IGF-I and IGF-I-binding proteins in rats with adjuvant-induced arthritis given recombinant human growth hormone

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Abstract

Adjuvant-induced arthritis in rats is associated with growth failure, hypermetabolism and accelerated protein breakdown. We have previously reported that adjuvant-induced arthritis in rats results in a decrease in body weight gain, pituitary GH mRNA, circulating GH and IGF-I together with an increase in serum IGF-binding proteins (IGFBPs). The aim of this study was to analyze the role of GH in the decrease in body weight and in the alterations in the IGF-I system observed in chronic inflammation.

Male Wistar rats were injected with complete Freund's adjuvant and 16 days later arthritic rats were injected daily with recombinant human GH (rhGH) (3 IU/kg s.c.) for 8 days; control rats received 250 µl saline. Arthritis significantly decreased body weight gain and serum IGF-I. These decreases were not due to the reduced food intake, since in pair-fed rats they were not observed. Furthermore, administration of rhGH to arthritic rats increased body weight gain without modifying food intake. To

further investigate the effect of GH administration, 14 days after adjuvant injection both control and arthritic rats were treated with 0, 1·5, 3 or 6 IU/kg of rhGH. GH treatment at the dose of 3 and 6 IU/kg significantly increased body weight gain in arthritic rats. GH administration, at the higher dose of 6 IU/kg, increased hepatic and serum concentrations of IGF-I in both control and arthritic rats. In control rats, rhGH at the three doses assayed increased circulating IGFBP-3. GH treatment in arthritic rats decreased IGFBP-1 and -2, and did not modify IGFBP-4. GH treatment at the dose of 3 IU/kg also decreased circulating IGFBP-3 in arthritic rats.

These data suggest that GH treatment can ameliorate the catabolism observed in adjuvant-induced arthritis, an effect mediated, at least in part, by modifications in the circulating IGFBPs.

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Introduction

Adjuvant-induced arthritis in rats, a chronic inflammatory illness, is associated with a decrease in body weight and cachexia (Roubenoff *et al.* 1997). As described in other catabolic diseases, an increase in the levels of circulating glucocorticoids and a decrease in food intake have been observed in arthritic rats (Harbuz & Lightman 1992, Roubenoff *et al.* 1997). Those effects seem to be secondary to the activation of the inflammatory response, since endotoxin administration in rodents induced similar responses, a reduction in body weight with an activation of the hypothalamic–pituitary–adrenal axis (Peisen *et al.* 1995).

Adjuvant-induced arthritis not only activates the adrenal axis, but also modifies other hormones from the somatotrophic axis. A decrease in serum growth hormone (GH) and pituitary GH mRNA has been described during the early phase before and after the disease develops

(Neidhart & Flückiger 1992, Selgas *et al.* 1997). We have recently reported that the decrease in GH mRNA is concomitant with a decrease in serum and hepatic insulin-like growth factor-I (IGF-I) that correlates with the decrease in body weight in arthritic rats (López-Calderón *et al.* 1999).

The inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 are able to inhibit pituitary GH secretion (Bernton *et al.* 1987, Walton & Cronm 1989, Peisen *et al.* 1995), and they have also been shown to inhibit hepatic IGF-I synthesis both *in vivo* and *in vitro* (Fan *et al.* 1995, Thissen & Verniers 1997). Thus, the decrease in IGF-I serum concentration in arthritic rats could be secondary to alterations in pituitary GH or be due to an inhibitory effect of cytokines at the hepatic level.

The decrease in GH secretion and in circulating IGF-I levels during chronic inflammation is associated with high levels of IGF-binding sites in serum (Soto *et al.* 1998, López-Calderón *et al.* 1999). As serum IGF-binding

proteins (IGFBPs) in certain situations can be growth inhibitors (Rechler & Clemmons 1998), the alterations in circulating IGFBPs can play an important role in the decrease in body weight observed in chronic inflammation. In humans, growth retardation is a serious problem in juvenile chronic arthritis (JCA). Low levels of IGF-I have been repeatedly reported in JCA and in rheumatoid arthritis (Davies *et al.* 1994, Foppiani *et al.* 1998). Normal and reduced GH secretion have been observed in rheumatoid arthritis (Butenandt *et al.* 1974, Woo 1994). Nevertheless, recombinant human GH (rhGH) administration to children with JCA partially counteracts the adverse effects of this disease on growth and metabolism (Davies *et al.* 1994, Touati *et al.* 1998). GH and IGF-I are essential hormones in stimulating protein synthesis and body weight, and abnormalities in the IGF-I and IGFBPs have been associated with catabolic states (Bentham *et al.* 1993). These data indicate that the wasting syndrome observed in adjuvant-induced arthritis (Roubenoff *et al.* 1997) can be, in part, secondary to the alterations in GH, IGF-I and IGFBPs. The aim of this work was to analyze if rhGH administration to arthritic rats was able to reverse the effect of this disease on body weight and on IGF-I and IGFBPs. The results suggest that rhGH, although it does not improve the inflammatory illness, can prevent the alterations in body weight and IGF-I and IGFBPs induced by chronic arthritis.

Material and Methods

Male Wistar rats were purchased from Charles River (Barcelona, Spain). Animals were maintained under controlled temperature (20–22 °C) and light conditions (lights on from 0730 to 1930 h), and housed three or four per cage. Food and water were freely available. The experimental procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals.

In the first experiment, the effect of experimental arthritis on food intake was examined. Arthritis was induced in rats by an i.d. injection of complete Freund's adjuvant (1 mg heat-inactivated *Mycobacterium butyricum*; Difco Laboratories, Detroit, Michigan, USA) at the base of the tail. Control animals were injected with vehicle (paraffin oil). We have previously observed that 14 days after adjuvant injection arthritic rats start to develop signs of inflammation. Therefore, at this time rats were divided into three groups: control, arthritic and pair-fed. Body weight, food intake and the arthritis index scores (see below) were examined daily. Food intake per cage was calculated by measuring the difference between the initial and the remaining amount of pellets in the feeder, and expressed as grams per rat per 100 g body weight. The same amount of food consumed by the arthritic rats was given to the pair-fed group the following day. Assessment

of arthritis was performed by measuring the arthritis index of each animal, which was scored by grading each paw from 0 to 4. Grading was determined as: 0, no erythema or swelling; 1, slight erythema or swelling of one or more digits; 2, swelling of the entire paw; 3, erythema and swelling of the ankle; 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16 (Tanaka *et al.* 1996). On day 22 after adjuvant injection rats were killed and blood was allowed to clot and was centrifuged and serum stored at –20 °C until IGF-I and IGFBPs assays were performed.

In the second experiment, the effect of rhGH administration to arthritic rats was examined. On day 16 after the adjuvant injection, arthritic rats were divided into two groups, one received 3 IU/kg s.c. of rhGH (Saizen, Serono, Italy) and the second group was injected with 250 µl saline. All rats were weighed daily from day 16 until day 23 after adjuvant injection. Arthritis index scores were also examined daily. On day 23 all animals were killed by decapitation and trunk blood was collected in tubes at 4 °C. The blood was allowed to clot and was centrifuged and serum stored at –20 °C until IGF-I and IGFBPs assays were performed.

In a third set of experiments, we tested the effect of administering different doses of rhGH to arthritic and control rats. Both arthritic and control rats were divided into four groups that were treated with 0, 1.5, 3 or 6 IU/kg of rhGH s.c. daily at 0900 h, from day 14 to 22. Rats were injected, weighed and the arthritis index score examined daily. Animals were killed by decapitation between 1130 and 1230 h and trunk blood was collected in cooled tubes, allowed to clot and was centrifuged and serum stored at –20 °C until IGF-I and IGFBPs analyses were performed. The liver was removed, dissected, frozen and stored at –20 °C until IGF-I assay.

Serum IGF-I concentrations were measured by a double-antibody RIA previously described (Soto *et al.* 1998). Hepatic IGF-I was extracted as described by Torres-Aleman *et al.* (1992). Samples were homogenized in 1 M acetic acid, boiled for 20 min, and lyophilized. Levels of IGF-I were expressed in terms of IGF-I A52-EPD-186 standard (Eli Lilly & Company, Madrid, Spain). The intra-assay coefficient of variation was 8%. Samples from one experiment were run in the same assay.

Protein content was measured by Bradford's method (1976).

Western ligand blot of IGFBPs

Western blots were prepared as previously described (Hossenlop *et al.* 1986). Two microliters of sera were diluted in sample buffer and boiled for 2 min at 100 °C, and submitted to electrophoresis on 1% SDS-12.5% acrylamide gels under non-reducing conditions. Following electrophoresis, proteins were transferred onto

nitrocellulose sheets (Hybond-C extra, Amersham International, Amersham, Bucks, UK). After transfer the nitrocellulose sheets were dried and blocked for 1 h with 5% non-fat dry milk, 0.1% Tween (Sigma, Madrid, Spain), in Tris-buffered saline. The nitrocellulose membranes were incubated overnight at 4 °C with ^{125}I -labeled IGF-I (5×10^5 c.p.m./ml), and blots were exposed at -80 °C to X-ray film (Kodak X-Omat AR, Eastman Kodak, Rochester, NY, USA). Autoradiographs were analyzed by densitometric scanning using a PC-Image (Foster Findlay Associates Ltd, Newcastle, UK) VGA24 program for Windows. To obtain semiquantitative data for statistical analysis, each gel was run with six serum samples from each experimental group and three with a pool of control samples. The density of the IGFBP bands in each lane was expressed as the percentage of the mean density of control sera.

Statistical analysis

All data are presented as the mean $\pm$ S.E.M. Comparisons between means were made by one-way or two-way ANOVA and subsequent Duncan's multiple range test. Comparisons between two groups were performed using Student's *t*-test. Simple linear regression was used to determine dose-related responses to treatment with different GH doses. Significance was assumed at $P < 0.05$.

Results

Experiment 1

Arthritic rats consumed 10% less food than control rats ($P < 0.01$). As seen in Fig. 1, the daily body weight gain in arthritic rats was significantly lower than that observed in the control group throughout the entire experiment ($P < 0.01$). This decrease does not only seem to be due to the decrease in food intake, since body weight gain in the pair-fed rats only decreased during the first 2 days of food restriction. Twenty-two days after adjuvant injection, serum concentrations of IGF-I were significantly lower in arthritic than in the control animals (1095 ± 55 vs 1287 ± 45 ng/ml, $P < 0.05$). However, pair-fed rats had similar serum IGF-I concentrations to control rats (1323 ± 60 ng/ml).

Experiment 2

On day 16 after adjuvant injection, before GH administration started, the mean body weight was lower in arthritic than in control rats (171 ± 6.2 vs 236 ± 3.8 g, $P < 0.01$). During the 8 days of the experiment, arthritic rats injected with saline gained less body weight than controls, and this decrease in body weight gain was concomitant with a significant ($P < 0.01$) decrease in food

Figure 1 Daily body weight gain in arthritic (AA), control (C) and pair-fed (PF) rats. Values are means $\pm$ S.E.M. for at least nine rats per group. * $P < 0.05$, ** $P < 0.01$ vs control; Duncan's multiple range test.

intake (Fig. 2). GH administration to arthritic rats induced an increase in body weight gain, higher than in the arthritic rats injected with saline ($F_{1,88} = 38$, $P < 0.01$). The stimulatory effect of GH treatment on body weight was not due to an increase in food intake, since over the 8 days of treatment food intake was similar in both groups of arthritic rats, independent of GH treatment (Fig. 2). GH administration to arthritic rats did not modify the splenomegaly or the arthritis index score observed in the arthritic rats injected with saline (Table 1).

Arthritis decreased serum concentrations of IGF-I, and GH treatment was not able to increase serum concentrations of IGF-I in arthritic rats (data not shown). There

Figure 2 Effect of adjuvant-induced arthritis and administration of rhGH (3 IU/kg) on body weight gain and mean food intake per rat. AA=arthritic rats injected with saline, AA GH=arthritic rats receiving GH, C=controls injected with saline. Values are means $\pm$ S.E.M. for at least seven rats per group. ** $P < 0.01$ vs control; Duncan's multiple range test.

Table 1 Effect of GH administration to arthritic rats on relative splenic weight and arthritis index scores. Data are expressed as the mean ± S.E.M.

	<i>n</i>	Treatment	Spleen (mg/100 g)	Arthritis scores
Control	10	—	323 ± 12	—
Arthritic	7	Saline	667 ± 23**	11 ± 0.7
Arthritic	7	GH	617 ± 28**	11 ± 0.6

P*<0.05, *P*<0.01 compared with control. One-way ANOVA followed by the Duncan test.

was no difference in serum concentrations of glucose between arthritic rats injected with GH or saline (120 ± 2.0 vs 115 ± 5.2 mg/dl).

Figure 3 Representative Western ligand blot of IGFBPs in 2 µl serum from control rats injected with saline (C) or arthritic rats injected with saline (AA) or with rhGH (AA+GH). IGFBPs were separated by SDS-PAGE on a 12.5% gel, transferred to nitrocellulose, ligand blotted with ¹²⁵I-IGF-I and visualized via autoradiography; approximate molecular masses of each band are indicated on the right. The 47, 33 and 24 kDa IGFBPs represent IGFBP-3, IGFBP-1 and -2 and IGFBP-4 respectively.

Western ligand blot analysis of rat serum IGFBPs (Fig. 3) showed the characteristic pattern with a band of an apparent molecular mass of 47 kDa identified as IGFBP-3, a group of two bands of 33 kDa (IGFBP-1 and -2) and a 24 kDa band (probably IGFBP-4). As shown in Fig. 3, arthritis increased the serum IGFBPs of 47, 33 and 24 kDa (*P*<0.01), and GH administration to arthritic rats reversed the increase in 47 and 33 kDa IGFBPs, whereas the arthritis-induced increase in 24 kDa IGFBP was only partially reversed by GH administration.

Experiment 3

When administered to control rats, rhGH had no effect on body weight gain (Fig. 4). In contrast, in arthritic rats, rhGH treatment increased body weight gain at the doses of 3 IU/kg (F1,120=16, *P*<0.01) and 6 IU/kg (F1,113=13, *P*<0.01). As described in the result of the first experiment, GH administration to arthritic rats, at the three doses studied, did not modify the splenic weight or the arthritis scores (data not shown).

As seen in Fig. 5, the stimulatory effect of GH on serum IGF-I in control rats was dose related (F1,34=8.4, *P*<0.01), the increase being statistically significant in the rats that received 3 or 6 IU/kg of GH. In the arthritic rats, rhGH administration also induced an increase in serum concentrations of IGF-I in a dose-dependent manner (F1,32=4.9, *P*<0.05), but the increase was only significant in the group that received 6 IU/kg of rhGH (Fig. 5). A dose-dependent increase in hepatic concentrations of IGF-I was observed in the rats injected with rhGH whether arthritic (F1,32=5.4, *P*<0.05) or not (F1,34=7.79, *P*<0.01). In both control and arthritic rats, the

Figure 4 Effect of rhGH treatment at different doses over 8 days on body weight gain in control and arthritic rats. Values are means ± S.E.M. for at least eight rats per group.

Figure 5 Changes in serum and hepatic concentrations of IGF-I in control (C) and arthritic rats (A) after administration of varying rhGH doses. Values are means $\pm$ S.E.M. for at least seven rats per group. Two-way ANOVA revealed that arthritis decreases ($F_{1,66}=112$, $P<0.01$) and GH administration increases ($F_{1,66}=3.22$, $P<0.05$) serum concentrations of IGF-I. Hepatic concentrations of IGF-I were also decreased by arthritis ($F_{1,64}=21$, $P<0.01$) and increased by GH treatment ($F_{1,64}=4.4$, $P<0.01$). * $P<0.05$, ** $P<0.01$ vs respective group injected with saline; Duncan's multiple comparison test.

increase in hepatic concentrations of IGF-I was significant in the groups injected with 6 IU/kg of rhGH (Fig. 5).

Figure 6 illustrates the effect of rhGH administration to control rats on serum concentrations of IGFBPs. GH increased the serum concentrations of IGFBP-3 in the control rats that received 1.5, 3, or 6 IU/kg, whereas IGFBP-1 and -2 and the 24 kDa IGFBP were not modified by any of the GH doses used in the study (Fig. 6). Arthritis increased serum concentrations of IGFBP-3 (119 ± 5 vs 97 ± 6 , $t=2.87$, $P<0.01$), IGFBP-1 and -2 (142 ± 7 vs 99 ± 7 , $t=4.29$, $P<0.01$), and IGFBP-4 (114 ± 2 vs 99 ± 4 , $t=3.4$, $P<0.01$). GH treatment reverted the effect of arthritis on IGFBP-3 in the rats that received 3 IU/kg. Serum concentrations of IGFBP-1 and -2 were also decreased by GH administration in all the doses analyzed. GH administration did not modify IGFBP-4 in the arthritic rats (Fig. 7).

Discussion

As we have recently reported, arthritic rats showed a decrease in body weight and in circulating IGF-I levels, which appears to be inversely related to the increase in serum binding activity of the IGFBPs (López-Calderón *et al.* 1999). The results of this study demonstrate that the decrease in serum concentrations of IGF-I in adjuvant-induced arthritis cannot be secondary to the anorexia, since pair-fed rats had no modifications in circulating IGF-I. Those effects could be related to the inflammatory response since similar modifications were observed in rats chronically injected with endotoxin (Soto *et al.* 1998).

These data are in accordance with those observed in patients with rheumatoid arthritis who have an increased resting energy expenditure and increased protein breakdown (Roubenoff *et al.* 1994). Also, an association between the activity of the inflammatory cytokine TNF- α and weight loss in arthritic rats was found (Roubenoff *et al.* 1997).

Our data show that the arthritis-induced decrease in body weight gain can be, in part, prevented by GH treatment. The stimulatory effect of rhGH on body weight gain in arthritic rats is not due to an increase in food intake. Similarly, in hypophysectomized rats, GH administration produced dose-dependent increases in body weight, without increasing food intake (Clark *et al.* 1985). An increased efficiency of food conversion in rhGH-treated rats could account for the effect of GH on body weight in the arthritic rat. This effect could be due to an increase in IGF-I synthesis or to a metabolic effect of GH by itself.

Although GH administration did not reverse the arthritis-induced decrease in serum IGF-I, it stimulated serum and hepatic IGF-I in the arthritic rats as in the controls, at the dose of 6 IU/kg. The dose-dependent increase in hepatic and serum IGF-I with rhGH administration indicates that in arthritic rats the liver is able to respond to GH by synthesizing this hormone. Thus, the decrease in GH secretion observed in the arthritic rat could be responsible for the decrease in circulating IGF-I and body weight gain. Nevertheless, we cannot exclude other causes in the decrease in circulating IGF-I in chronic inflammation. GH resistance has been described during the inflammatory response in rats (Defalque *et al.* 1999). However, GH administration in septic rats increases visceral protein content (Okamura *et al.* 1989), and in

Figure 6 The effect of different doses of rhGH administered over 8 days on serum IGFBPs in control rats. Data from six individual rats were quantified by densitometry and expressed as percentage of the mean value in control rats treated with saline. * $P < 0.05$, ** $P < 0.01$ vs control group injected with saline (open bars); Duncan's multiple comparison test.

septic patients GH ameliorates the catabolic state by increasing protein synthesis and IGF-I levels (Voerman *et al.* 1992). In addition, rhGH administration to children with rheumatoid arthritis increases height velocity and plasma levels of IGF-I (Davies *et al.* 1997).

High doses of GH in patients with critical illnesses have been associated with increased mortality (Takala *et al.* 1999). Similar data have been reported in rats after acute endotoxin administration (Liao *et al.* 1997). Although both

Figure 7 The effect of different doses of rhGH administered over 8 days on serum IGFBPs in adjuvant-induced arthritic rats. Data from six individual rats were quantified by densitometry and expressed as percentage of the mean value in control rats treated with saline. * $P < 0.05$, ** $P < 0.01$ vs arthritic group injected with saline (open bars); Duncan's multiple comparison test.

acute lipopolysaccharide (LPS)- and adjuvant-induced arthritis stimulate the inflammatory response, the endocrine responses are not the same. Acute LPS administration induces hypoglycemia (Soto *et al.* 1998) and this effect is exacerbated by GH administration (Liao *et al.* 1997). However, chronic LPS administration (Soto *et al.* 1998)

and adjuvant-induced arthritis (López-Calderón *et al.* 1999) are not associated with hypoglycemia. Furthermore, both chronic LPS- and adjuvant-induced arthritis increased all the IGFBPs, whereas acute LPS injection only increased the 33 kDa IGFBP (Soto *et al.* 1998).

The fact that the dose of 3 IU/kg stimulates body weight gain without increasing serum concentrations of IGF-I, suggests that the effect of rhGH on body weight is not only mediated by IGF-I. Other studies indicate that GH is more potent than IGF-I administration in promoting growth (Fielder *et al.* 1996). In the arthritic rats, the decrease in GH, IGF-I and body weight gain is associated with an increase in the IGFBPs. These data indicate that the bioavailability of IGF-I is further reduced in these rats. Disregulation in IGFBP-3 and other IGFBPs appears to be involved in arthritic disorders (Martel-Pelletier *et al.* 1998). Whereas it is accepted that IGFBP-1 and -2 production may contribute to impaired metabolism in clinical conditions where cytokines production is increased (Fan *et al.* 1995, Benbassat *et al.* 1999), the role of IGFBP-3 is less known. IGFBP-3 is elevated in rats in a different catabolic situation as acute renal failure (Bohé *et al.* 1998). Furthermore, children with chronic renal failure are often growth retarded despite normal serum levels of GH and IGF, and recent studies suggest that the excess IGFBPs in serum may contribute to growth failure (Powell *et al.* 1998). The increase in IGFBP-3 in arthritic rats can be secondary to the increased cytokines release in this disease, since TNF- α and interferon- γ have been shown to increase IGFBP-3 production (Katz *et al.* 1995, Rozen *et al.* 1998).

In contrast to that observed in serum IGF-I, all administered doses of rhGH normalize serum concentrations of the 33 kDa band IGFBP corresponding to IGFBP-1 and -2. Taking into account that GH is one of the regulators of IGFBP-1/2, these data can be due to the correction of the GH deficiency in the arthritic rats treated with rhGH. Similarly, hepatic IGFBP-1 synthesis is enhanced in hypophysectomized rats and down-regulated by GH administration (Seneviratne *et al.* 1990). The fact that rhGH decreases IGFBP-3 in arthritic rats is unexpected, since GH has been shown to increase serum concentration of IGFBP-3. It is unlikely that in our study GH treatment was not efficacious, since rhGH in control rats increases serum concentration of IGFBP-3 at all doses used in our study. In contrast, in arthritic rats, rhGH at the dose of 3 IU/kg decreases serum IGFBP-3, and this decrease is concomitant with an increase in body weight gain. Similarly, GH administration to JCA children improved growth velocity and increased the IGF-I/IGFBP-3 ratio (Touati *et al.* 1998).

There is evidence that free IGFBP-3 can inhibit cell growth *in vitro*, and this inhibitory effect can be attenuated by IGF-I (Oh 1997). Furthermore, the antiproliferative action of antiestrogens is correlated with suppression of circulating IGF-I and upregulation of IGFBP-3 (for

review see Rosen & Pollak 1999). All these data indicate that IGFBP-3 is an important regulator of cell growth, and inhibits it through mechanisms both dependent on and independent of IGF-I binding. The ability of rhGH to improve body weight gain in arthritic rats and its correlation with circulating IGFBP-3 suggest an important role of this IGFBP in the catabolic response induced by chronic inflammation.

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