EXTENDED REPORT

Leptin consumption in the inflamed joints of patients with rheumatoid arthritis

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Background: Leptin has been shown to participate in bone remodelling and leptin substitution reported to have a protective effect in experimental septic arthritis.
Objective: To assess leptin levels in inflamed joints and plasma of patients with RA.
Material and methods: Leptin concentrations were assessed in matched blood and synovial fluid samples from 76 patients with RA. Blood samples from 34 healthy subjects acted as additional controls. Results were analysed and correlated with duration and activity of RA, *x* ray changes, and treatment at time of sampling.
Results: In patients with RA, leptin levels were significantly higher in plasma than in synovial fluid samples obtained simultaneously and higher than in control samples. Plasma and synovial fluid leptin levels correlated strongly. Locally in the joint, leptin levels were related to WBC count. Such a relation was not seen in the bloodstream. Leptin levels were not related to sex, age, or disease duration. Difference between leptin levels in plasma and synovial fluid was greater in non-erosive arthritis (5.1 (SEM 1.2) *v* 3.7 (0.9) ng/ml, p=0.006), than in patients with erosive joint disease (6.2 (1.0) *v* 5.4 (0.8) ng/ml, NS). Methotrexate treatment was associated with relatively high plasma leptin levels, while treatment with other DMARDs was associated with lower leptin levels than in patients receiving no DMARD treatment (p=0.0005).
Conclusions: Leptin production was significantly increased in patients with RA compared with healthy controls. Synovial fluid leptin levels were significantly lower than in matched plasma samples, suggesting an in situ consumption of this molecule.

Leptin is a cytokine-like 16 kDa peptide produced mostly by adipose tissue and regulating food intake, basal metabolism, and the β-oxidation of fatty acids. When binding to its receptor(s) located in hypothalamic nuclei occurs, leptin is an important trigger of adaptive mechanisms during starvation leading to down regulation of thyroid and reproductive functions and stimulation of hypothalamus. In healthy subjects, leptin levels in blood are proportional to the body fat mass.[1 2] Leptin has recently been recognised as a modulator of inflammatory and immune responses[3] by virtue of its interaction with leptin receptors expressed by peripheral blood mononuclear cells, vascular endothelial cells, smooth muscle cells, and osteoblasts [4 5] Interacting with its receptor in various tissues, leptin acts as a growth factor. Indeed, leptin participates in bone formation by stimulating osteoblastic cell proliferation and formation of mineralised nodules in primary osteoblasts and osteosarcoma cells.[6 7] Leptin facilitates proliferation of human endothelial cells supporting angiogenesis and neovascularisation.[8] Leptin has a dual role in inflammation. On the one hand, it activates monocyte/macrophage cells and potentiates production of the proinflammatory cytokines, tumour necrosis factor α (TNFα), interleukin (IL)6,[9] and directs T cell differentiation to Th1 phenotype, expressing interferon γ and IL2. On the other hand, it expresses certain anti-inflammatory properties by releasing IL1 receptor antagonist.[10 11] We have previously shown that supplementation with leptin alleviated the course of staphylococcal sepsis.[12] In addition, subjects with higher plasma leptin levels had a better prognosis for survival of sepsis and septic shock.[13]

In rheumatoid arthritis (RA) it has been suggested that hypothalamic-pituitary dysregulation is an important pathogenic mechanism.[14 15] An increase in corticotrophin releasing hormone production and low levels of secreted cortisol seems to be crucial for the onset of RA. A regulatory loop exists between the hypothalamus-pituitary axis and levels of circulatory leptin. Indeed, leptin administration during starvation reduces hypercortisolaemia,[16] while treatment with glucocorticoids up regulates leptin levels.[17 18] In addition, stimulation of hypothalamic centres by intracerebral injection of leptin diminishes response to proinflammatory stimuli exerted by lipopolysaccharide and TNFα. The role of leptin for the development of arthritis has been assessed in two experimental studies. Leptin deficient ob/ob mice were partly protected from antigen induced arthritis,[19] because they developed less synovial tissue proliferation and less humoral response to the injected antigen. In contrast, leptin supplementation to outbred mice reduced the severity of joint destruction in septic arthritis.[12]

In this study we show that leptin levels are increased in patients with RA compared with healthy controls. Moreover, leptin levels in synovial fluid are reduced as compared with matched plasma samples, suggesting local consumption of this molecule in the joint cavity.

PATIENTS AND METHODS

Patients

Plasma and synovial fluid samples were collected from 76 patients who attended the rheumatology clinics, at Sahlgrenska University Hospital in Göteborg, for acute joint effusion. RA was diagnosed according to the American College of Rheumatology criteria.[20] At the time of synovial fluid and

Abbreviations: BSA-PBS, bovine serum albumin-phosphate buffered saline; CRP, C reactive protein; DMARDs, disease modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; GABA, γ-aminobutyric acid; IL, interleukin; MTX, methotrexate; RA, rheumatoid arthritis; TNFα, tumour necrosis factor α; WBC, white blood cell

blood sampling all the patients were receiving non-steroidal anti-inflammatory drugs. Disease modifying antirheumatic drugs (DMARDs) were used by 45 patients, of whom 28 used methotrexate (MTX) alone or in combination with other DMARDs (two with sulfasalazine, three with cyclosporin, five with TNFα inhibitors); five used sulfasalazine (one in combination with cyclosporin); six used parenteral or oral gold salt compounds; three used cyclosporin A (one in combination with leflunomide); two patients were treated with azathioprine (one in combination with etanercept); and the remaining patient received hydroxychloroquine. Six of the 45 patients were treated with the inhibitors of TNFα—four patients received infliximab (three in combination with MTX, one with MTX/cyclosporin), and two patients received etanercept (one in combination with MTX, and one in combination with azathioprine). The remaining 31 patients had no DMARD treatment at the time of blood and synovial fluid sampling. Twenty four patients used oral glucocorticoids (2.5–10 mg/day, mean 6.5 mg), of whom 11 were also treated with MTX and six with other DMARDs. Seven patients received monotherapy with oral glucocorticosteroids.

Recent radiographs of the hands and feet were obtained for all the patients. The presence of bone erosions, defined as the loss of cortical definition at the joint, was recorded in proximal interphalangeal, metacarpophalangeal, carpus, wrist, and metatarsophalangeal joints. The presence of one erosion was sufficient to fulfil the requirement of an erosive disease. The presence of rheumatoid factor of any of the immunoglobulin isotypes was considered as positive.

Collection and preparation of samples

Synovial fluid was obtained by arthrocentesis, aseptically aspirated, and transmitted into sodium citrate (0.129 mol/l; pH 7.4) containing tubes. In most cases synovial fluid was obtained from knee joints. Three samples were from elbows and six samples were obtained from shoulder joints. Blood samples were simultaneously obtained from the cubital vein and directly transferred into sodium citrate medium. Blood samples from 34 healthy subjects (aged 18–67, mean 42 (SEM 7) years) were used as the control group. Collected blood and synovial fluid samples were centrifuged at 800 *g* for 15 minutes, aliquoted, frozen, and stored at –20°C until use.

Laboratory measures of disease activity

Serum levels of C reactive protein (CRP) were measured by standard nephelometry, with established normal range 0–5 mg/l. The erythrocyte sedimentation rate (ESR) was measured by the Westergren method having normal range of 0–20 mm/1st h. White blood cell (WBC) counts in blood and synovial fluid were obtained with a microcell counter F300 (Sysmex, Toa, Japan). Synovial fluid samples were treated with hyaluronidase before the cell count. The level of polymorphonuclear cells in synovial fluid was expressed as a percentage of the overall number of WBCs. The level of folic acid in blood samples was assessed by a chemoluminescence method using Folat ready Pack (Bayer Corporation, Pittsburg, PA) on ADVIA Centauer automatic line. The established normal range is 0.79–54.4 nmol/l.

Leptin levels

Leptin levels were determined by a sandwich enzyme linked immunosorbent assay (ELISA) using matched antibody pairs (R&D systems, Stockholm, Sweden). Briefly, 96 well polystyrene dishes (Nunc, Denmark) were coated with capture antibodies and left overnight at room temperature. After washing, the plates were blocked with bovine serum albumin-phosphate buffered saline (BSA-PBS) containing 5% sucrose. Matched samples of plasma and synovial fluid were introduced into the parallel strips, at a dilution of 1:10 in BSA-PBS. Horseradish peroxidase labelled detection antibodies and corresponding substrate were used for colour development. Double wave length reading at 450 and 570 nm was used, and the difference of absorbances was calculated. The absorbance values obtained were compared with serial dilution of recombinant leptin and presented as ng/ml.

Statistical analysis

The level of leptin in the blood and synovial fluid samples was expressed as mean (SEM). Leptin levels in the patient blood samples and in those of healthy controls were compared. For further comparison patient material was stratified according to the radiological findings (erosive RA *v* non-erosive RA). The difference in the blood and synovial fluid leptin levels between the groups was calculated separately with the Mann-Whitney U test. Comparisons of the matched blood and synovial fluid samples were analysed by the paired *t* test. For an evaluation of the possible influence of current treatment on the leptin levels, patient material was stratified according to DMARD treatment (treated *v* untreated), and further stratified according to treatment with folic acid antagonist (MTX *v* other). Comparison in the group pairs was performed with the Mann-Whitney U test. For the simultaneous comparison of leptin levels in more than two groups the equality of variance F test was used. Interrelation between the leptin levels and duration of the joint disease, age, folic acid, WBC count, and CRP was calculated using the Spearman correlation coefficient. For all evaluations, values of $p<0.05$ were considered significant.

RESULTS

Clinical and demographic data of the patients at presentation

Table 1 presents the clinical and demographic data of the patient and control groups. Matched samples of synovial fluid and blood were collected from 76 patients with RA—49 patients had destructive joint disease (erosive RA), and the remaining 27 patients had no changes of the joints as judged by the recent radiological examination of the hands and feet (non-erosive RA). The patients with erosive RA were, as

Table 1 Clinical and demographic characteristics of patients with rheumatoid arthritis and of healthy controls

	RA, erosive (n=49)	RA, non-erosive (n=27)	Healthy controls (n=34)
Age (years), mean (SEM) [range]	64 (12) [30–85]	54 (20) [20–83]	42 (14) [18–67]
Sex, male/female	20/29	8/19	12/22
Duration of the disease (years), mean (SEM)	14 (1)	8 (2)	–
Rheumatoid factor, +/–	45/4	7/20	N/A
Treatment with DMARD:			
MTX	23	5	–
Other DMARDs	11	6	
None	15	16	
Oral glucocorticosteroids	16	8	

N/A, not assessed.

Table 2 Leptin levels in patients with RA and in healthy controls. Results are shown as mean (SEM)

	RA, erosive (n=49)	RA, non-erosive (n=27)	Healthy controls (n=34)
Leptin level (ng/ml)			
Plasma	6.2 (1.0)	5.1 (1.2)	2.0 (0.6)*
Synovial fluid	5.4 (0.8)	3.7 (0.9)†	
White blood cell count ($\times 10^9$/ml)			
Blood	8.1 (0.4)	7.5 (0.5)	
Synovial fluid	13.3 (2.5)	8.4 (2.0)	
C reactive protein (mg/ml)	45.4 (6.7)	35.9 (9.0)	N/A

*p=0.0019, patients with RA versus healthy controls; †p=0.006 plasma versus synovial fluid, non-erosive RA. N/A, not assessed.

Table 3 Influence of methotrexate treatment on leptin levels. Results are shown as mean (SEM) unless otherwise stated

	Treated with MTX (n=28)	Treated with other DMARD (n=17)	Non-treated (n=31)
Disease duration (years)	13.9 (1.7)	8.8 (1.6)	11.5 (1.8)
Erosive disease (No (%))	23 (82)	11 (65)	15 (48)
Oral glucocorticoids (No (%))	11 (39)	6 (35)	7 (22)
C reactive protein (mg/l)	46.3 (6.9)	49.8 (16.8)	36.0 (8.9)
Folic acid (nmol/l)	28.5 (4.9)	33.5 (9.5)*	14.2 (3.1)
Leptin levels (ng/ml)			
Plasma	5.9 (1.2)†	3.4 (0.3)	6.8 (1.2)‡
Synovial fluid	5.1 (1.0)	3.5 (1.3)	5.1 (0.9)

*p=0.003 DMARD treated versus non-treated; †p=0.002 MTX treated versus other DMARD treated; ‡p=0.024 plasma versus synovial fluid, non-treated group.

expected, significantly more often positive for rheumatoid factor in blood (45/49 *v* 7/27, p<0.0001), had longer duration of joint disease (p=0.002), and were more often receiving MTX treatment (23/49 *v* 5/27, p=0.013) than the group with non-erosive RA. No difference in glucocorticoid treatment between the groups was seen. Although the group with erosive RA tended to be older, the patient groups and the controls did not differ significantly in age or sex.

Increased levels of leptin in blood of patients with RA

Plasma of patients with RA contained significantly higher leptin levels than that of the control group (5.8 (SEM 0.7) ng/ml *v* 2.0 (0.6) ng/ml, p=0.037) (table 2). There was a significant decrease of leptin levels in joint fluid as compared with plasma in the patient group studied (5.8 (0.7) ng/ml *v* 4.8 (0.5) ng/ml, p=0.019). Leptin levels in plasma and synovial fluid of patients with RA showed close correlation (r_s=0.86, p=0.0001). Leptin levels in plasma were not related either to the age or the sex of patients with RA and control subjects. Leptin levels in synovial fluid correlated with synovial WBC counts (r_s=0.47, p=0.021), whereas such a correlation was not found for the leptin levels in plasma. Leptin levels in plasma were not related to the levels of CRP.

Correlation between leptin levels and clinical characteristics of patients with RA

A comparison of leptin levels was made in patients with RA stratified according to the erosivity of joint disease (table 2). Leptin levels in the plasma of patients with erosive and non-erosive joint disease did not differ significantly (6.2 (1.0) *v* 5.1 (1.2), NS), whereas in synovial fluid the leptin levels were higher in patients with erosive RA than in those with non-erosive RA (5.4 (0.8) *v* 3.7 (0.9), respectively, p=0.047). Patients with non-erosive RA displayed a more pronounced difference between the levels of leptin in plasma and its relative reduction in synovial fluid samples (p=0.006), while in the group of patients with erosive RA the difference between leptin levels in plasma and synovial fluid was negligible. Leptin levels correlated with the duration of the joint disease (r_s=0.47, p=0.017). Both in plasma and synovial fluid samples, leptin levels gradually increased with the duration of RA, being highest in the patients with disease lasting over 20 years (plasma 8.4 (1.6) ng/ml, synovial fluid 6.6 (1.2) ng/ml). This increase of leptin levels was, however, not related to the age of patients (the mean age of the group of patients with RA of over 20 years' duration was 59.9 (3.0) years).

Influence of treatment on leptin levels

For further analysis, patients were stratified according to their treatment at the time of sampling (table 3). Patients treated with DMARDs other than MTX had significantly lower leptin levels in plasma than those having no immune modulating treatment (3.4 (0.3) *v* 6.8 (1.2), p=0.0005). The difference between leptin levels in plasma and synovial fluid of patients receiving no DMARD at the time of sampling was significant (p=0.024), while leptin levels in plasma and synovial fluid of DMARD treated patients were similar. Interestingly, patients receiving MTX treatment had significantly higher plasma and synovial fluid leptin levels than the patients receiving other DMARDs (p=0.002). The clinical characteristics of the patients treated with MTX and with other DMARDs, such as the incidence of erosive disease and the levels of inflammation assessed as CRP level and WBC count, were similar and could not be the reason for the difference in leptin levels between the groups. To assess whether the level of folic acid might be responsible for the increase of leptin levels in patients treated with MTX, plasma folate levels in patients with RA and controls were compared (table 3). The folate levels were similar in patients with RA and healthy controls (patients with RA 22.6 (3.0) nmol/l, controls 23.2 (4.0) nmol/l). However, when patients with RA were separated into those treated and those not treated with DMARDs, the non-treated group had significantly lower plasma folate levels (14.2 (3.1) *v* 30.0 (4.3) nmol/l, p=0.003). The MTX treated group did not differ from patients treated with other DMARDs (28.5 (4.9) *v* 33.5 (9.5) nmol/l, NS).

DISCUSSION

Our study showed (*a*) significantly increased leptin levels in the plasma of patients with RA compared with healthy subjects and concomitantly, (*b*) a significant decrease of synovial fluid leptin levels compared with matched plasma samples. Leptin levels in synovial fluid correlated with the cellularity of the synovial fluid samples, implying local uptake/degradation of leptin in arthritis. Interestingly, the most clear cut difference in leptin levels between plasma and synovial fluid was found in patients with RA with the non-erosive joint disease. A positive association between the consumption of leptin in the synovial cavity and the absence of bone destruction may be viewed as leptin mediated down regulation of the erosive process in the joints.

In favour of this suggestion is the knowledge that leptin induces IL1 receptor antagonist production.[10 11] Treatment of patients with RA with IL1 receptor antagonist has been recently proved to stop the joint destructive process.[21] Additionally, chondrocytes and fibroblasts are the two cell types sensitive to leptin stimulation and responding with increased proliferation.[7 22] On the other hand, incubation of cell cultures in TNFα or IL1β enriched media suppresses leptin production.[23 24] Increased levels of these cytokines locally in the synovial fluid of patients with RA may down regulate local production of leptin inside the joint, aggravating leptin deficiency.

This study shows that leptin levels in plasma are influenced by the use of DMARDs. Indeed, high leptin levels were found in patients treated with MTX, whereas patients receiving other DMARDs had significantly lower leptin levels (table 3). High leptin levels were not dependent on the age, disease duration, or activity of RA (judged as levels of CRP and ESR). Two mechanisms of MTX mediated increase of leptin levels may be proposed. MTX is a folic acid antagonist, being recognised as one of the most efficient drugs against chronic destructive arthritides, which can prevent the development of cartilage degradation and joint destruction. MTX competes with folic acid for transport into the cell using the reduced folate carrier protein and for binding to dehydrofolate reductase, resulting in depletion of products for biosynthesis of thymidylate and purines.[25] MTX is known to block γ-aminobutyric acid (GABA) and to affect neuronal activity.[26 27] In contrast, leptin stimulates activity in promelanocortin/GABA neurons.[28] Interestingly, treatment with another GABA antagonist, valproic acid, is also associated with a rise in leptin levels.[29 30] Taken together, these findings indicate reciprocal central stimulation of leptin production in MTX treated patients, mediated in the hypothalamus. However, one should be aware of other possibilities for the increase in leptin levels because the patient group with RA who were not receiving any DMARD treatment showed a similar tendency.

Administration of glucocorticoids is known to influence the leptin levels in circulation.[17 18] We found no difference in leptin levels between the users and non-users of glucocorticoids among the patients with RA. This may possibly be explained by the relatively low doses of glucocorticoids provided, or the prolonged treatment time, or both. Folic acid substitution is regularly provided during long term MTX treatment in RA.[31 32] Folic acid supplementation used in weight reducing programmes correlated positively with an increase in leptin levels.[33] However, in our patients the folate levels in plasma of those treated with MTX were similar to the levels of those treated with other DMARDs and could not be the reason for the significant difference in leptin levels between these two groups.

In conclusion, our study demonstrated a significant increase of circulating leptin levels in patients with RA compared with healthy controls. Local consumption of leptin in the joint cavity was associated with non-erosive joint disease, suggesting that leptin has a protective role against the destructive course of RA.

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