Leptin enhances MMP-1, MMP-3 and MMP-13 production in human osteoarthritic cartilage and correlates with MMP-1 and MMP-3 in synovial fluid from OA patients

A. Koskinen¹, K. Vuolteenaho¹, R. Nieminen¹, T. Moilanen¹², E. Moilanen¹

¹The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland; ²Coxa Hospital for Joint Replacement, Tampere, Finland.

Abstract

Objective

In the present study, we investigated the role of adipocytokine leptin in the pathogenesis of osteoarthritis (OA) by measuring its effects on matrix metalloproteinase (MMP) production in human OA cartilage. In addition, the correlations between leptin and MMP concentrations in synovial fluid from OA patients were studied.

Design

Cartilage tissue obtained from leftover pieces of total knee replacement surgery from patients with OA was used in the experiments. Production of collagenases MMP-1, MMP-8 and MMP-13, and stromelysin-1 (MMP-3) in the cartilage was measured by immunoassay and the signalling pathways were explored by pharmacological means. In addition, synovial fluid samples were collected from 84 OA patients undergoing knee replacement surgery. The concentrations of leptin and MMPs in synovial fluid were measured by immunoassay.

Results

Leptin alone and in combination with IL-1β enhanced production of MMP-1, MMP-3, and MMP-13 in human OA cartilage, while MMP-8 concentrations remained undetectable. The effects of leptin on MMP-1, MMP-3 and MMP-13 production were mediated through transcription factor NF-κB, and through protein kinase C and MAP kinase pathways. Interestingly, leptin concentrations in synovial fluid from OA patients correlated positively with MMP-3 (r=0.51, p<0.001) and MMP-1 (r=0.41, p<0.001) levels.

Conclusions

To our knowledge, this is the first study to show that leptin up-regulates MMP-1 and MMP-3 production in human OA cartilage and correlates positively to MMP-1 and MMP-3 in synovial fluid from OA patients. The findings suggest that leptin has catabolic effects in OA joints by increasing MMP production in cartilage.

Key words

leptin, adipokines, osteoarthritis, matrix metalloproteinases, cartilage
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.

Anna Koskinen, MD
Katriina Vuolteenaho, MD, PhD
Riina Nieminen, PhD, Assoc. Prof.
Teemu Moilanen, MD, PhD, Assoc. Prof.
Eeva Moilanen, MD, PhD, Professor

This study was supported by the Research Programme on Nutrition, Foods and Health (ELVIRA) of the National Technology Agency in Finland, the Competitive Research Funding of the Pirkanmaa Hospital District, the Scandinavian Rheumatology Research Foundation and by Tampere Graduate School in Biomedicine and Biotechnology.

Please address correspondence and reprint requests to:
Prof. Eeva Moilanen,
The Immunopharmacology Research Group,
University of Tampere School of Medicine,
33014 Tampere, Finland.
E-mail: eeva.moilanen@uta.fi

Received on July 16, 2010; accepted in revised form on October 18, 2010.
© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2011.

Competing interests : none declared.

Introduction
Leptin is a hormone that was initially found to be synthesised by white adipocytes and to regulate food intake and energy expenditure (1). Obese people have increased circulating levels of leptin as compared to non-obese individuals (2), and in murine models leptin deficiency causes morbid obesity (1). However, due to leptin resistance in hypothalamus, the increased levels of leptin in the circulation of obese subjects fail to induce expected responses, i.e. reduced food intake and body weight (3). In addition to the regulatory function in energy homeostasis, leptin plays a role in other physiological functions such as neuroendocrine function, reproduction, angiogenesis and bone formation. More recently, leptin has been recognised as a cytokine-like hormone with pleiotropic actions also in immune response and inflammation (4-6).

Osteoarthritis (OA) is the most common joint disease worldwide. It is characterised with progressive degeneration of articular cartilage, limited intra-articular inflammation, pain, stiffness and loss of mobility. The etiology of OA is not established but the risk factors are well known including obesity as one of the main risk factors. Traditionally obesity has been thought to contribute to the development of OA by increased load on the weight-bearing joints. However, it does not offer full explanation for the connection between obesity and OA because obesity is also connected to OA of hand joints (7-9). This suggests that obesity is associated with the development of OA also by systemic/metabolic influence, in addition to biomechanical effect.

Recent studies provide evidence for leptin as a possible link between obesity and OA. Leptin has been shown to be produced by chondrocytes (10-14) as well as by other tissues in the joint including synovium, infrapatellar fat pad, osteophytes, meniscus and bone (10-12, 15). Leptin is found in synovial fluid (SF) (10, 12, 13, 16, 17), and its levels in synovial fluid have been reported to be higher in OA patients than in controls (16). Leptin concentration in SF, and leptin mRNA expression in OA cartilage have been found to correlate positively with BMI (10, 13) and the levels of leptin and leptin receptor (Ob-Rb) expression in human OA cartilage have been found to be related to the severity of cartilage destruction (10, 13). Griffin et al. recently showed that extreme obesity in leptin deficient mice does not increase the incidence of OA as compared to lean wild type mice (18). A recent study by Ding et al. demonstrated an association of serum leptin level to cartilage volume loss in 190 randomly selected subjects (19). These findings further support the role of leptin in the pathogenesis of OA. There is also some evidence of increased leptin levels in patients with radiographically severe OA as compared to control patients and patients with radiographically less severe OA (16, 20).

Cartilage destruction in OA is due to dis-equilibrium between the catabolic and anabolic factors produced by chondrocytes and other cells in the joint (21). Chondrocytes play an important role in the degenerative processes by releasing inflammatory and destructive mediators including proinflammatory cytokines, nitric oxide (NO), prostaglandins and catabolic enzymes (22, 23). Matrix metalloproteinases (MMPs) are extracellular matrix degrading enzymes known to have essential role in the pathogenesis of OA. IL-1β and TNF-α are considered as the main mediators inducing synthesis and secretion of MMPs, NO and proinflammatory cytokines (21).

Recent studies have shown that also leptin might be a factor that regulates chondrocyte functions. We have previously demonstrated that leptin induces IL-6, IL-8, NO and prostaglandin E2 (PGE2) production, and expression of inflammatory enzymes iNOS and COX-2 in human OA cartilage, and, that leptin-induced IL-6, IL-8 and COX-2 expression is mediated by NO (24). Exogenous leptin has also been reported to enhance IL-1β, MMP-9 and MMP-13 production, and downregulation of leptin with small interference RNA has been found to inhibit MMP-13 expression in human OA cartilage (13, 25).

In the present study, we investigated the effect of leptin on the production of four matrix metalloproteinases centrally involved in cartilage damage in OA,
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.

i.e. collagenases MMP-1, MMP-8 and MMP-13, and stromelysin MMP-3, in human OA cartilage. These metalloproteinas are produced by chondrocytes and are present in diseased cartilage. The collagenases are able to cleave type II collagen, which is the predominant collagen type present in cartilage, while MMP-3 is the major enzyme that cleaves aggrecan and it is also able to activate other MMPs. The association of increased production of these MMPs with cartilage damage has been established (21, 26-28). In addition, signalling pathways mediating leptin induced MMP expression were evaluated by pharmacological means. We found out that leptin enhances the production of MMP-1, MMP-3 and MMP-13 in OA cartilage. In order to evaluate the clinical relevance of this finding, leptin and MMP levels were measured in the SF from OA patients, and leptin was found to correlate with MMP-1 and MMP-3.

Materials and methods

Materials

Reagents were obtained as follows: recombinant human leptin and IL-1β were purchased from R&D systems (Minneapolis, MN, USA); SP600125, SB220025, AG 490 and WHI-P154 were from Calbiochem (Merck, Darmstadt, Germany); PD 98059 was from Promega (Madison, WI, USA); Ro 31-8220 was from Alexis Corporation (Lausen, Switzerland); MG 132 was from Tocris Bioscience (Ellisville, MO, USA); and PDTC was from Sigma Chemical Co (St Louis, MO, USA).

Patients

Samples were obtained from 96 OA patients (63 female and 33 male, BMI 30.2±0.6 kg/m², age 70.6±0.9 years, mean±SEM) undergoing total knee replacement surgery in Tampere University Hospital and the patients gave their written approval.

Tissue cultures

Cartilage tissue was obtained from the left over pieces of total knee replacement surgery. The samples were washed with phosphate buffered saline (PBS). Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus, and patellar surfaces were removed aseptically from subchondral bone with a scalpel and cut into small pieces (about 2x2x2 mm). Three randomly selected cartilage cubes were placed in one well of a 6-well plate and incubated in 3mL of tissue culture medium [Dulbecco’s modified Eagle’s medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, and penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL)]; all obtained from Invitrogen, Carlsbad, California, USA] at 37°C in 5% carbon dioxide atmosphere for the time indicated.

In the first two series of experiments, OA cartilage samples from 7 patients were used. Explants were incubated with leptin (0.1 μg/ml or 10 μg/ml) alone or in combination with IL-1β (10 pg/ml) for 48h. In the third series of experiments, explants from subsequent 5 patients were incubated for 48 hours with leptin (10 μg/ml) alone or with leptin in combination with each of the following signalling pathway inhibitors added into the culture media together with leptin: SP600125 10 μM (JNK inhibitor), SB220025 0.5 μM (p38 inhibitor), PD98059 10 μM (Erk1/2 inhibitor), AG490 10 μM (JAK2 inhibitor), WHI-P154 10 μM (JAK3 inhibitor), Ro 31-8220 1 μM (PKC inhibitor), MG132 10 μM (NF-κB inhibitor) and PDTC 100 μM (NF-κB inhibitor). Due to limited amount of cartilage that can be obtained from one donor, we used only one concentration of each inhibitor in the experiments. The concentrations used in the present experiments, were selected on the basis of the studies previously carried out in our laboratory, and on data collected from literature (31-33). The stock concentrations of the inhibitors were made in DMSO (when appropriate), and all treatments as well as controls contained same concentration of the diluent (final concentration of DMSO 1:1000 in all wells). After the experiments the cartilage explants were weighed and the results were counted per mg of cartilage. Aliquots of the culture media were kept at -20°C until assayed. Concentrations of MMP-1, MMP-3, MMP-8 and MMP-13 were determined in the culture medium by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, MN, USA).

Synovial fluid samples

Synovial fluid samples were punctured aseptically in the operation room before knee replacement surgery from 84 OA patients. The samples were stored at -70°C until assayed. MMP-1 and MMP-13 concentrations in the synovial fluid were determined by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, MN, USA), and leptin and MMP-3 concentrations were assessed by Enzyme-linked immuno-sorbent assay (R&D Systems, Inc, Minneapolis, USA).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the results was calculated by paired t-test or by repeated measures analysis of variance with Bonferroni post-test when appropriate. Pearson’s r was used to analyse the correlations. R ≥0.3 or r ≤-0.3 was considered to indicate a meaningful correlation (r=0.3 to 0.5 or r= -0.5 to 1.0 indicating medium or strong correlations, respectively, according to Cohen (34)). p-values <0.05 were considered as significant. Standard multiple regression modeling was used to evaluate the relationships between MMPs and leptin in synovial fluid from OA patients. The independent variables entered into the models were gender, BMI, age and leptin, and MMP-1 or MMP-3 as dependant variable. Multi collinearity was
assessed with tolerance and variance inflation factor methods. Instat (GraphPad Software, La Jolla, California, USA) and SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) were used in the statistical analysis.

Results
The effects of leptin on MMP-1, MMP-3, MMP-8 and MMP-13 production in human OA cartilage
OA cartilage produced low levels of MMP-1, MMP-3 and MMP-13 in the tissue culture medium, and their production was significantly increased when leptin (10 μg/ml) was added into the culture (Fig. 1). Lower concentration of leptin (0.1 μg/ml) had a slight enhancing effect on MMP-1 and MMP-13, but it did not reach statistical significance. The effect of leptin was also investigated in the presence of low concentration of IL-1β (10 pg/ml) mimicking the inflammatory condition in OA joints. Leptin enhanced MMP-1, MMP-3 and MMP-13 production also in the presence of IL-1β (Fig. 2). MMP-8 concentrations remained undetectable in control samples and under leptin or and IL-1β treatment with the detection limit of 90 pg/ml.

Signalling pathways involved in the leptin-induced MMP-1, MMP-3 and MMP-13 production
The signalling pathways involved in leptin-stimulated MMP-1, MMP-3 and MMP-13 production in OA cartilage were studied by pharmacological means. Inhibitors of transcription factor NF-κB, MG 132 (10 μM) and PDTC (100 μM), protein kinase C inhibitor Ro 31-8220 (1 μM), and JNK inhibitor SP600125 (1 μM) inhibited leptin-induced MMP-1, MMP-3 and MMP-13 production in a statistically significant manner (Fig. 3). In addition, leptin-induced MMP-1 and MMP-13 production was inhibited by p38 inhibitor SB220025 (0.5 μM). MMP-13 and to a lesser extent MMP-3 production was also reduced by JAK3 inhibitor WHI-P154 (10 μM). Erk1/2 inhibitor PD 98059 (10 μM) and JAK2 inhibitor AG 490 (10 μM) did not have statistically significant effect on leptin-induced MMP-1, MMP-3 or MMP-13 production.

The relationships between leptin and MMP-1, MMP-3 and MMP-13 in synovial fluid from OA patients
Because leptin enhanced MMP-1, MMP-3 and MMP-13 production in OA cartilage in vitro, the concentrations of leptin and these MMPs were determined in synovial fluid from OA patients to investigate if there is a relationship between leptin and MMPs in vivo. Leptin concentrations (20.4±2.2 ng/ml) correlated positively with MMP-3 (r=0.51, p<0.001, 823.2±73.3 ng/ml) and MMP-1 (r=0.41, p<0.001, 16.7±1.5 ng/ml) levels (Fig. 4). MMP-13 remained undetectable in synovial fluid with the detection limit of 90 pg/ml. In the standard multiple regression model,
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.

leptin and female gender, but not age or BMI, were significant determinants of MMP-1; and leptin, but not gender, age or BMI, was a significant predictor for MMP-3 in synovial fluid. Standardised beta was 0.320 ($p=0.023$) for leptin predicting MMP-1, and 0.380 ($p=0.006$) for leptin predicting MMP-3 (Table I). In these models, leptin explained 5.2% and 7.3% (square of semipartial correlation coefficient) of variation in MMP-1 and MMP-3 concentration, respectively.

Discussion

Obesity is a major risk factor for OA, which has been explained by increased load on the weight-bearing joints, and by systemic factor(s) associated with obesity. Adipokines, especially leptin, are present in OA synovial fluid (10, 12, 13, 16, 17), and chondrocytes express functional receptors for leptin (13, 35). In the present study, we investigated the action of leptin on OA cartilage by measuring its effects on the production of the matrix metalloproteinases that play a central role in the pathogenesis of osteoarthritis. To our knowledge, this is the first study to show that leptin upregulates the production of MMP-1 and MMP-3 in human OA-cartilage. We also found that leptin enhances MMP-13 production in OA-cartilage which is supported by the previous findings (13, 36). The levels of MMP-8 remained under detection limit in our study. Further, we investigated if the relation between leptin and MMPs could be translated to an in vivo situation. Interestingly, we found a positive correlation between leptin and MMP-1 and MMP-3 levels in synovial fluid from OA patients. These findings support the idea of leptin having a role in regulating MMP production in OA cartilage.

The present results together with recently published findings provide evidence for leptin, either produced in the joint or transported there from the circulation, as a factor in the pathogenesis of OA. Leptin was recently found to enhance the production of proinflammatory/destructive mediators NO, IL-6, IL-8, IL-1β and PGE$_2$ in articular chondrocytes (13, 24, 37-39), and to decrease proliferation of cultured human chondrocytes (13). On the other hand, Figenschau et al. demonstrated increased proliferation of chondrocytes and enhanced synthesis of proteoglycans and collagen under leptin exposure (35) suggesting that leptin has also anabolic properties in the joint or that the catabolic effects of leptin trigger compensatory anabolic responses which is typical for the early OA process. Dumond et al. reported that leptin injection to rat knee led to increased synthesis of insulin-like growth factor 1 (IGF-1), transforming growth factor β.
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.

Leptin enhances MMPs in OA cartilage / A. Koskinen et al. (TGFβ) and leptin itself (10). While in a study by Bao et al. (40) intra-articular leptin was found to increase the expression of MMP-2, MMP-9, cathepsin D, collagen II, ADAMTS -4 and -5 genes and to reduce bFGF and proteoglycan expression. The somewhat contradictory effects of leptin may be explained by differences in experimental conditions, by possible differences in responses between healthy and OA cartilage and by differences between animal species used in the experiments and vs human cartilage. The current findings support the catabolic role of leptin in human OA joints through increased MMP expression in OA cartilage and positive correlations between leptin and MMP-1 and MMP-3 concentrations in synovial fluid from OA affected joints.

Leptin transduces its signal by binding to leptin receptor (OB-Rb). Intracellular signalling mechanisms which leptin has been described to activate include JAK-STAT pathway, phosphoinositide 3-kinase (PI3K), nuclear factor κB (NF-κB), protein kinase C (PKC) and mitogen-activated protein kinases (MAPK), the latter including extracellular signal-regulated kinase (Erk1/2), p38 kinase and c-jun N-terminal kinase (JNK) (6). In OA, MAPK pathways and NF-κB are known to be activated in response to inflammatory cytokines to enhance MMP expression (41). We have reported previously that leptin enhances the production of the proinflammatory mediators NO, IL-6, IL-8 and PGE2 in OA cartilage through activation of NF-κB and JNK pathways. In addition, p38, Erk1/2, PKC and JAK3 were involved in some responses (24). Tong et al. reported that leptin increases IL-8 production in human synovial fibroblasts via JAK2/STAT3- and IRS1/PI3K/Akt/NF-κB-dependent manner (42). Otero et al. have shown in mouse chondrogenic ATDC5 cell line and in human primary chondrocytes that leptin when given together with IL-1β or IFNγ, but not alone, induced NO production and nitric oxide synthase II (NOSII) expression through pathways involving JAK2, PI3K, MEK-1 and p38 (37-39). Figenschau et al. reported that leptin binding to Ob-Rb resulted in activation of STAT1 and STAT5 in cultured human articular chondrocytes (35). To our knowledge, the signalling pathways activated by leptin to enhance MMP production in cartilage have not been studied before. According to our present results, leptin-induced MMP-1, MMP-3 and MMP-13 production is mediated through NF-κB, PKC and JNK pathways. Also, activation of p38 kinase is involved in MMP-1 and MMP-13 production, and JAK3 pathway mediates MMP-3 and MMP-13 production. JAK2 and Erk1/2 inhibitors had no effect on the production of the MMPs studied.

Leptin has previously been shown to enhance production of NO, IL-6, IL-8 and PGE2 in human OA cartilage (4, 23, 37, 39). In addition to their other effects in OA joints, IL-6 (21, 43) and IL-8 (44) have been also reported to enhance MMP production, and NO has been shown to activate MMPs (45). Therefore it is possible that these me-
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.

Table I. Standard multiple regression predicting synovial fluid MMP-1 and MMP-3 by gender, age, BMI and synovial fluid leptin.

<table>
<thead>
<tr>
<th></th>
<th>Standardised beta for predicting synovial fluid MMP-1 (ng/ml)</th>
<th>p-value</th>
<th>Standardised beta for predicting synovial fluid MMP-3 (ng/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>0.262</td>
<td>0.021</td>
<td>0.118</td>
<td>0.277</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.038</td>
<td>0.718</td>
<td>0.033</td>
<td>0.751</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.027</td>
<td>0.838</td>
<td>0.127</td>
<td>0.329</td>
</tr>
<tr>
<td>SF leptin (ng/ml)</td>
<td>0.320</td>
<td>0.023</td>
<td>0.380</td>
<td>0.006</td>
</tr>
</tbody>
</table>

In the study, we measured leptin concentration of 20.4±2.2 ng/ml (mean±SEM) ranging from 2.4 to 101 ng/ml in synovial fluid from OA-patients, and that is comparable to previously published data (10, 12, 13, 16, 17). In the in vitro studies, leptin concentrations starting from the highest levels found in OA synovial fluid (100 ng/ml) going to 10 ug/ml were needed to enhance MMP production. That raises a question if the in vitro findings could be translated to an in vivo situation in OA joints. That could be supported by the observations that cultured cartilage explants in general require elevated levels of exogenously added protein mediators compared to physiologically levels to be responsive. High molecular size proteins (MW for leptin is 16 kDa) are not easily diffusible into the cartilage matrix, and therefore the concentration of exogenously added proteins likely remains clearly lower in the vicinity of the chondrocytes in tissue culture than that added into the culture medium (46). In the physiological situation, chondrocytes are thought to produce a significant proportion of the leptin present in the synovial fluid, and respond to it in an autocrine/paracrine manner. That is the case, leptin concentrations in the vicinity of the chondrocytes are most likely much higher than those measured in synovial fluid. To clarify if there might be an association between leptin and MMPs in OA joints in vivo, we also measured leptin and MMP levels in synovial fluid samples. Interestingly, leptin correlated positively with MMP-1 and MMP-3 suggesting that leptin may enhance the production of these MMP enzymes also in vivo.

MMP-1, MMP-3 and MMP-13 are involved in matrix degradation in OA (27, 47). MMP-3 is also used as a biomarker of cartilage degradation in OA patients (48). In the present study, leptin correlated with MMP-1 and MMP-3 in synovial fluid, whereas MMP-13 remained below the detection limit. Although leptin has been reported to be associated with BMI, leptin but not BMI was found to be a significant determinant of MMP-1 and MMP-3 in SF according to standard multiple regression modeling. To our knowledge, the positive correlations between synovial fluid leptin and MMP-1 and MMP-3 have not been reported previously, and they support the view of leptin as an intra-articular factor involved in the pathogenesis of OA.

In conclusion, OA cartilage was shown to respond to leptin by producing increased amounts of MMP-1, MMP-3 and MMP-13 enzymes which have a central role in the pathogenesis of cartilage degradation in OA. The enhancing effect of leptin in inducing MMP-1, MMP-3 and MMP-13 expression was dependent on activation of transcription factor NF-κB, and on PKC and JNK pathways in human OA cartilage, and activation of p38 kinase was involved in MMP-1 and MMP-13 production. The clinical significance of the novel finding of leptin as an MMP enhancing factor in cartilage was supported by the positive correlation between leptin and MMP-1, and MMP-3 in synovial fluid from OA patients. These findings support the role of leptin as a factor in the pathogenesis of osteoarthritis through increased MMP production in OA affected joints.

Acknowledgements

The excellent technical assistance of Ms. Meiju Kuukkonen, Mrs. Marja-Leena Lampén, Mrs. Maria Nousiemies and Mrs. Ella Lehto, and the skilful secretarial help of Mrs. Heli Määttä are greatly acknowledged.

References

9. YUSUF E, NELISSEN RG, IOAN-FACSNAY A et al.: Association between weight or body mass index and hand osteoarthritis: a system-
Leptin enhances MMPs in OA cartilage / A. Koskinen et al.