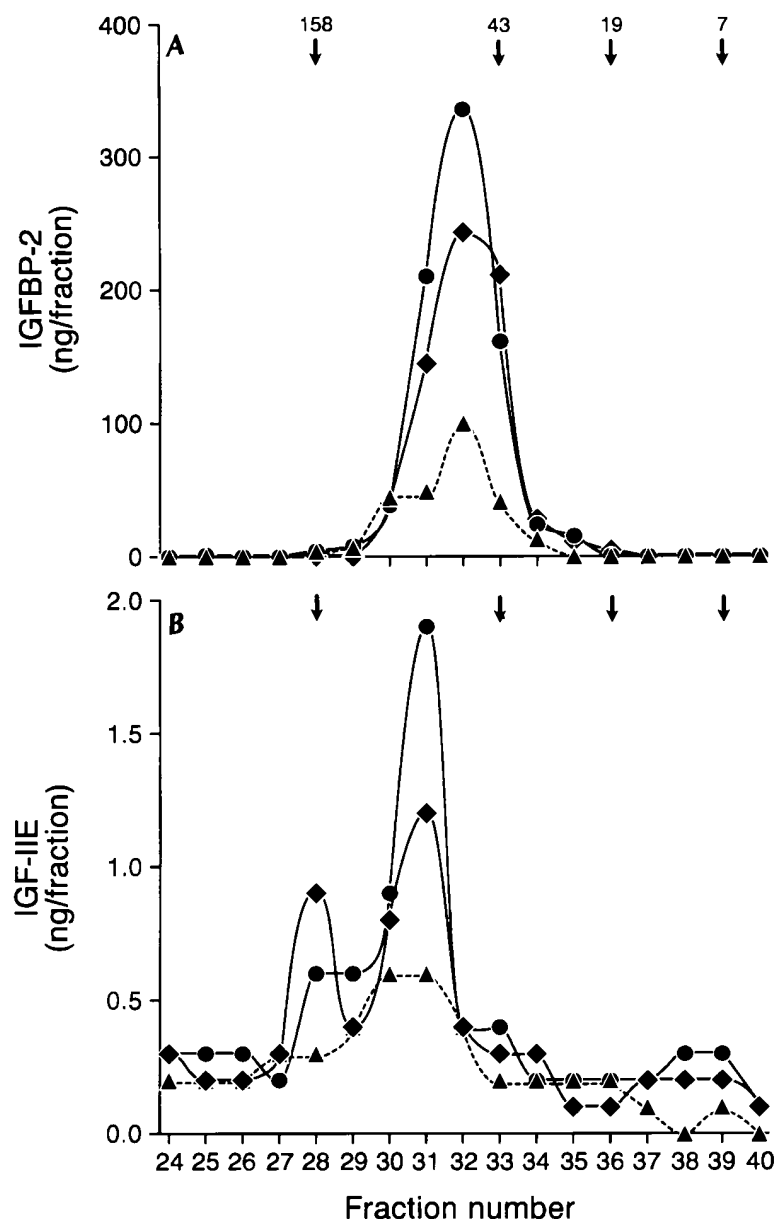


Figure 4. S-200 column chromatography at neutral pH. Sera from two HCAO patients (◆ and ●) and a control subject (▲) were filtered on a S-200 column. Fractions were assayed for IGFBP-2 (A) and IGF-II by RIA (B). The arrows indicate the elution volume of proteins used as molecular size markers, with the numbers indicating the size in kD.

( $P < 0.005$ ), and from  $0.98 \pm 0.08$  to  $2.06 \pm 0.12\%$  ( $P < 0.005$ ) in the 10-d cultures. As shown in Fig. 6, however, whereas IGFBP-2 markedly inhibited (by  $\sim 80\%$ ) IGF-II stimulation of osteoblast proliferation in the 1-d cultures, the IGF-II/IGFBP-2 complex was as effective as IGF-II in stimulating osteoblast proliferation in the 10-d cultures. IGFBP-2 alone had no effect on osteoblast proliferation under either condition (data not shown).

## Discussion

Our studies demonstrate that HCAO, which is characterized by a marked increase in bone mass in adult life (1–8), is associated with elevated serum levels of IGF-II and IGFBP-2, with little or no alteration in IGF-I and -II levels or in their serum-binding profiles. Our longitudinal data spanning 3 yr in one of the patients provide support for a cause-and-effect relation-

ship between the elevated IGFBP-2 and IGF-II levels and the increase in bone formation. In addition, our *in vitro* studies show that IGF-II promotes the binding of rhIGFBP-2 to the extracellular matrix produced by human osteoblasts, and that in the presence of this extracellular matrix, the IGF-II/IGFBP-2 complex is as effective as IGF-II alone in stimulating osteoblast proliferation. Taken together, these data provide a potential mechanism for the targeting of IGFs to bone in HCAO, with a subsequent stimulation of osteoblast function.

Increases in both IGFBP-2 and IGF-II may be required for the stimulation of bone formation observed in HCAO patients. Thus, patients with hepatitis C but without osteosclerosis or patients with Paget bone disease had elevated IGF-II immunoreactivity but normal IGFBP-2 levels. IGFBP-2 levels are elevated after long-term fasting (35), in association with certain tumors (36), and in advanced cirrhotic liver disease (37). However, none of these conditions are associated with

Table III. Specific  $^{125}$ I-labeled rhIGFBP-2 Binding to the Extracellular Matrix of Human Osteoblasts

	Percent specific binding
$^{125}$ I-labeled rhIGFBP-2 alone	$0.73 \pm 0.08$
$^{125}$ I-labeled rhIGFBP-2 + IGF-I	$1.31 \pm 0.11^*$
$^{125}$ I-labeled rhIGFBP-2 + IGF-II	$3.13 \pm 0.30^\ddagger$

\* $P = 0.01$  versus  $^{125}$ I-labeled rhIGFBP-2 alone; and  $^\ddagger P = 0.002$  versus  $^{125}$ I-labeled rhIGFBP-2 alone.

osteosclerosis as found in the HCAO patients. Finally, as part of the present study, we tested the hepatitis C genotype of four of the seven HCAO patients, and failed to observe a common genotype or novel strain of hepatitis C (data not shown).

It should be noted that the measurements of IGF-IIIE in serum ( $\sim 10$ – $20$  kD) were based on standard curves using the E-II<sub>69–84</sub> fragment (1.8 kD). Furthermore, although the antibody generated against E-II<sub>69–84</sub> specifically recognizes the IGF-IIIE prohormone, it does not appear to have full access to this epitope in the larger IGF-IIIE precursor form (24, 38). A recombinant IGF-IIIE that consists of IGF-II plus the first 21 amino acids of the E-peptide domain is only 3–17% as effective an antigen as the E-II<sub>69–84</sub> peptide in the IGF-II E-domain RIA (24, 38). Thus, on a molar basis, conservative estimates of circulating levels of intact IGF-IIIE in HCAO patients would equal or exceed levels of IGFBP-2 ( $\sim 24$  nM).

The only other known disorder associated with both IGFBP-2 and IGF-IIIE elevations is NICTH (22, 23, 25). NICTH patients develop severe hypoglycemia, and although bone mass or bone turnover have not been assessed in these individuals, they have not been reported to have osteosclerosis. Conversely, none of the patients with HCAO has had clinically evident hypoglycemia. Indeed, two of the HCAO patients had diabetes mellitus and received therapy with either an oral agent or with insulin (5, 8), and serum-fasting glucose concentrations in at least six of the patients have not been low (Whyte, M.P., unpublished data).

The primary cause of hypoglycemia in NICTH appears to

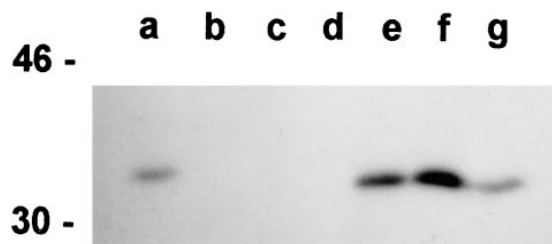


Figure 5. Binding of rhIGFBP-2 to extracellular matrix produced by human osteoblasts. Extracellular matrix was prepared from confluent human osteoblast cultures as described in Methods. Binding was carried out overnight at  $4^\circ\text{C}$ , and IGFBP associated with the extracellular matrix determined by Western ligand blotting. Lane a: exogenous rhIGFBP-2 alone; lane b: exogenous IGF-I alone; lane c: exogenous IGF-II alone; lane d: exogenous insulin alone; lane e: rhIGFBP-2 + IGF-I; lane f: rhIGFBP-2 + IGF-II; lane g: rhIGFBP-2 + insulin. Migration positions of molecular size markers (in kD) are indicated on the left.

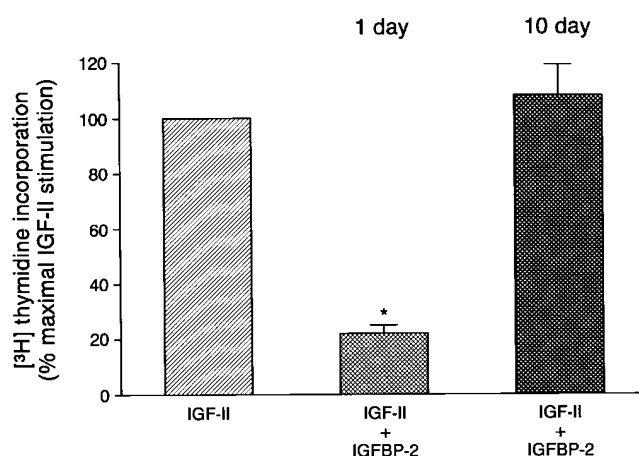


Figure 6. Effects of IGFBP-2 on IGF-II (both at 10 nM) stimulation of osteoblast proliferation in cells cultured either for 1 or 10 d before stimulation with IGF-II or IGF-II + IGFBP-2.  $^3\text{H}$ Thymidine incorporation was determined as described in Methods. Data are expressed as the percent of maximal IGF-II stimulation (mean  $\pm$  SEM of three experiments). \* $P < 0.001$  versus IGF-II alone.

be the effect of tumor-derived IGF-IIIE to directly and indirectly impair formation of the 150-kD ternary IGF/IGFBP-3/ALS complex (i.e., by decreasing growth hormone and growth hormone-dependent ALS) (22, 23, 25). In contrast, formation of the 150-kD complex is not impaired in HCAO, with all of IGF-I and the majority of IGF-II remaining tightly bound to IGFBP-3 in the ternary complex, as in the case of normal serum. It is attractive to speculate that if, in HCAO, IGF-IIIE and IGFBP-2 are produced by the same tissue (i.e., the liver) and rapidly form a complex, then the normal IGF/IGFBP pattern might not be disturbed, explaining an absence of hypoglycemia.

Our studies also demonstrate that the IGFBP-2 and IGF-IIIE in HCAO sera circulate together in a 50-kD complex. Several lines of evidence suggest that in this form, IGFBP-2 may facilitate the transport of IGF-IIIE or IGF-II to skeletal tissue. Prosser et al. (39) have shown that the concomitant infusion of IGFBP-2 with IGF-I or -II in goats increased the clearance of IGF-I and -II, and at the same time reduced the transfer of IGFs into breast milk. They postulated that IGFBP-2 facilitates the transit of the IGFs out of the circulation, and away from the mammary epithelium, potentially towards some other tissue. The findings in the HCAO patients and our in vitro data suggest that this other tissue may well be the skeleton. Our demonstration that IGFs, and especially IGF-II, enhance IGFBP-2 binding to the extracellular matrix produced by human osteoblasts provides a potential mechanism for the selective transport of IGF-IIIE (and possibly IGF-II) to the bone matrix of HCAO patients. Our in vitro studies are similar to recent observations of Arai et al. (32), who showed that the IGF/IGFBP-2 complex was able to bind the extracellular matrix produced by fibroblasts. These investigators further demonstrated that binding of IGFBP-2 was principally to glycosaminoglycans, which are abundant in the bone matrix (32).

We also found that under conditions in which extracellular matrix is present, the IGF-II/IGFBP-2 complex is as effective as IGF-II in stimulating osteoblast proliferation. In contrast to

our findings, several previous studies (40–43) indicate that IGFBP-2 can inhibit IGF action. There are, however, important differences between these studies and our in vivo and in vitro findings. All of the previous studies demonstrating an inhibitory effect of IGFBP-2 on IGF-I stimulation of osteoblast function used a 10-fold or greater molar excess of IGFBP-2 relative to IGF-I (40–43). Our findings suggest that circulating levels of intact IGF-II in HCAO patients would equal or exceed levels of IGFBP-2; hence, IGFBP-2 would not be in significant molar excess in HCAO patients. In the in vitro study in Fig. 6, we used an equimolar amount of IGFBP-2 and IGF-II, and IGFBP-2 was not inhibitory in the 10-d cultures. This is, in fact, consistent with previous studies in the rat fetal calvarial system, where equimolar amounts of IGFBP-2 did not inhibit IGF-I action (40). Thus, both the concentration of IGFBP-2 used and the presence or absence of extracellular matrix appear to be important factors in determining IGFBP-2 effects on IGF bioavailability. A similar situation appears to be present in the case of IGFBP-3, which can inhibit IGF action (44), but IGFBP-3 at low concentrations (45) or cell-associated IGFBP-3 (46) is not inhibitory for IGF-I effects. In addition, IGFBP-5 binding to extracellular matrix results in decreased affinity of IGFBP-5 for IGFs, thus increasing the bioavailability of IGFs (47). Clearly, more studies are needed to better define the role of extracellular matrix in modulating the effects of IGFBP-2 on IGF action.

Finally, our studies have obvious potential implications for the treatment of osteoporosis (3, 10, 12). Whereas several anti-resorptive drugs, including estrogen, calcitonin, bisphosphonates, and selective estrogen receptor modulators are now available to treat osteoporosis, the ability to achieve large, sustained increases in bone mass remains an elusive goal. As noted earlier, HCAO is associated with marked increases in bone mass in adult life. Our data indicate that these increases in bone mass may be related to specific abnormalities in the IGF/IGFBP system. However, the notion of using IGFs to treat osteoporosis is not a novel one (48, 49). Previous studies in humans using IGF-I treatment have found increases in serum levels of markers of bone formation (48, 49). However, this approach is limited by the multiple systemic effects of IGF-I, including hypoglycemia. In an attempt to circumvent this problem, recent animal studies have used a combination of IGF-I and IGFBP-3, thus permitting administration of higher doses of IGF-I, and some increase in bone formation indices was noted (50, 51). Our findings with the HCAO patients, however, suggest that even more efficient transfer of IGFs to the skeleton could potentially be accomplished using IGFBP-2. If further studies validate this hypothesis, the insights provided by this rare but fascinating disorder may lead to the development of novel strategies to not just prevent, but actually reverse the loss of bone in patients with osteoporosis.

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