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Original article

Growth hormone resistance and inhibition of somatomedin activity by excess of insulin-like growth factor binding protein in uraemia

Werner F. Blum[1], Michael B. Ranke[1], Klaus Kietzmann[1], Burkhardt Tönshoff[2], and Otto Mehls[2]

[1] University Children's Hospital, Tübingen, Federal Republic of Germany
[2] University Children's Hospital, Heidelberg, Federal Republic of Germany

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Abstract. Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) were studied in children with end-stage renal failure (ESRF, $n = 31$) and chronic renal failure ($n = 11$) with residual glomerular filtration. Somatomedin bioactivity in patient sera was found to be decreased while IGF-I and IGF-II levels by radio-immunoassay (RIA) were normal. In contrast, IGFBP-1 and IGFBP-3 levels (measured by RIA) were markedly increased in uraemia. Excess IGFBP was shown to be able to bind IGF by determination of the free IGF binding capacity. Using high-performance liquid chromatography a shift of the IGFBP-3 profile to low molecular weight components could be demonstrated in ESRF. Affinity cross-linking experiments showed that these low molecular weight IGFBP-3 immunoreactive forms are biologically active. In normal urine only IGFBP-3 forms smaller than 60 kDa were detected with a major peak at 12–20 kDa. Removal of excessive IGFBP from patient sera by affinity chromatography on an IGF-II Sepharose column resulted in a significant increase in somatomedin bioactivity. Model calculations on the interaction of IGF and IGFBP using empirical data suggested a reduction of IGF secretion in uraemia by an order of magnitude. It is concluded: (1) that renal failure causes an accumulation of low molecular weight IGFBP, (2) that the resulting excess of IGFBP acts as a somatomedin inhibitor, and (3) that in uraemia there is a relative growth hormone resistance with respect to IGF production.

Key words: Insulin-like growth factor – Binding protein – Growth hormone – Chronic renal failure – Uraemia

Introduction

Growth retardation is a common problem in children with chronic renal failure (CRF). Various factors are possibly involved in this phenomenon including abnormalities in the growth hormone (GH)-somatomedin axis. Although serum levels of GH [1] and insulin-like growth factors (IGFs) were found to be normal or slightly elevated in these patients [2, 3], serum somatomedin bioactivity (SmBA) is decreased [4, 5]. This decrease was attributed to the presence of somatomedin inhibitors [6].

The principal somatomedins are IGF-I, which mediates the growth-promoting effect of GH, and IGF-II, which is less GH dependent and whose physiological role is still obscure. Unlike most peptide hormones somatomedins are bound to specific carrier proteins (IGF binding proteins, IGFBPs) in the circulation.

The IGFBPs constitute a family of proteins. To date at least three classes of IGFBPs can clearly be distinguished (IGFBP-1, IGFBP-2, IGFBP-3) [7] with a high degree of sequence homology (about 30%–40%) [8–12]. The predominant IGFBP in the circulation in post-natal life is IGFBP-3 [13, 14]. In contrast to the other IGFBPs it has the unique property of associating after binding of IGF-I or IGF-II, with an acid-labile non-binding subunit (IGFBP-3α) resulting in a high molecular weight complex (120–150 kDa) [15]. Its regulation is most prominently subject to the GH secretory status showing a positive linear correlation with the logarithm of the GH secretion integrated over time [16].

To further elucidate a possible pathogenetic role of IGFs and their binding proteins in impaired growth in children with renal failure, IGF-I, IGF-II, IGFBP-1, and IGFBP-3 were measured by radio-immunoassay (RIA) in these patients. The SmBA and free IGF binding capacity were also studied. Computer calculations were performed to obtain some information on the secretion rate of IGFs in uraemic patients compared with normal controls.

Patients and methods

Patients

Patients suffering from renal failure due to various causes were divided into two groups: children with end-stage renal failure (ESRF, $n = 31$)

Offprint requests to: W. F. Blum, Universitätskinderklinik, Rümelinstrasse 23, W-7400 Tübingen, Germany

who were on dialysis, and patients with CRF ($n = 11$) who had still some residual glomerular filtration. The mean chronological age was 10.5 ± 4.8 years (range 2.3–16.5 years) and 7.3 ± 3.1 years (range 1.7–12.8 years, respectively. Blood samples from all patients were taken in the morning in a cross-sectional manner for biochemical analysis.

Assays

Somatomedin bioassay. SmBA was measured by sulphate incorporation into porcine costal cartilage [17]. Values were corrected for elevated sulphate concentrations in uraemic sera if necessary.

IGF-I RIA. The assay was performed after acid-ethanol extraction or acidic gel chromatography of sera utilizing the high-affinity antibody of Underwood and Van Wyk [18]. Both extraction methods gave identical results.

IGF-II RIA. IGF-II was measured in acid-ethanol extracts using a specific antiserum for the C-peptide domain [19]. Interference of residual IGFBP could be completely blocked by addition of excess IGF-I as described before [19].

IGFBP-1 RIA. Placental protein 12 (pp 12), which is identical to IGFBP-1, and anti-pp12-serum were a gift from Dr. Hans Bohn (Behringwerke, Marburg, FRG). They were used for establishing a specific RIA that does not cross-react with IGFBP-3.

IGFBP-3 RIA. The acid-stable binding subunit of the large GH-dependent binding protein (IGFBP-3β) was isolated from human plasma Cohn fraction IV and a specific RIA for this protein was developed which recognizes the high molecular weight complex [14]. No cross-reaction was observed with IGFBP-1.

Free somatomedin-binding capacity (SmBC). Serial dilutions of sera were incubated with ^{125}I-IGF-II (20,000 cpm) for 2 h at room temperature. Unbound tracer was precipitated with excess IGF-II antiserum [19] and donkey anti-rabbit-IgG after incubation at 4° C for 1 h. The bound radioactivity was measured in the supernatant after centrifugation. Dilution curves of normal sera and sera from patients with CRF were virtually parallel. Therefore, SmBC was related to a normal serum pool defining 1 unit as the free SmBC of 1 ml of normal serum.

Exclusion chromatography

Serum samples (20 µl) from normals and patients with ESRF or concentrated urine samples were chromatographed under high-performance liquid chromatography (HPLC) conditions on a TSK-G 4000 SW column (7.5×600 mm) at room temperature. Fractions of 0.5 ml were collected at a rate of 0.25 ml/min and assayed for IGFBP-3 and IGFBP-1 by RIA [14].

Affinity cross-linking

Serum samples (25 µl) were incubated with either radiolabelled IGF-I or IGF-II (200,000 cpm) for 90 min and bound IGF tracer was covalently cross-linked with disuccinimidyl suberate [20]. Labelled IGFBP was precipitated with specific antisera for IGFBP-3 or IGFBP-1 using a second antibody. The pellets were dissolved in sample buffer containing 0.1 mol/l dithiothreitol and analysed by polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5%–15% gradient gels followed by autoradiography.

Extraction of free SmBC by affinity chromatography

In order to remove free IGFBP not being occupied by IGF, control sera and sera from patients (1 ml) were passed over an IGF-II-Sepharose column (1×1 cm) which was prepared by covalent coupling of 0.5 mg pure IGF-II to activated CH-Sepharose 4B (Pharmacia, Uppsala, Sweden). As verified by the SmBC assay the unoccupied IGFBP was completely removed by this procedure. Dilution of the samples was accounted for on the basis of protein content.

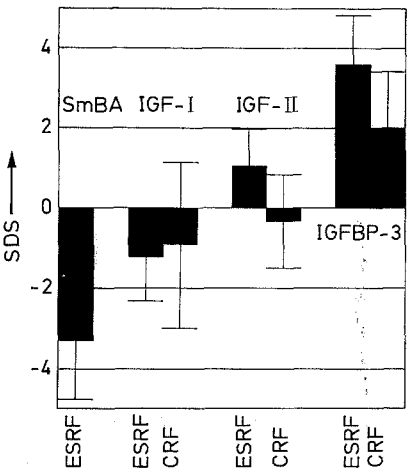

Fig. 1. Serum somatomedin bioactivity (*SmBA*), insulin-like growth factor-I (*IGF-I*), *IGF-II* and IFG binding protein-3 (*IGFBP-3*) in patients with end-stage renal failure (*ESRF*) and chronic renal failure (*CRF*) with residual renal function. Except for SmBA ($n = 11$) the number of patients in each group was 31 for ESRF and 11 for CRF. Values are given as standard deviation scores (*SDS*) because of their age dependence

Results

SmBA and IGF-I, IGF-II and IGFBP-3 levels in children with ESRF and CRF with residual glomerular filtration are shown in Fig. 1. To account for age-dependence values are given as standard deviation scores.

SmBA was subnormal (-3.30 ± 1.47 SD) in all individuals tested ($n = 11$). IGF-I was in the lower normal range (ESRF, -1.23 ± 1.09 SD; CRF, -0.92 ± 2.05 SD), while IGF-II was slightly elevated or normal (ESRF, 1.04 ± 0.93 SD; CRF, -0.34 ± 1.17 SD). IGFBP-3 was clearly supranormal in ESRF (3.58 ± 1.26 SD) or on average in the upper normal range in CRF (1.97 ± 1.44 SD).

Serum levels of IGFBP-1 in patients with ESRF and CRF in comparison with normal age- and sex-matched controls are given in Fig. 2. In both groups IGFBP-1 was markedly increased in most patients. Compared with normal controls, IGFBP-1 was relatively more increased than IGFBP-3. However in absolute terms, IGFBP-1 levels (range 0.048–0.465 mg/l) were still only a small percentage of the IGFBP-3 concentration (range 2.31–9.81 mg/l).

The ratio of IGFs, in particular IGF-I to IGFBP, is important. In normals a high linear correlation was found between the sum of serum IGF-I and IGF-II and IGFBP-3 ($r = 0.91$), whereas the correlation between serum IGF-I and IGFBP-3 was exponential [21, 22]. In renal failure, however, there was a marked deviation from the normal correlation curves to higher IGFBP-3 levels for both serum concentration of IGF-I plus IGF-II versus IGFBP-3; this was even more pronounced for IGF-I versus IGFBP-3 [22].

The question of whether this excess of IGFBP is only immunoreactive material giving high levels in the RIA or

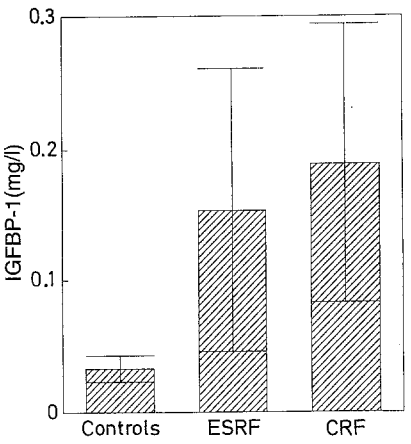

Fig. 2. Serum IGFBP-1 in ESRF (n = 31) and CRF (n = 11) compared with age and sex-matched controls (n = 42). Levels are given as mean ± SD

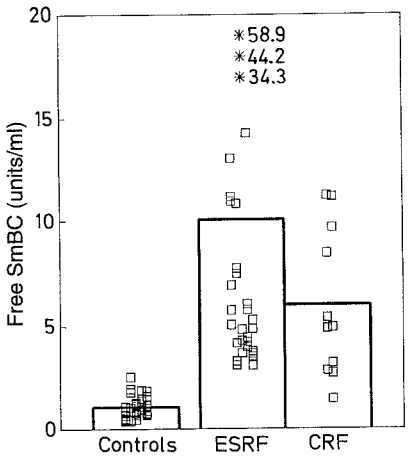

Fig. 3. Free somatomedin binding capacity (*SmBC*) in ESRF (n = 31), CRF (n = 11), and age- and sex-matched controls (n = 42). The values are expressed as units μ/ml, where 1 unit refers to the binding capacity of a serum pool of normal young adults. The columns show the mean of SmBC in each group

whether it is biologically active in the sense that it is able to bind IGF, was investigated by determination of the free IGF-II binding capacity (SmBC) in patients and in age- and sex-matched controls (Fig. 3). SmBC was clearly increased in ESRF with excessively high values in some patients. On average, this increase was somethat lower in CRF.

IGFBP-3 is present in normal serum mainly as a high molecular weight complex (Fig. 4) of about 120–150 kDa. However, when sera from patients with ESRF were subjected to HPLC exclusion chromatography, most of the immunoreactive IGFBP-3-like material eluted in the range of 60–20 kDa showing two major peaks at about 55 and 25 kDa. In normal urine only immunoreactive IGFBP-3 components of less than 60 kDa were detected with a major peak at 12–20 kDa (Fig. 4). IGFBP-1 (measured by RIA) eluted as a single peak in both serum and urine at about 30 kDa (data not shown).

The molecular weight of IGFBP components was further studied by affinity cross-linking of IGF-I and

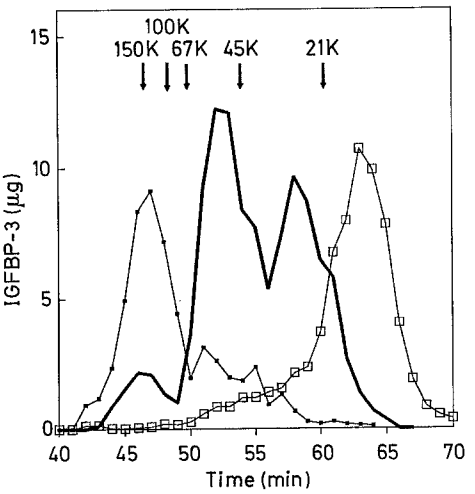

Fig. 4. Representative elution profiles of immunoreactive IGFBP-3 in normal serum (-■-), uraemic serum (■) and normal urine. High-performance liquid chromatography was performed on a TSK G-4000 SW column. The *arrows* indicate the peak positions of the molecular weight marker proteins

IGF-II to sera from patients with ESRF, and precipitation of the labelled complexes with specific anti-IGFBP-1 and anti-IGFBP-3 sera. Analysis of the precipitates by SDS-PAGE and autoradiography revealed radiolabelled bands at 45, 40, 30, 20, and 15 kDa for IGFBP-3 and 34 kDa for IGFBP-1 after subtraction of the molecular weight of the label. An additional radiolabelled band was found at about 35 kDa which could not be precipitated by either IGFBP-1 or IGFBP-3 antiserum (not shown).

To test the hypothesis that the excess of IGFBP over IGF may have an inhibitory effect on somatomedin activity, serum samples from controls (n = 3) and patients with ESRF (n = 3) were passed over an IGF-II-Sepharose column. Removal, was almost complete as verified by the assay for free SmBC. After extraction of free SmBC the SmBA increased from 0.97±0.13 to 1.22±0.37 units in the control samples and from 0.48±0.12 to 0.97±0.42 units in ESRF. Although this increase was clear in each individual experiment, it did not reach statistical significance due to the small sample number.

Discussion

SmBA (measured by sulphate incorporation into porcine costal cartilage) was significantly decreased in patients with ESRF. This finding is in line with earlier studies [4–6]. In contrast, serum IGF-I and IGF-II levels were found to be in the normal range when taking into account interference of IGFBPs with the RIAs; this is in agreement with the results of Powell et al. [2, 3].

To further investigate the discrepancy between subnormal SmBA and normal IGF levels by RIA, IGFBPs were studied using various techniques. Quantitation of serum IGFBP-1 and IGFBP-3 by RIA revealed clearly elevated levels compared with normal controls. Similar findings were reported by others [13, 23], although absolute values differed between the various groups which might be due to the use of different standards. This is particularly true for

IGFBP-1. Very high levels were found by Lee et al. [23], whereas the levels in our study were lower by an order of magnitude. Indeed, the normal range established by the currently used RIA was in excellent agreement with the age-dependent range reported by Hall et al. [24] and also compares well with the basal levels observed by others [25, 26]. Although the relative increase of serum IGFBP-1 in renal failure was clearly higher than the increase of IGFBP-3, IGFBP-1 levels still did not make up more than a small percentage of the concentration of IGFBP-3 in renal failure. Besides IGFBP-1 and IGFBP-3 other IGFBPs, in particular IGFBP-2, may also be present in the circulation of uraemic patients [27], as a band visualized by affinity cross-linking experiments could not be precipitated by either IGFBP-1 or IGFBP-3 antisera. Quantitatively, however, IGFBP-3 and related compounds appear to be predominant. Therefore, from our findings it has to be concluded that the excess of IGFBP-3-related peptides rather than IGFBP-1 plays a major role in the possible inhibition of IGF activity in contrast to a previous report [23].

Due to the very short metabolic half-life of free IGF [28] the molar ratio of total IGF (IGF-I plus IGF-II) and IGFBP-3 is approximately 1·0 in normal individuals [13]. In uraemia, however, the situation is unusual, because a large excess of IGFBP-like material is present. This means, that there is a relative deficiency of IGF which is even more pronounced for IGF-I than IGF-II.

The question of whether the excess of IGFBP, as determined by the IGFBP-1 and IGFBP-3 RIA, is merely immunoreactive material or whether it is bioactive, being able to bind IGF, was studied by measuring the free IGF-II binding capacity. The fact that free SmBC was increased in renal failure by an order of magnitude compared with normals suggests that the excess of IGFBP is, in fact, biologically active. Increased IGF binding in uraemia was also reported by other groups using dextran-coated charcoal for separation of bound and unbound tracer [3, 29]. The relatively small increase of free SmBC in these studies may be related to the incomplete exclusion of low molecular weight IGFBP forms from adsorption to dextran-coated charcoal, or alternatively to differences in patient selection.

In normal serum most of the IGFBP-3 is present as a high molecular weight complex. In contrast, most of the immunoreactive IGFBP-3 in uraemia was found to be present as low molecular weight forms by HPLC exclusion chromatography. The results of these studies compare well with previous findings showing that small IGFBP forms are increased in CRF [27, 29]. These findings were confirmed by the affinity cross-linking experiments demonstrating the presence of low molecular weight IGFBP-3 forms in uraemia. Moreover, and most importantly, it could be shown that these low molecular weight IGFBP-3 forms are biologically active, that is, they are able to bind IGF and may well contribute to the excess of free SmBC in uraemia.

The increase of IGFBP-1 and low molecular weight forms of IGFBP-3 in uraemia may be due to various factors. By HPLC exclusion chromatography of urine from normal individuals only IGFBP-1 and IGFBP-3 forms of less than 60 kDa could be detected with a major peak of the latter at 12–20 kDa, which possibly represents fragments. It may be concluded that renal filtration plays an important role in the clearance of small IGFBP forms while the large IGFBP-3 complex remains in the circulation. If renal function is impaired the low molecular weight IGFBP forms accumulate leading finally to an excess of IGFBP. Further support of this hypothesis comes from the finding that in adult patients with ESRF serum IGFBP-3 (measured by RIA) decreased dramatically after kidney transplantation, from very high to very low levels within 1 day of surgery (data not shown).

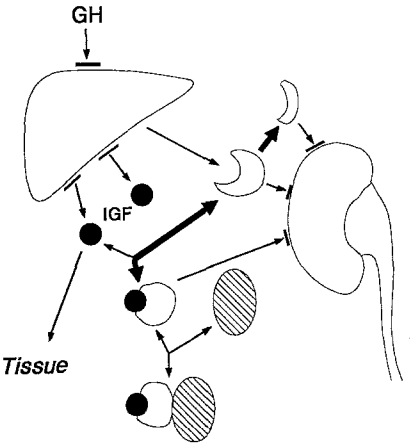

Fig. 5. Hypothetical model of the production of IGF and IGFBP-3 by the liver, their clearance by the kidneys and their interaction. In renal failure the stimulatory effect of growth hormone (*GH*) on IGF (●) and IGFBP-3 (○) production by the liver is impaired. The IGF/IGFBP-3 complex binds the acid-labile non-binding subunit (▨) to form the high molecular weight ternary complex which is no longer subject to renal filtration, while IGFBP-3 or smaller fragments are cleared from the circulation by the kidneys. In renal failure, however, their clearance is impaired resulting in an accumulation of these compounds. High levels of IGFBP shift the chemical equilibrium towards bound IGF which causes, in combination with decreased IGF production rates, a decrease of free IGF that is available for acting on the target tissues

Both inhibitory and stimulatory effects of IGFBP-1 and IGFBP-3 on IGF were found depending on the experimental conditions used. From a theoretical point of view it is most pertinent that sequestration of IGF by an excess of IGFBP results in an inhibition of IGF activity. Indeed, removal of excessive IGFBP by affinity chromatography resulted in an increase of SmBA. Hence the excess of IGFBP in uraemia acts as a somatomedin inhibitor. Besides excessive concentrations of various IGFBP forms other small molecular weight somatomedin inhibitors have been described in uraemia that await further characterization [6].

One might assume that a normal secretion rate of IGF should fill up free IGFBP binding sites resulting in elevated levels of IGF-I and IGF-II in uraemia. However, as this is not the case, we concluded that IGF production is decreased. Indeed, applying a mathematical model, which describes the interaction of IGF and IGFBP to the uraemic situation under steady state conditions using empirical data for calculation [22, 30], IGF secretion rates were estimated to be diminished by an order of magnitude in this pathological situation (data not shown). Most interestingly, calculated IGF secretion rates in normal controls ($n = 220$,

0.91 ± 0.86 nmol/l per min) were well in the range obtained by Guler et al. [28] using an experimental approach, suggesting that the simplifying assumptions did not grossly limit the applicability of the model to empirical situations.

The major consequence of this theoretical conclusion, that in renal failure IGF secretion rates are decreased, is that there must be some kind of GH resistance with respect to IGF production as GH levels are normal or elevated [1, 4]. At this moment the molecular mechanism of this resistance remains unclear. Theoretically, it may include structural alterations of the GH molecule, alterations at the GH receptor level or even impaired post-receptor events.

In summary, the following hypothetical situation may be schematically depicted on the basis of the current findings (Fig. 5). Circulating IGF-I, IGF-II, and IGFBP (at least IGFBP-1 and IGFBP-3) are mainly produced in the liver. Free IGF-I or IGF-II bind to the IGFBP-3 binding subunit which is then able to bind the non-binding subunit IGFBP-3α (which is present in excess) to constitute the high molecular weight complex [15]. This large complex is no longer subject to renal filtration and remains in the circulation. In chemical equilibrium IGFs are released from this complex to act on their target cells, possibly by mediation of other IGFBPs. Low molecular weight IGFBP forms are cleared from the circulation by the kidneys. In renal failure, however, their clearance is impaired and therefore, they accumulate in the circulation leading finally to an excess of IGFBP over IGF. The excess of IGFBP shifts the chemical equilibrium to bound IGF thereby lowering free, biologically available IGF. A second mechanism that reduces free IGF is the low secretion rate. This may be particularly true for IGF-I. As a result of low levels of free IGF, IGF action at the target cell level is diminished.

This hypothesis may outline one mechanism among others that contribute to the pathogenesis of growth retardation in CRF. Moreover, it provides a rationale for GH therapy in these patients. Relative GH resistance may be overcome by administration of high doses of GH resulting in a pronounced increase of IGF-I, and, as a consequence, an improvement in longitudinal growth [31, 32].

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