CLINICAL STUDY

Inflammation is a modulator of the insulin-like growth factor (IGF)/IGF-binding protein system inducing reduced bioactivity of IGFs in cystic fibrosis

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Abstract

Objective: In inflammatory bowel diseases, increased serum interleukin (IL)-6 levels are associated with high serum insulin-like growth factor-binding protein 2 (IGFBP-2) levels, and cytokines modify the insulin-like growth factor (IGF)/IGFBP system in models *in vitro*. In cystic fibrosis (CF) the IGF/IGFBP system has not been extensively studied, and relationships with proinflammatory cytokines have not been explored. The aim of this study was to investigate the IGF/IGFBP system and verify changes dependent on IL-1 β , IL-6, tumour necrosis factor α (TNF α), and insulin.

Methods: Eighteen subjects with CF (mean age 26.6 ± 1.1 years) and 18 controls, comparable for age, sex, and body mass index, were enrolled. Serum IGF-I, IGF-II, IGFBP-2, IGFBP-3, IL-1 β , IL-6, TNF α , insulin and C-peptide were measured. Different molecular forms of IGFBP-2 and IGFBP-3 were investigated by Western immunoblotting. The patients were analysed as a whole and as two subgroups depending on established clinical criteria (Swachman–Kulczycki score).

Results: Patients had higher serum concentrations of IL-1 β , IL-6, TNF α and IGFBP-2 than controls. Serum concentrations of IGF-I and IGF-II were significantly lower and insulin and C-peptide levels significantly increased in CF compared with healthy controls whereas IGFBP-3 serum concentrations were similar, with comparable IGF-I/IGFBP-3 and decreased IGF-I/IGFBP-2 and IGF-II/IGFBP-2 molar ratios. From correlation analysis we detected a significant positive correlation between IGFBP-2 and IL-6 and a negative correlation between IGFBP-2 and IGFBP-3.

Conclusions: Our findings suggest that inflammation is an important modulator of the IGF/IGFBP system with an overall reduction in IGF bioactivity in CF.

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Introduction

Impaired growth and delayed pubertal development are often described in chronic inflammatory diseases. Much data in the literature refer to inflammatory bowel diseases (1–4). Few papers have studied modifications of growth factors in cystic fibrosis (CF). Cellular growth and proliferation, differentiation and apoptosis are mainly dependent on the effect of the growth hormone (GH)/insulin-like growth factor (IGF)/IGF-binding protein (IGFBP) axis. Growth delay in CF has been attributed to calorie and protein malnutrition due to anorexia, malabsorption and increased resting energy expenditure (5, 6). Generally, most of these patients have catch-up growth during treatment on dietary management consisting in high caloric food intake (7, 8). However, at least 20–30% of CF patients present with childhood growth delay and/or weight deficit (9–12). In CF, serum IGF-I

levels have been described to be normal (13) or below the normal range for age and it has been suggested that this could be secondary to liver dysfunction, impaired glucose tolerance, pubertal delay or chronic inflammation (9, 14).

IGF-I and IGFBP-3 serum concentrations in CF show a trend to increase and normalize after antibiotic treatment (15), suggesting that infection and inflammation might also play a role in regulating the IGF/IGFBP system. The finding of low IGF-I serum concentrations lead to the hypothesis of peripheral GH resistance. However, in 1999 (16) a study in adult patients with CF reported normal spontaneous GH secretion and release after stimulation with arginine. The report a few years later of low serum IGF-I, increased IGFBP-3 proteolysis and poor growth in transgenic mice overexpressing interleukin (IL)-6 supports the hypothesis that inflammatory mediators *per se* could modulate the IGF/IGFBP system (17, 18).

In CF the inflammatory response to chronic inflammation is similar to that elicited by lipopolysaccharide and is mediated mainly by cytokines as tumour necrosis factor α (TNF α), IL-1, IL-6 and IL-8 (19). We have previously shown *in vitro* that IL-1 β and IL-6 modify IGFBP secretion into conditioned media (20), and *in vivo*, in inflammatory bowel diseases, that IGFBP-2 concentrations are increased together with IL-6, IL-1 and C-reactive protein (21).

The aim of this study was to investigate whether inflammation in CF was related to changes in the IGF/IGFBP system. We investigated whether IL-1 β , IL-6, TNF α or insulin were related to changes in IGF-I, IGF-II, IGFBP-2 and IGFBP-3.

Materials and methods

Subjects

Eighteen consecutive patients in our CF clinic (26.6 ± 1.2 years, 6 males and 12 females) and 18 control subjects (28.0 ± 1.2 years, 4 males and 14 females), were enrolled into the study. Clinical details of patients and controls are shown in Table 1. The two groups were comparable for chronological age, sex and body mass index (BMI) and all had attained pubertal stage 5 according to the criteria of Marshall and Tanner (22, 23). The diagnosis of CF had been confirmed in all by genetic analysis and a sweat test. All had exogenous pancreatic insufficiency that required enzymatic supplementation and were treated with on-going antibiotic therapy. Two patients had been taking steroids for at least 3 months (0.15 mg/kg per day of prednisone) and none were receiving azitromycin at the time of the study. One patient was excluded from the study for being diabetic and having signs of liver dysfunction. All but one patient tested positive for *Pseudomonas aeruginosa* infection, and one also had *Burkholderia cepacia* infection. C-reactive protein serum concentrations were assayed in all patients at the time of the study.

Auxology observations

Height and weight were measured in all subjects, and BMI was calculated accordingly. BMI, height and target height are expressed as standard deviation scores (SDS) using Italian reference data (24).

Pulmonary function

Standard pulmonary function tests were performed the same day as the blood samples were taken. For statistical analysis the forced expiratory volume in 1 s (FEV1) was expressed as a percentage of the reference value (25).

Subgroups of subjects

The patients were subdivided into two groups according to the Shwachman–Kulczycki scoring system (general activity, physical examination, nutrition and X-ray findings) (26). Group 1 were patients ($n = 10$) with scores of 71–100 (mildly affected) and group 2 were patients ($n = 8$) with scores of 41–70 (severely affected) (26). Control subjects were enrolled from young physicians training at our medical school and were healthy at the time of the study.

Assays

Blood samples were drawn after an overnight fast between 0800 and 0900 h. The blood was kept on ice until centrifugation at 2000 g/min for 10 min at 4°C . The serum was then aliquoted and stored at -80°C until assayed. Total serum IGF-I was measured using a RIA method (Nichols Institute Diagnostics, CA, USA). The coefficients of variation (CVs) were 3% (intra-assay) and 8.4% (inter-assay), respectively. Prior to assaying, serum samples were acid-ethanol-precipitated to avoid interference of binding proteins. Serum IGF-II and IGFBP-3 were measured using an immunoradiometric assay method (Diagnostic System Laboratories, Webster, TX, USA). The CVs were 5.3 and 1.9% (intra-assay) and 8.7% and 3.9% (inter-assay), respectively. Serum IGFBP-2 was assayed

Table 1 Clinical data of patients and controls.

	CF	Controls
Number (males, females)	18 (6, 12)	18 (4, 14)
Chronological age (years)	26.6 ± 1.2	28.0 ± 1.2
BMI SDS	-0.3 ± 0.2 (range: $-1.7 - +1.2$)	0.1 ± 0.2 (range: $-1.8 - +1.6$)
Height SDS	$-0.7 \pm 0.2^*$	0.5 ± 0.2
Target height SDS	-0.4 ± 0.3	-0.1 ± 0.2
Total protein (g/dl)	5.7 ± 0.1	
Glucose (mg/dl)	0.8 ± 0.1	0.7 ± 0.2
AST (IU/l)	22.3 ± 2.6	
ALT (IU/l)	24.5 ± 3.9	

BMI SDS, body mass index expressed as standard deviation score (SDS); total protein (NI. 6–8.2 g/dl); AST, aspartate aminotransferase (NI. $<40 \text{ IU/l}$); ALT, alanine aminotransferase (NI. $<40 \text{ IU/l}$); fasting serum glucose (NI. 60–110 mg/dl); NL, normal light. Data are expressed as mean $\pm$ s.e.m.

* $P < 0.05$ versus controls.

using an RIA method (Diagnostic System Laboratories, Inc. Webster, Texas, USA). The CVs were 4.7% (intra-assay) and 7.2% (inter-assay).

To transform IGF-I, IGF-II, IGFBP-2 and IGFBP-3 from ng/ml to nM we multiplied the single values by 0.131, 0.134, 0.035 and 0.035, respectively. Serum IL-6 and IL-1 β were measured using an ultrasensitive ELISA method (Quantikine HS; R&D Systems, Minneapolis, MN, USA). The CVs were 3.8 and 6.9% (intra-assay) and 9.9% and 10.3% (inter-assay).

Serum TNF α was measured using an ultrasensitive ELISA method (Biosource International, Camarillo, CA, USA). The CVs were 11.9% (intra-assay) and 12.1% (inter-assay). Serum insulin and C-peptide were measured using a chemiluminescence method by Diagnostic Products Corporation (Los Angeles, CA, USA) for reading by Immulite2000. The intra-assay CVs were 6.5 and 7.8%, respectively. The inter-assay CV for insulin was 7.1%. The inter-assay CV for C-peptide was not calculated.

Different molecular forms of IGFBP-2 and -3 in the sera from all the patients and controls were assessed by Western immunoblotting. Equal aliquots of serum were subjected to electrophoresis on 12.5% acrylamide gels (27). The proteins were then transferred to nitrocellulose membranes and probed with specific antibodies against IGFBP-2 and -3 (Upstate Biotechnology, NY, USA; 1:2000) at 4°C overnight as previously described (20, 21). The bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK).

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) test followed by Scheffé's test for the growth factors. The IL data were not normally distributed and were analysed using Kruskal–Wallis test. The correlation analysis was performed using Pearson's or Spearman's linear regression analyses. $P < 0.05$ was considered significant. Data are expressed as means $\pm$ S.E.M. unless otherwise stated.

Ethical approval

Informed consent was obtained from the subjects and/or the parents as appropriate. The study was approved by the local Ethics Committee (University of Parma Medical School).

Results

Results in males and females were analysed together because no significant difference was found between the sexes in patients or controls. Serum concentrations of IL-1 β , IL-6, TNF α , insulin, C-peptide, IGF-I, IGF-II, IGFBP-2 and IGFBP-3 are shown in Table 2. Serum

Table 2 Comparison of cytokines, insulin, C-peptide, IGF-I, IGF-II, IGFBP-2 and IGFBP-3 serum concentrations in CF patients and in controls.

	CF	Controls
IL-1 β (ng/l)	1.0 $\pm$ 0.4*	0.8 $\pm$ 0.4
IL-6 (ng/l)	5.0 $\pm$ 0.8*	1.3 $\pm$ 0.3
TNF α (μ g/l)	1.7 $\pm$ 0.3*	0.9 $\pm$ 0.1
Insulin (IU/l)	11.5 $\pm$ 2.4*	6.1 $\pm$ 0.7*
C-peptide (mg/l)	2.8 $\pm$ 0.6*	1.9 $\pm$ 0.1
IGF-I (μ g/l)	231.7 $\pm$ 16.1*	302.2 $\pm$ 26.7
IGF-II (μ g/l)	1027.4 $\pm$ 57.2*	1264.7 $\pm$ 63.7
IGFBP-2 (μ g/l)	520.5 $\pm$ 72.9*	290.9 $\pm$ 41.2
IGFBP-3 (μ g/l)	4210.6 $\pm$ 205.4	4717.6 $\pm$ 304.9

* $P < 0.05$ versus controls.

IL-1 β , IL-6 and TNF α levels were significantly higher in CF patients compared with control subjects.

Serum

Insulin was significantly higher in the patients with respect to controls, and in the patient groups levels were higher in group 2 patients (severely affected, with lower Shwachman–Kulczycki scores) compared with group 1 (mildly affected): 18.8 $\pm$ 4.8 versus 7.1 $\pm$ 1.5 IU/l, respectively ($P < 0.05$). All controls had normal values (normal range, 4–25 IU/l; Fig. 1). Serum C-peptide, similarly to insulin, was higher in the patients than in the controls, with higher values in the group 2 compared with the group 1 patients: 4.3 $\pm$ 1.2 versus 2.0 $\pm$ 0.3 mg/l, respectively ($P < 0.05$).

Serum IGF-I and IGF-II levels were significantly lower in the patients than in the controls, whereas serum IGFBP-2 was significantly higher in patients with CF

Figure 1 Insulin serum levels in CF subjects and controls, based on Shwachman–Kulczycki score. Group 1 patients are mildly affected, whereas group 2 patients are affected severely. Data are means $\pm$ S.E.M. * $P < 0.05$ versus controls.

than in controls. The immunoblot analysis showed that intact IGFBP-2 was increased (Fig. 2A). Serum IGFBP-3 concentrations were similar in patients and controls. The immunoblot analysis did not show differences in the molecular forms between patients and controls (Fig. 2B).

Molar ratios

The IGF-I/IGFBP-2 and IGF-II/IGFBP-2 molar ratios were significantly lower in the patient group: 11.6 ± 2.1 and 3.2 ± 0.6 respectively versus 23.1 ± 4.2 and 5.4 ± 0.7 in controls ($P < 0.05$).

Correlation analysis

Results from the whole group are reported in Table 3. In the control subjects, a positive correlation between IGF-I and IGFBP-3 was shown ($P = 0.022$; $r = 0.57$).

In the patients, IL-6 correlated positively with C-reactive protein ($P < 0.001$; $r = 0.77$) and with IGFBP-2 ($P = 0.03$; $r = 0.64$; Fig. 3). IGFBP-2 correlated negatively with IGFBP-3 in the whole group ($P = 0.025$; $r = 0.54$).

Discussion

This study shows that CF patients have higher serum concentrations of IL-1 β , IL-6, TNF α and IGFBP-2 compared with normal controls. Serum concentrations of IGF-I and IGF-II were significantly lower than in healthy controls, with insulin and C-peptide levels being increased. We also detected a significant positive correlation between serum IL-6 and IGFBP-2 concentrations.

For the first time we describe relationships between cytokines and the IGF/IGFBP system in CF patients, even if these must be regarded as preliminary data owing to the small number of patients enrolled in the study. Moreover, this is a cross-sectional study so we

cannot comment on the natural progression of these relationships over time, and can put forward only hypotheses.

The serum concentrations of pro-inflammatory cytokines in our patients were similar to previously published data (19). Serum IGF-I levels were significantly lower in CF patients than in controls. At variance with previous studies this did not seem to be dependent on malnutrition or anorexia (9, 14, 28, 29) as the BMI values of these patients were normal and similar to those of controls, and total serum protein concentration was normal. Other authors have hypothesized that low serum IGF-I concentrations could be dependent on insulin hyposecretion in CF. Taylor *et al.* (28) described a progressive reduction of IGF-I and IGFBP-3 related to declining BMI, and deteriorating pulmonary function, and suggested that progressive insulin deficiency might be contributing to this. Ripa *et al.* (29) further supported this hypothesis by showing a high frequency of insulin hyposecretion with increased serum IGFBP-1 levels, normal IGF-I and reduced growth velocity in CF. However, this group of patients had basal insulin levels similar or greater than controls. Furthermore, the low IGF-I serum levels cannot be ascribed to impaired liver function (14, 16) as liver function tests were normal in all the subjects. In the case of impaired liver function insulin clearance would be reduced; however, C-peptide, which is metabolized by the kidneys, showed a similar pattern to insulin, suggesting that this is very unlikely. Thus, a state of latent insulin resistance that worsens with deteriorating clinical conditions is possible (30). Insulin concentrations in our patients could be increased because of increased serum levels of TNF α reducing the function of the glucose transporter GLUT4 (31), which is associated with reduced glucose disposal rate and insulin resistance. However, we did not have specific data on glucose tolerance, as this was not the aim of this study. Alternatively, other mechanisms leading to altered insulin receptor signalling that have been reported could be involved: IL-6 has also been

Figure 2 Serum IGFBP-2 (A) and IGFBP-3 (B) levels in cystic fibrosis patients and in controls. Data are means $\pm$ S.E.M. * $P < 0.05$ versus controls. Beneath each graph is the corresponding Western immunoblot. Serum from controls is marked as C, and from cystic fibrosis subjects with CF. These subjects were comparable for age, sex, BMI and pubertal stage. Molecular mass (Mr) markers are shown on the left in kDa. IGFBP-2 was intact, and molecular forms of IGFBP-3 were similar in all subjects.

Table 3 Correlation analysis results in the entire group of subjects.

	Variable	<i>r</i>	<i>P</i> value
IGF-I	IL-6	−0.44	0.014
	IGFBP-2	−0.39	0.018
	IGFBP-3	+0.63	<0.001
IGF-II	IGFBP-3	+0.49	0.004
IGFBP-2	IGFBP-3	−0.37	0.031

Figure 3 The correlation between serum IL-6 and IGFBP-2 in CF patients ($P = 0.03$; $r = 0.64$).

suggested to interfere with insulin action (32). Furthermore, based on very recent literature, IGF-I concentrations seem to affect insulin sensitivity, and GH and IGF-I replacement seem to be capable of improving insulin sensitivity in diabetes and CF (33–35).

Recently, it has been observed that IGF-I concentrations progressively decrease with age whereas IL-6 tends to increase, with a possible link to morbidity in the elderly (36). In light of this, data from our study suggest that this combination could be harmful also in subjects with CF, who present similar concentrations of IL-6 and IGF-I at a much younger age. Serum IGF-II levels have not been investigated previously and showed a similar pattern as IGF-I. IGFBP-2 concentrations were definitely increased in the patients and correlated positively with IL-6. Furthermore, the pattern was similar to that of $\text{TNF}\alpha$. We previously described this increase in patients with inflammatory bowel diseases (21). These data suggest that IGFBP-2 on the one hand behaves as an acute phase protein, and on the other that the IGF/IGFBP system is modulated by some proinflammatory cytokines. It could be suggested that the serum levels could be altered in the two patients treated with oral steroids; however, it has been shown that 3 months of treatment with steroids reduce, not increase, IGFBP-2 serum levels (37).

IGFBP-3 serum concentrations were similar in the patients and controls although they tended to be lower in patients with the most severe clinical state, and we did not find any relationship with insulin at variance with previously published data (28, 29).

This helps to explain why the IGF-I/IGFBP-3 molar ratios were similar in CF and healthy subjects. The IGF-I/IGFBP-2 and IGF-II/IGFBP-2 molar ratios were strongly reduced in CF, which would confirm a possible impaired compensatory mechanism for free IGF-I and IGF-II in CF.

Moreover, we did not find different molecular forms of IGFBP-2 and IGFBP-3 in patients and controls that could suggest increased proteolysis. The negative relationship between IGFBP-3 and IGFBP-2, both in the whole group of subjects enrolled in the study and in the patients, further strengthens the hypothesis that overall IGF bioactivity is blunted as the two peptides seem to go in opposite directions; if IGFBP-3 decreases, possibly increasing free IGF-I, IGFBP-2 increases, binding and reducing IGF-I bioavailability. The data of this study therefore suggest that the IGF/IGFBP system is modulated to reduce IGF-I and IGF-II bioactivity and thus their anabolic and pro-insulin activity.

In conclusion, it can be speculated that chronic inflammation is an important modulator of the IGF/IGFBP system in CF, and represents a possible mechanism for peripheral resistance to GH. This is in consideration of the fact that the Shwachman–Kulczycki score uses general activity, a physical examination, nutrition and X-ray findings. Nutritional status in this patient group was very good and cannot have played an important role, whereas inflammatory cytokines were significantly increased and correlated with growth factors. The overall data suggest a dysregulation which influences bioactivity of IGFs. Furthermore, these data prompt considerations of alternative or additional treatments in CF. They support the use of anti-inflammatory drugs such as ibuprofen (38), possibly new anti-cytokine drugs (39), and even GH and IGF-I.

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