Arthritis-induced increase in serum levels of IGF-binding protein-3 in rats is secondary to the decrease in its proteolytic activity

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Abstract

Adjuvant-induced arthritis is a chronic inflammatory illness that induces a catabolic state, with a decrease in pituitary GH and hepatic IGF-I synthesis. We have previously observed an increase in serum IGF-binding protein-3 (IGFBP-3) in arthritic rats, and found that GH administration prevents the increase in circulating IGFBP-3 in arthritic rats. The aim of this work was therefore to study IGFBP-3 synthesis in the liver as well as its proteolysis in serum as the two possible causes of the increased circulating IGFBP-3 in arthritic rats. The effect of recombinant human GH (rhGH) administration was also analysed. Adult male Wistar rats were injected with complete Freund’s adjuvant or vehicle, and 14 days later they were injected s.c. daily until day 22 after adjuvant injection with rhGH (3 IU/kg) or saline. Three hours after the last GH injection, all rats were killed by decapitation. Arthritis increased serum IGFBP-3 levels (P<0.01). The increase in serum IGFBP-3 levels in arthritic rats seems to be due to decreased proteolysis (P<0.01) rather than to an increased synthesis, since liver IGFBP-3 mRNA content was not modified by arthritis. GH administration to control rats resulted in an increase in both hepatic IGFBP-3 mRNA content and in serum IGFBP-3 levels in spite of the increase in IGFBP-3 proteolysis in serum. In arthritic rats, GH treatment did not modify liver IGFBP-3 synthesis, but it increased serum proteolysis of IGFBP-3, leading to a serum concentration of IGFBP-3 similar to that of control rats. Furthermore, there was a negative correlation between circulating IGFBP-3 and its proteolytic activity in the serum of adjuvant-induced arthritic rats. These data suggest that in chronic arthritis the increase in IGFBP-3 serum concentration is secondary to a decrease in proteolytic activity, rather than to an increase in hepatic IGFBP-3 gene expression.


Introduction

Rat adjuvant-induced arthritis (AIA) is a well-established model of rheumatoid arthritis (RA) (Langman et al. 1990, Wooley 1991). Histologic changes, including leukocyte invasion and synovial cell activation, precede clinical symptoms such as joint swelling and weight loss (López-Bote et al. 1988, Halloran et al. 1996). The decrease in body weight in AIA induces a catabolic state (Roubenoff et al. 1987) that may result, at least in part, from a lack of anabolic hormones such as insulin-like growth factor-I (IGF-I) and growth hormone (GH). A decrease in serum 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 reported that the decrease in GH mRNA is concomitant with a decrease in serum and hepatic IGF-I, which correlates with the decrease in body weight in AIA (López-Calderón et al. 1999).

The bioactivity of IGF-I is regulated by IGF-I binding proteins (IGFBPs), where IGFBP-3 is the main carrier of circulating IGF-I and, together with the acid-labile subunit (ALS), it forms a high molecular weight ternary complex. The ternary complex is thought to increase the half-life of circulating IGF-I and to control the access of IGF-I to extravascular target tissues (Binoux & Hossenlopp 1988, Martin & Baxter 1992). IGFBP-3 also plays an important role in the cellular environment, where it may both inhibit and potentiate IGF-I-stimulated DNA synthesis (De Mellow & Baxter 1988, Conover 1992). IGFBP-3, as well as IGF-I and ALS, are regulated by GH. Thus, IGFBP-3 levels are decreased in GH deficiency and increased in acromegaly (Baxter & Martin 1986, Blum et al. 1990). Moreover, in GH-deficient dwarf rats and...
hypophysectomized rats, GH induces IGFBP-3 and restores the 150 kDa ternary complex (Gargosky et al. 1994, Fielder et al. 1996). However, we have observed during chronic inflammation that the decrease in GH secretion and in circulating IGF-I levels is associated with high levels of IGFBP-3 in rat serum (Soto et al. 1998, López-Calderón et al. 1999). Furthermore, an increase in plasma IGFBP-3 has also been recently reported in RA patients (Neidel 2001).

The mechanisms by which IGFBP-3 activity can be regulated is through its hepatic synthesis, since the liver has relatively high levels of IGFBP-3 mRNA (Albiston & Herington 1992) or through its proteolysis. This last modulation of IGFBP-3 occurs during pregnancy in humans (Giudice et al. 1990, Hossenlopp et al. 1990), rats (Davenport et al. 1990) and mice (Fielder et al. 1990). The generated fragments bind IGF with lower affinity compared with intact IGFBP-3, resulting in an increased bioavailability of IGF (Blat et al. 1994). Increased proteolysis of IGFBP-3 has also been observed under conditions of physiological stress including post-surgery (Frost et al. 1993), diabetes (Bang et al. 1994) and cancer (Frost et al. 1993).

We have previously observed an increase in serum IGFBP-3 in arthritic rats, and found that GH administration prevents this increase (Ibáñez de Cáceres et al. 2000). Therefore, the aim of this work was to study the mechanism responsible for the increase in serum IGFBP-3 in arthritic rats, by evaluating hepatic synthesis and proteolysis of IGFBP-3 in the serum. The effect of recombinant human GH (rhGH) administration was also analysed.

Materials and Methods

Animals
Male Wistar rats were housed three or four per cage with free access to food and water, under constant conditions of temperature (20–22 °C) and light (lights on from 0730 to 1630 h). The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals. Control and arthritic rats were purchased from Charles River (Barcelona, Spain). Arthritis was induced by an intradermal injection of a suspension of 1 mg heat-killed Mycobacterium butyricum in incomplete Freund’s adjuvant into the tail base. Control animals were injected with vehicle (para-Freund’s adjuvant). Fourteen days after adjuvant injection, control and arthritic animals were divided into two groups, one injected daily with 3 IU/kg rhGH s.c. (Saizen, Serono, Italy) and the other group received 250 µl saline, until day 22 after adjuvant injection. 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, anklyosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16. Rats with a severity score below 6 were excluded from the study (two rats of 20 injected with adjuvant). Arthritis score was 12·5 ± 0·6 (means ± s.e.m.) on day 22 in arthritic rats injected with saline. On day 22, all rats were killed by decapitation, 2·5 h after the last rhGH or saline injection. Trunk blood was allowed to clot, centrifuged and serum stored at −20 °C until IGF-I, IGFBP-3 and proteolysis were measured. The liver was removed, dissected, frozen in liquid nitrogen and stored at −80 °C until RNA extraction was performed.

IGF-I determination by radioimmunoassay (RIA)
Total IGF-I concentrations were measured by a double-antibody RIA (Soto et al. 1998) using the antibody NIDDK UB2–495 distributed by the Hormone Distribution Program of NIDDK through the National Hormone and Pituitary Program, a gift from Dr Underwood and Dr Van Wj. Serum IGFBPs were removed by acid–ethanol extraction. 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%, all samples were run in the same assay.

Western ligand blot
Western blots were prepared as previously described (Hossenlopp et al. 1986). Two microlitres of sera were diluted in sample buffer and boiled for 2 min at 100 °C, loaded onto 1% SDS–12–5% polyacrylamide gels, and electrophoresed under non-reducing conditions. Proteins were transferred onto nitrocellulose sheets (Hybond-C extra; Amersham International plc, Amersham, Bucks, UK). The membranes were dried and blocked for 1 h with 5% non-fat dry milk, 0·1% Tween (Sigma, St Louis, MO, USA), in Tris-buffered saline. Membranes were probed overnight at 4 °C with 125I-labelled IGF-I (5 × 105 c.p.m./ml). The nitrocellulose sheets were then washed, dried and exposed at −80 °C to X-ray film (Kodak X-Omat AR; Eastman Kodak, Rochester, NY, USA) and to two intensifying screens for 1–4 days according to the signal obtained. The signals of the film were quantified by densitometry using a PC-Image VGA24 program for Windows. The density of the IGFBP bands in each lane was expressed as the percentage of the mean density of sera from control rats injected with saline.

Assay for IGFBP-3 proteolytic activity
Sera samples were assayed for their ability to fragment radiolabelled IGFBP-3 as described by Lamson et al. (1991). Serum samples (5 µl) were mixed with 15 000
IGFBP-3 proteolysis in arthritic rats

C.P.M. $^{125}$I-ngIGFBP-3 (recombinant human non-glycosylated IGFBP-3 [rhIGFBP-3], Diagnostic System Laboratories, Webster, Texas, USA, iodinated using the chloramine T method) in a total volume of 30 µl in 0·05 M phosphate buffer, pH 7·4. The mixture was incubated for 18 h at 37 °C and the reaction was stopped by boiling the mixture with 7 µl 5 × SDS loading buffer before loading onto a 12·5% SDS-polyacrylamide gel and run overnight. Gels were fixed and dried (Bio-Rad gel drying system-543, Madrid, Spain) and exposed to X-ray film at −80 °C for 2–3 days. Proteolysis was measured by densitometry of intact IGFBP-3 remaining in each lane and expressed as a percentage of control rat intact IGFBP-3.

Northern blot analysis of hepatic IGFBP-3 mRNA

Total liver RNA was extracted by the guanidine thiocyanate method using a commercial reagent (Ultraspec RNA; Biotex Laboratories, Houston, TX, USA). The extracted RNA was dissolved in diethylpyrocarbonate water, 0·1% SDS and quantified at 260 nm; RNA integrity was confirmed by agarose gel electrophoresis. For Northern blotting, 20 µg denatured RNA from each liver were separated by formaldehyde–agarose gel electrophoresis, transferred to nylon membranes (Hybond-N+; Amersham International plc) and fixed by u.v. cross-linking (Fotodyne, Hartland, WI, USA). The rat IGFBP-3 probe corresponded to nucleotides of rat cDNA described by Albiston and Herington (1990). The $^{32}$P-labelled RNA antisense probe was generated from linearized PEGEM 4Z plasmid with [$\alpha^{32}$P]cytidine triphosphate (Nuclear Ibérica, Madrid, Spain) and T7 RNA polymerase (Roche Molecular Biochemicals, Barcelona, Spain). Prehybridization was performed for 30 min at 68 °C in ULTRAhyb buffer (Ambion, Austin, TX, USA) followed by hybridization for 16 h at the same temperature with 5 × 10$^6$ c.p.m./ml IGFBP-3 labelled riboprobe, in the same buffer. The membranes were washed twice with 2 × SSC, 0·1% SDS at 68 °C for 10 min, and twice with 0·1 × SSC, 0·1% SDS at 68 °C also for 10 min. To verify loading, control hybridization was performed with a 28S DNA probe labelled with $^{32}$P-dCTP by random primer (Roche). The membranes were exposed at −80 °C for 2–7 days. The intensities of autoradiograph signal levels were measured and expressed as the percentage of the mean intensity of the control group injected with saline.

Statistical analysis

Statistics were computed using the statistics program STATGRAPHICS plus for Windows. Data are presented as means ± s.e.m. and were analysed by two-way ANOVA followed by Duncan’s multiple range test. Correlation between two variables was calculated by linear regression. Statistical significance was set at $P<0·05$.

Results

Arthritis induced a dramatic decrease in body weight gain (15·4 ± 2·2 g/7 days vs 61 ± 1·7, means ± s.e.m., $P<0·01$), and GH administration increased body weight gain in arthritic rats (25·3 ± 2·5 g/7 days, $P<0·01$), but not in control rats. Arthritis induced a significant decrease in serum concentrations of IGF-I (1113 ± 59 ng/ml vs 1373 ± 65, $P<0·05$), and GH administration did not modify circulating IGF-I in control or arthritic rats. Similarly, GH treatment did not modify the arthritis index scores (data not shown).

The IGFBP-3 mRNA content in the liver is represented in Fig. 1: arthritis did not modify IGFBP-3 gene expression in the liver. GH administration increased the
IGFBP-3 mRNA content in the liver in control rats \((P<0.01)\), but not in arthritic rats.

As shown in Fig. 2, the IGF-I-binding activity of IGFBP-3 in serum was increased in arthritic rats \((P<0.01)\). GH administration had different effects: it increased the IGF-I-binding activity of IGFBP-3 in the serum of control animals, but GH administration decreased this binding activity \((P<0.01)\) in arthritic rats.

The IGFBP-3 proteolytic activity in rat serum was different from that observed in humans (Fig. 3). In normal human serum, the 29 kDa band corresponding to the added intact IGFBP-3 was more intense than in normal rat serum, suggesting that IGFBP-3 proteolysis activity was less active in normal human serum than in normal rat serum. The IGFBP-3 fragments corresponding to 21.5 and 17 kDa bands were increased and the intact IGFBP-3 band was decreased in pregnant human serum as a result of increased IGFBP-3 proteolysis. In pregnant rat serum, the proteolytic activity was also increased in comparison with normal rat serum. However, in normal and pregnant rat serum, the higher IGFBP-3 proteolysis was not associated with an increase in the 21.5 and 17 kDa bands, suggesting different protease activities in both species. The proteolysis in rat serum presumably results in more extensive fragmentation generating smaller fragments, which then run off the bottom of the gel.

Proteolysis was decreased in arthritic rats \((P<0.01)\), reaching values almost similar to normal human serum (Fig. 4). GH treatment had the opposite effect, since it increased the proteolytic activity in serum in both control and arthritic rats \((P<0.01)\). In control rats injected with saline and in both groups of arthritic rats, there was a negative correlation between the IGFBP-3 proteolytic activity in serum and the serum concentration of IGFBP-3 \((r=0.58, F_{1,15}=7.6, P<0.05)\). The rats that had the lowest serum concentration of IGFBP-3 had the highest IGFBP-3 proteolytic activity in serum. However, in the control rats treated with GH there was no correlation between IGFBP-3 in serum and its proteolysis (Figs 2 and 4).

**Discussion**

In this study we have analyzed the proteolytic activity for circulating IGFBP-3 and its hepatic synthesis in arthritic
rats as the two possible causes of the increase in serum IGFBP-3. The results showed that the high serum levels of IGFBP-3 in arthritic rats appear to be largely due to decreased proteolysis of IGFBP-3, since hepatic synthesis of IGFBP-3 was not changed.

As we have already described in arthritic rats (Ibáñez de Cáceres et al. 2000), concentrations of IGFBP-3 in serum are increased and GH treatment prevents this increase. These changes in serum protein concentrations did not correlate with the hepatic synthesis of IGFBP-3, since liver IGFBP-3 mRNA content was not modified by arthritis and the synthesis remained constant in response to GH. Similarly, liver IGFBP-3 mRNA in humans was not changed after GH treatment (Olivecrona et al. 1999). The results of the present study contrast with several studies that indicate the GH dependency of circulating IGFBP-3 in humans (Baxter & Martin 1986, Blum et al. 1990, Olivecrona et al. 1999) and rats (Gargosky et al. 1994, Fielder et al. 1996, Lemmey et al. 1997), and its hepatic synthesis in the rat (Lemmey et al. 1997). However, we found this GH dependency of IGFBP-3 synthesis in control rats in which GH treatment increased hepatic IGFBP-3 mRNA content and serum concentrations of IGFBP-3.

It is not clear whether GH acts directly in stimulating IGFBP-3 synthesis or is mediated by IGF-I produced in the hepatocytes under GH control (Villafuerte et al. 1994). Scharf et al. (1996) reported that IGF-I is a potent stimulator for the synthesis of IGFBP-3 and ALS, in co-cultures of Kupffer cells and hepatocytes in adult rats, and that GH did not exert direct effects on IGFBP-3 synthesis, but stimulated the expression of ALS. In adult female rats, serum IGFBP-3 as well as hepatic IGFBP-3 mRNA levels increased after IGF-I treatment (van Neck et al. 2000). In contrast, IGF-I mRNA levels and IGFBP-3 mRNA in the liver were reduced in hypophysectomized rats, and GH treatment increased both of them (Domene et al. 1993). However, in GH-deficient dwarf rats, it has been reported (Lemmey et al. 1997) that GH treatment increased liver IGFBP-3 mRNA levels, whereas IGF-I had no effect, which implies that GH might have a direct function in the regulation of IGFBP-3 synthesis.

In control rats, GH treatment increases the hepatic IGF-I synthesis (López-Calderón et al. 2001), which correlates with the increase in IGFBP-3 mRNA, suggesting a GH–IGF-I dependency of IGFBP-3 synthesis. However, we previously found a decrease in hepatic IGF-I gene expression in arthritic rats (López-Calderón et al. 2001) and IGFBP-3 mRNA levels in the liver were unchanged. Moreover, GH treatment in arthritic rats resulted in an increase in hepatic IGF-I synthesis that was not accompanied by an increase in IGFBP-3 synthesis. The results from the arthritic rats suggest that rat liver biosynthesis of IGFBP-3 is not only dependent on the GH–IGF-I system, but that other mechanisms can also be involved.

In patients with RA, IGFBP-3 was elevated in plasma and synovial fluid compared with osteoarthritis, while IGF-I did not differ significantly between the two groups (Neidel et al. 1997). However, some authors found increased IGFBP-3 in synovial fluid of RA patients (Fernihough et al. 1996), while serum IGFBP-3 levels were decreased (Fernihough et al. 1996, Lemmey et al. 2001). The discrepancies between these authors could be due to the degree of disease activity, to the pharmacological treatment or to the degree of serum IGFBP-3 proteolysis. Some of these authors measured IGFBP-3 proteolysis in synovial fluid of patients with RA, as discussed below. But none of them measured IGFBP-3 proteolysis in serum, probably because in normal human serum
IGFBP-3 proteolysis is negligible as can be observed in Fig. 3 of this study and which has also been observed by other authors (Whellams et al. 2000). In addition, in RA patients, the synovial fluid concentrations of IGFBP-3 were positively correlated with synovial fluid levels of interleukin-1β and tumour necrosis factor-α (Neidel et al. 1997). Also, Fernhough et al. (1996) found a significant correlation between levels of IGFBP-3 in the synovial fluid and serum levels of C-reactive protein in RA patients. These data indicate that inflammatory products could induce IGFBP-3 gene expression and release from other tissues, such as endothelial cells, which could mediate the changes in serum concentrations of IGFBP-3 in arthritic rats.

The proteolysis of IGFBP-3 was decreased in the serum of arthritic rats, which correlates with the observed increase in serum concentrations of this protein. Similarly, in the synovial fluid of patients with RA, the proteolysis of IGFBP-3 was decreased (Fernhough et al. 1996, Whellams et al. 2000) and the levels of IGFBP-3 were increased (Fernhough et al. 1996, Tava et al. 1996, Whellams et al. 2000). Inverse correlation between plasma IGFBP-3 levels and proteolysis of IGFBP-3 has also been found in patients after surgery, but show a reduction in circulating IGFBP-3 as a result of IGFBP-3-specific proteases (Davenport et al. 1992). Although a variety of catabolic states has been characterized by the presence of increased IGFBP-3 proteolysis (Davenport et al. 1992, Cotterill et al. 1996), this does not always occur, as we have observed in arthritic rats and humans. Also, in patients with anorexia nervosa, another catabolic condition, the IGFBP-3 proteolytic activity was not increased, but decreased levels of circulating IGF-I and IGFBP-3 were found (Stoving et al. 1999).

During inflammation, data in vivo and in vitro suggest that neutrophil proteases, cathepsin G and elastase, may potentially act as IGFBP proteases (Gibson & Cohen 1999). These proteases do not seem to play a major role in AIA, since we found decreased IGFBP-3 proteolysis despite an increase in the percentage of neutrophils in blood and synovial tissue (Szekanecz et al. 2000).

Our results support the GH dependency of IGFBP-3 proteolysis, since proteolysis of IGFBP-3 was decreased in arthritic rats, and we found an increased proteolytic activity of IGFBP-3 in serum in both control and arthritic rats after GH treatment. Data in the literature about the GH dependency of proteolytic activity towards IGFBP-3 are unclear. After major surgery in humans the degree of IGFBP-3 proteolysis (Skjaerbaek et al. 1996) described in GH-deficient patients including pediatric cases (Yamada et al. 1999), in Laron syndrome (Vaccarello et al. 1993) and in patients with diabetes mellitus (Cheetham et al. 1998). However, in experimental animals such as dwarf mice or hypophysectomized rats, a strong proteolytic activity has been reported (Koedam et al. 1998). In contrast, Rutishauer et al. (1993) described the fact that serum from normal rats contains a proteolytic activity against IGFBP-3 which disappears after hypophysectomy and reappears after infusion of GH. Similarly, we found an increase in IGFBP-3 proteolysis in arthritic rats after GH administration.

Furthermore, the results of Rutishauer et al. (1993) suggest that the proteolytic activity for IGFBP-3 is specifically dependent on GH, but by a different mechanism than through the induction of IGF-I, because administration of IGF-I had no effect on the IGFBP-3 proteolytic activity in the hypophysectomized rat. Accordingly, in patients with Laron dwarfism characterized by GH insensitivity and very low levels of IGF-I (Laron et al. 1980), an increased serum IGFBP-3 proteolytic activity has been described (Cotterill et al. 1992, Fielder et al. 1992).

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