EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 3, 243-250, June 2004

Growth impairment of primary chondrocyte cells by serum of rats with chronic renal failure

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Accepted 17 May 2004

Abbreviations: BUN, blood urea nitrogen; CRF, chronic renal failure; FCS, fetal calf serum; GH, growth hormone; GHBP, GH binding proteins; IGF-I, insulin-like growth factor-I; IGFBP, IGF binding protein; MTT, methylthiazol tetrazolium; SFM, serum free medium

Abstract

Insulin-like growth factor (IGF)/IGF binding protein (IGFBP) abnormalities may be important in the pathogenesis of growth failure in chronic renal failure (CRF). We induced experimental CRF by 5/6 nephrectomy in Sprague Dawley rats (100 g) and observed for 2 weeks comparing with sham-operated pair-fed control rats (Sham- C). CRF rats gained 30% less height than Sham- C rats ($P < 0.01$). Serum IGFBP profiles by Western ligand blot revealed that IGFBP4 was elevated two fold in CRF rats ($P < 0.01$ vs. Sham-C). However, IGFBP4 mRNA levels in liver or skeletal muscle were not different in two groups. To determine if the increase of serum IGFBP4 in CRF retarded the growth of cartilage, epiphyseal chondrocytes were isolated from CRF or control rats and cultured in the presence of control or CRF rat sera. Incubation with 10% CRF serum reduced proliferations of normal chondrocytes and L6 rat skeletal muscle cells. In contrast, 10% CRF serum did not inhibit the growth of CRF chondrocytes. Rat sera from two groups were separated into two different fractions, high (>10 kDa, containing IGFBPs) and low (<10 kDa, containing free IGF) molecular weight fractions using a gel filtration column. Both fractions obtained from CRF sera decreased the growth of control chondrocytes up to 40% compared with those from control sera. We suggest that the pathogenesis of growth failure in CRF may be involved in the increase of circulating IGFBP4 as well as the unidentified small molecular weight uremic serum factors which block the growth of chondrocytes in growth plate.

Keywords: chondrocytes; CRF; growth failure; IGFBP4; rat; serum factor

Introduction

Growth retardation is a serious problem found in children with chronic renal failure (CRF). Several factors have been identified as contributors to impaired linear growth and they include protein and calorie malnutrition, metabolic acidosis, growth hormone resistance, anemia, and renal osteodystrophy (Kuizon and Salusky, 1999). For the treatment of these children, supraphysiological concentration of growth hormone (GH) is administered although there is no evidence of GH deficiency in CRF patients (Mehls *et al.,* 2002). In fact, normal or elevated serum GH concentrations are commonly reported in CRF.

The growth promoting effect of GH is largely mediated by insulin-like growth factor-I (IGF-I). IGF-I is released from liver after GH stimulation and the serum IGF-I mediates mitogenic growth of cartilage and bone cells. Therefore, maintenance of the serum GH/IGF-I axis is thought to be important in normal growth, and alteration of this axis is considered as one of the factors for the growth impairment in CRF (Tonshoff *et al.,* 1990). GH bioactivity may be controlled by plasma GH binding proteins (GHBP) and GH receptor levels (Tonshoff *et al.,* 1994). Plasma level of GHBP was increased and GH receptor level in liver was decreased in CRF, which might be causes of GH resistance. Furthermore serum IGF-I and IGF-II levels were slightly decreased and IGFBP levels were increased in CRF patient sera possibly due to the reduced renal clearance (Mehls *et al.,* 1992). Increase of serum IGFBPs could reduce the bioavailability of free IGF resulting in growth impairment in CRF (Richmond *et al.,* 2001).

Six IGFBPs with high binding affinity to IGFs and

several IGFBP-rPs with low affinity have been identified from human and rat serum, several body fluids, and cell lines and their cDNAs were all cloned although the roles of individual IGFBP have not been understood clearly (Kim *et al.,* 1997; Hwa *et al.,* 1999). The suggested roles of IGFBPs in circulation are following: 1) IGFBPs inhibit receptor binding of IGF resulting in inhibition of biological activity, 2) BPs may serve as a transporter of IGF to the cell, 3) IGF-IGFBP complexes may interact with the cell surface, 4) free IGFBP itself has biologically active (Oh *et al.,* 1995). The profiles of IGFBPs are different in different biological fluids. In human serum, IGFBP1, IGFBP2 and IGFBP3 are predominantly present while IGFBP1, IGFBP3 and IGFBP4 are in rat serum (Donovan *et al.,* 1989; Oh *et al.,* 1993b). IGFBP4 was first identified as an inhibitory IGFBP on human osteoblast proliferation (LaTour *et al.,* 1990) and further characterized as growth-inhibitory binding proteins in the growth cartilage (Wagner *et al.,* 2000; Kiepe *et al.,* 2001).

In the present study, IGFBPs in rat CRF model were investigated in order to obtain a better understanding the role of IGFBP on growth defect in CRF patient. The effects of the CRF and control sera on the growth of rat epiphyseal chondrocytes were studied. The results could provide us a direct evidence for the inhibitory effects of serum components on the growth of growth plate chondrocytes.

Materials and Methods

Animals

Male Sprague-Dawley rats (100 g) underwent 5/6 nephrectomy or sham operation. Sham-operated animals were pair-fed to nephrectomized rats and water was provided. Two weeks after the operation, serum, liver, skeletal muscle, and joints were obtained during nembutol anesthesia. Before and after the experiment the weight and nose to tail length of the rat were measured. Serum and urine creatinine, and blood urea nitrogen (BUN) level was determined as described previously (Mak and Pak, 1996).

Western ligand blot

Rat serum (2 μl) were separated on 12% SDS-PAGE and western ligand blot using ^{125}I-IGF-I and ^{125}I-IGF-II was performed as described (Hossenlopp *et al.,* 1986). Urine samples were concentrated (1:10) using Centricon-10 (Amicon Inc, MA) and 100 μl of concentrated urine was analyzed as described above. The nitrocellulose paper was autoradiographed and the bands were quantified by densitometry.

Immunoprecipitation

Serum samples (2 μl) were immunoprecipitated with anti-rat IGFBP4 antibody (kindly provided by Dr. R. Rosenfeld, Univ. of Oregon Health Science, OR) and separated on 12% SDS-PAGE. The gel was transferred onto nitrocellulose membrane, and then western ligand blot was performed as described above.

Northern blot analysis

Total RNA was isolated from different tissues as described by Chomzynski and Sacchi (Chomczynski and Sacchi, 1987; Eum *et al.,* 2003). RNA was separated on 1% agarose/2.4 M formaldehyde gels, transferred to Nitroplus 2,000 paper (Microseparation Inc., Wesrboough, MA), and subsequently hybridized with ^{32}P-labeled human IGFBP4 cDNA probe (1.2 kb *Xho*I fragment, a generous gift from Dr. R. G. Rosenfeld, Oregon Health Science Center) as described previously (Fong *et al.,* 1995). For normalization of even loading, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was also determined using cDNA probe (Pak *et al.,* 1996).

Serum fractionation

Pool of sera obtained from either control or CRF rats were fractionated into two different molecular weight parts using G200-Sephadex chromatography (Martin and Baxter, 1986). Collected fractions were lyophilized and reconstituted in water to the original volume. High and low molecular weight fractions were named as BP ($>$10 kDa) and IGF ($<$10 kDa) fractions, respectively.

Cell culture and primary epiphyseal chondrocyte culture

Rat epiphyseal chondrocytes were isolated as described previously. Briefly, cartilage from rat tibial growth plate was dissected, digested with collagenase (Type II and IV, Worthington Biochemicals, Freewood, NJ), washed with phosphate buffered saline and filtered though a 100 mm polypropylene mesh (Mak and Pak, 1996). Cell number was determined by counting on a hemocytometer and viability was determined by tryphan blue dye exclusion. In each isolation, cells from 4 rats per group were pooled. Subsequently, cells were suspended in complete media: 1:1 mixture of Dulbecco's minimum essential medium (DMEM) and Ham's F12 supplemented with gentamicin and 10% fetal calf serum (FCS) and grown until confluence on 100 mm culture dishes. The cells were then trypsinized and seeded in 24 well plates coated with poly-L-lysine at a density of 4×10^4 cells per well and allowed to attach to the plate in 10% FCS for 24

Table 1. Growth and blood chemistry of the CRF and sham-control rats.

Group	Initial weight (g)	Weight gain (g)	Initial length (cm)	Length gain (cm)	BUN[a] (mg/dl)	Serum creatinine (mg/dl)
CRF (n = 12)	99.1±2.1	32.2*±7.1	27.4±0.5	4.3*±0.40	35.8*±4.4	0.72*±0.09
Sham-C (n = 11)	99.7±2.2	57.5±4.3	27.0±0.3	6.3±0.7	12.5±1.3	0.41±0.05

[a]BUN, blood urea nitrogen. Data = Mean±SD. *: $P < 0.01$ vs Sham-C.

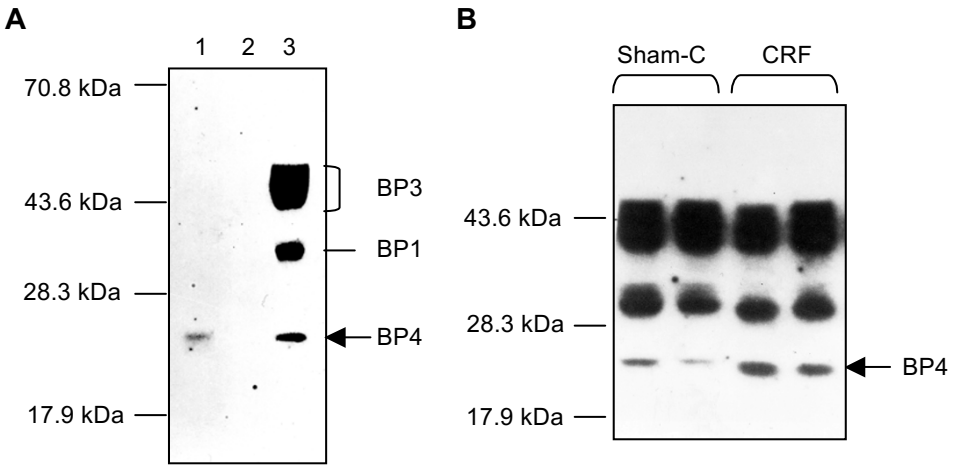

Figure 1. Western ligand blots of rat sera (A) Rat serum (2 μl) was either immunoprecipitated using anti-rat IGFBP4 antibody (lane 1) or control antibody (lane 2) separated on 12% SDS-PAGE Serum sample without immunoprecipitation is shown in lane 3 (B) Sera (2 μl) obtained from sham-operated control (Sham-C) and CRF (CRF) rats were separated on 12% SDS-PAGE. The gels were transferred onto nitrocellulose membrane, and incubated with ^{125}I-IGF-I and ^{125}I-IGF-II. The membrane was washed and autoradiographed. The position of IGFBP4 was marked with arrows.

h. Then the cells were washed and resuspended in serum free medium (SFM): 1:1 mixture of DMEM and Ham's F12 supplemented with gentamicin, 20 nM selenium and lipid emulsion containing lecithin, cholesterol, sphingomyelin, vitamin E and vitamin E acetate. L6 rat skeletal muscle cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For growth assessment, the cells were incubated with SFM for 48 h, and the test sera obtained from CRF and control rats were added into each well and incubated for 24 h. Cell number, ^{3}H-thymidine incorporation or mitogenic activity using methylthiazol tetrazolium (MTT) were determined as described (Mak and Pak, 1996).

Statistics

All growth studies were repeated 4-6 times using different pools of cells. Each pool of cells was obtained from 3-4 different rats of CRF and control, respectively. Unpaired t-test using unequal variance was used for statistical analysis. P values less than 0.05 was accepted as statistically significant.

Results

Renal failure of nephrectomized animals was confirmed by BUN (CRF, 35.8 ± 4.4 mgl/dl; Sham-C, 12.5 ± 1.3 mgl/dl) and serum creatinine (CRF, 0.72 ± 0.09 mgl/dl; Sham-C, 0.41 ± 0.05 mgl/dl) that were much higher ($P < 0.01$) than those of sham animals (Table 1). Weight and longitudinal growth of CRF and control rats are presented in Table 1. CRF rats grew 30% less than the controls ($P < 0.01$).

To determine the levels of IGFBPs in rat serum, Western ligand blots were carried out. As shown in Figure 1, IGFBP3 complexes (45 kDa), IGFBP1 (32 kDa) and IGFBP4 (24 kDa) were observed predominantly in rat serum. This profile is different from that of human in which IGFBP1 and IGFBP3 are major IGFBPs (LaTour *et al.,* 1990). The band of 24 kDa protein was confirmed as IGFBP4 by immunoprecipitation using antibody raised against rat IGFBP4

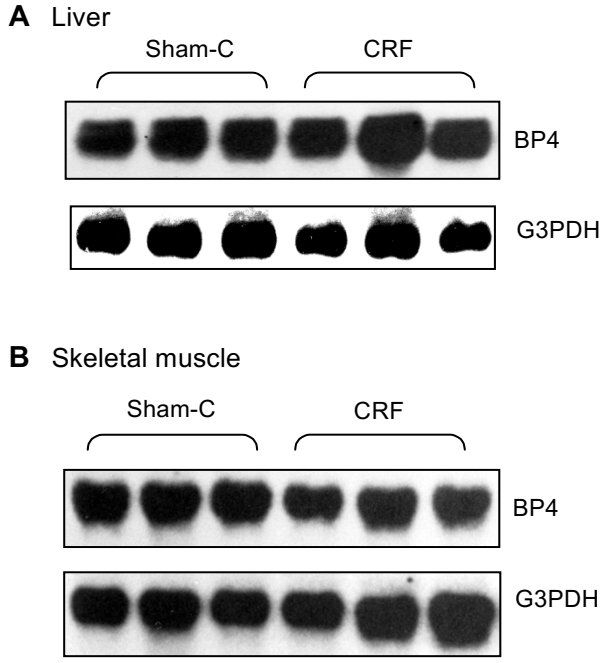

Figure 2. Northern blot analysis of IGFBP4. Total RNA was isolated from liver or skeletal muscle obtained from sham control (Sham-C) and CRF rats (CRF). Northern blot analysis was performed as described in Method section. The mRNA levels of IGFBP4 and G3PDH were quantified using densitometer and relative levels of IGFBP4 expression were determined to the level of G3PDH.

(lane1, Figure 1A). Figrue 1B showed that the levels of IGFBP4 were approximately 2 fold increased in CRF compared with those of controls ($P < 0.01$). The levels of other IGFBPs were not different in two groups.

To determine if the increase of serum IGFBP4 was resulted from its enhanced synthesis, the steady state levels of IGFBP4 mRNA in liver and skeletal muscle, which were major sources of IGFBP production, were determined by Northern blots. Figure 2 showed that IGFBP4 mRNA levels were not altered in CRF tissues. The result tells us that the IGFBP4 increase in CRF is not a result from the increased steady state mRNA levels in liver and skeletal muscle.

In order to determine whether the CRF serum has direct effects on the growth of cartilage, epiphyseal chondrocytes were isolated and the effects of CRF rat serum on proliferation of the chondrocyte cells in primary culture were studied. The serum obtained from CRF rats inhibited the incorporation of ^{3}H- thymidine ($P < 0.01$, Figure 3A) and the proliferation of control chondrocytes in cell number to the null ($P < 0.01$, Figure 3B) when 10% concentration was utilized. At 10% concentration, the CRF serum also blocked the growth of L6 rat skeletal muscle cells in aspects of cell number and thymidine incorporation (Figure 4). Interestingly, the CRF serum did not affect

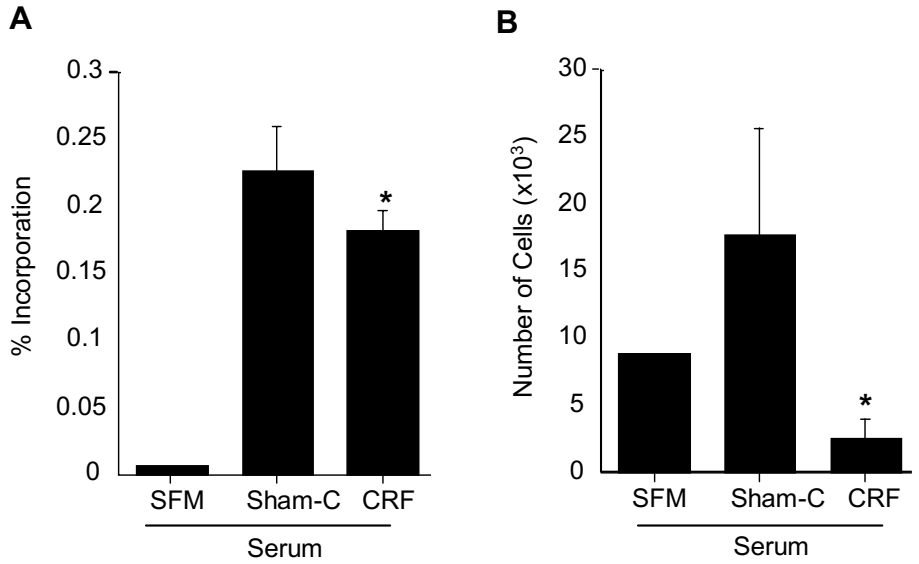

Figure 3. Effects of rat sera on the proliferation of normal chondrocytes. Epiphyseal chondrocytes were isolated from sham-control rats. The cells were cultured in SFM on 24-well plate in the presence of either 10% control (Sham-C) or CRF rat sera for 24 h. Cell proliferation was assessed by ^{3}H-thymidine incorporation (panel A) and counting cells using hemocytometer (panel B). For ^{3}H-thymidine incorporation, radiolabeled thymidine was added 18h prior to harvesting cells. % incorporation was calculated by radioactivity associated with cells divided with total radioactivity. *: $P < 0.01$ vs Sham-C.

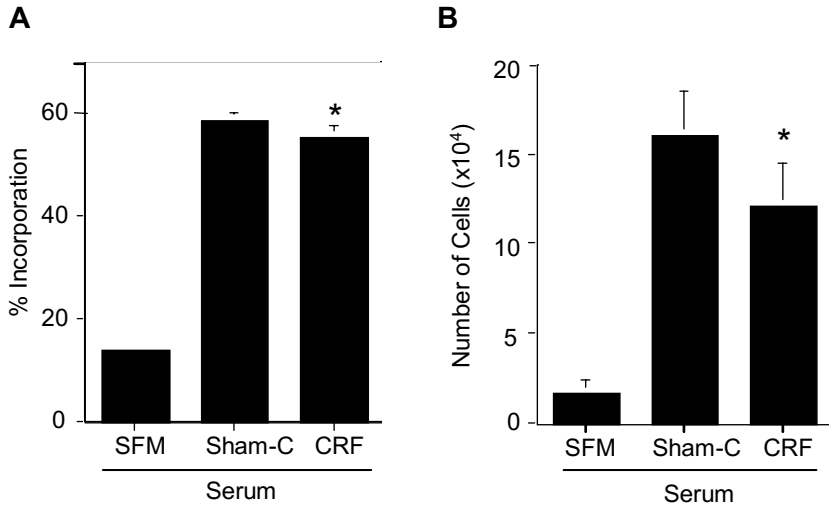

Figure 4. Effects of rat sera on the proliferation of L6 rat skeletal muscle cells. L6 cells were grown on 24-well plate and the effects of 10% control (Sham-C) or CRF rat sera in serum free media (SFM) were measured as described in Figure 3. *: $P < 0.01$ vs Sham-C

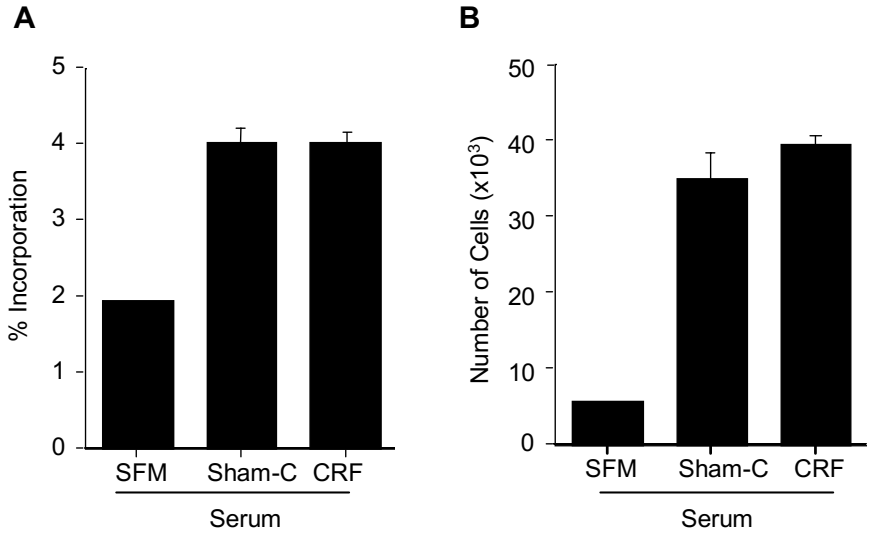

Figure 5. Effects of rat CRF serum on the proliferation of CRF chondrocytes Epiphyseal chondrocytes were isolated from CRF rats and the effects of 10% control (Sham-C) or CRF rat sera in serum free media (SFM) were examined as described in Figure 3.

the growth of CRF chondrocytes (Figure 5). MTT mitogenic assay showed that the CRF serum inhibited the growth of the control chondrocytes at 10%, probably above 5% concentration (Figure 6).

IGFBP4 was reported as an inhibitory protein on cell proliferation. Since we observed the IGFBP4 protein levels in CRF serum increased, IGFBP4 was thought to be a candidate component which might be responsible for growth failure in CRF. Therefore, the sera obtained from either CRF or control rats were partially purified into two different molecular weight fractions containing either IGFBP proteins or free IGFs using gel filtration chromatography. The obtained two fractions were named as BP ($>$10 kDa) and IGF ($<$ 10 kDa) fractions, respectively. Chondrocytes were then incubated with 10% of the BP or IGF fractions reconstituted after lyophilization. Unexpectedly, both fractions from CRF sera retarded the growth of the control cells to the similar extent (40%, $P < 0.05$) when the growth responses were compared to the

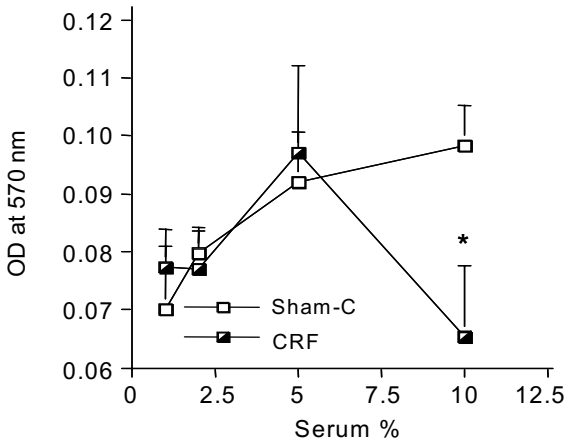

Figure 6. Dose-response of control (Sham-C) or CRF rat sera on the proliferation of normal chondrocytes determined by MTT assay. Epiphyseal chondrocytes were isolated from sham-control rats and the effects of different concentrations of control and CRF rat sera in serum free media (SFM) were examined by mitogenic MTT assay. *: $P < 0.01$ vs Sham-C at 10%.

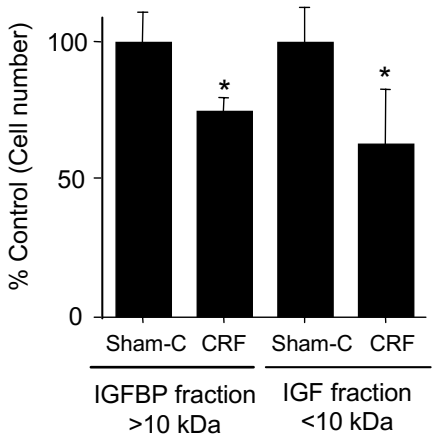

Figure 7. Effect of serum IGFBP and IGF fractions on the growth of normal chondrocytes. Epiphyseal chondrocytes were isolated from control rats and the effects of the IGFBP (>10 kDa) and IGF (<10 kDa) fractions obtained from sham-control (Sham-C) or CRF rat sera in serum free media (SFM) were examined as described in Figure 3.

fractions separated from the control rats (Figure 7). But both CRF serum fractions did not retard the proliferation of CRF cells (data not shown).

Discussion

Increase of IGFBPs in CRF patient serum has been noticed for a long time but the exact role of IGFBPs in CRF has not been clearly understood. This study demonstrated that the possible growth impairment effect of IGFBPs in CRF serum on primary culture of epiphyseal chondrocytes. Since human and rat IGFBP profiles were different, different IGFBP would be altered in rat models. As expected, IGFBP4 was increased in CRF rat serum rather than IGFBP3 which was increased in human CRF serum (Tonshoff *et al.,* 1994). IGFBP3 concentration in human CRF serum was increased due to the decreased serum and urine protease activity towards IGFBP3 (Lee *et al.,* 1994) but the mechanism of IGFBP4 increase in rat CRF serum have not been studied. As shown in Figure 2, IGFBP4 mRNA expression in liver and skeletal muscle was not changed in CRF rats compared to the controls. We observed that neither protease activity in rat CRF serum nor IGFBP4 clearance in urine was altered when they were determined by radiolabeled IGFBP4 degradation and urine western ligand blots (data not shown). Therefore, IGFBP4 in rat CRF serum seemed to be enhanced by unidentified mechanism. One of the possible steps might be the release of IGFBP4 from the local storage, such as IGFBP receptor, which was identified in IGFBP3 system (Oh *et al.,* 1993c). However, the presence of receptor for IGFBP4 has yet been reported.

It has been demonstrated that IGFBPs may be an important regulator of the cell growth. IGFBPs might inhibit the growth response of IGF through binding to IGF and reducing the bioavailabilty of IGF. Overexpression of IGFBP3 inhibited the growth of breast cancer cells (Oh *et al.,* 1993a; Oh *et al.,* 1995) and IGFBP4 inhibited human osteoblast and osteosarcoma cell proliferation (Mohan *et al.,* 1989). Since growth retardation in CRF patients and rat CRF models would be a result of serum IGFBP4 increase that might decrease or prevent IGF binding to its signaling receptor or IGFBPs might exert a direct effect on the growth of cartilage. Our results demonstrated that rat CRF serum inhibited the proliferation of normal chondrocyte primary cultures when it was present at 10% concentration and the CRF serum concentration lower than 10% did not inhibit the proliferation of the normal cells. Thus it was suggested that certain levels of inhibitory molecules in CRF serum were necessary for the effects. The same concentration (10%) of CRF serum did not inhibit the proliferation of the CRF chondrocytes. This is an interesting observation suggesting that CRF chondrocytes are different from normal chondrocytes in responding to exogenous stimuli. This may be because cells undergo an "adaptation" process to survive when the cells are exposed to CRF serum environment for a long time. The end-organ resistance of CRF chondrocytes to the growth stimulating peptides which we reported previously (Mak and Pak, 1996) would be one of adaptation processes.

To identify the nature of the inhibiting molecules,

probably IGFBP4, the CRF and control rat sera were fractionated into high and low molecular weight parts. High molecular weight fractions (>10 kDa) contained IGFBPs and IGF-IGFBP complexes, and the low molecular weight fractions (<10 kDa) did free IGF. This fractionation method was usually used for determining the concentration of free IGF in serum. Interestingly both fractions retarded proliferation of normal chondrocytes, but not of CRF cells. Cell proliferation inhibitory effects of the fractionated CRF serum were less than those of whole CRF serum. It is possible that inhibitory molecules in BP fractions might be either free IGFBP4 or IGF-IGFBP4 complexes. Inhibitory molecules in IGF fractions might be the unidentified novel small molecules present in CRF serum. Inhibition of chondrocyte proliferation of whole CRF serum would be additive or synergistic effects of those two high and low molecular weight molecules. Further studies are required to characterize the nature of the molecules in detail.

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