Authors:	Koskinen Anna, Juslin Sami, Nieminen Riina, Moilanen Teemu, Vuolteenaho Katriina , Moilanen Eeva
Name of article:	Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen activated protein kinase pathways
Year of publication:	2011
Name of journal:	Arthritis Research & Therapy
Volume:	13
Number of issue:	6
Pages:	R184
ISSN:	1478-6354
Discipline:	Natural sciences / Biochemistry, cell and molecular biology
Language:	en
School/Other Unit:	School of Medicine

Arthritis Research & Therapy

arthritis research&therapy

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Arthritis Research & Therapy 2011, **13**:R184 doi:10.1186/ar3512

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ISSN 1478-6354

Article type Research article

Submission date 24 August 2011

Acceptance date 11 November 2011

Publication date 11 November 2011

Article URL http://arthritis-research.com/content/13/6/R184

Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen activated protein kinase pathways

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Abstract

Introduction: Adiponectin is an adipokine that regulates energy metabolism and insulin sensitivity, but recent studies have pointed also to a role in inflammation and arthritis. The purpose of the present study was to investigate the association and effects of adiponectin on inflammation and cartilage destruction in osteoarthritis (OA).

Methods: Cartilage and blood samples were collected from 35 male OA patients undergoing total knee replacement surgery. Preoperative radiographs were evaluated by Ahlbäck classification criteria for knee OA. Circulating concentrations of adiponectin and biomarkers of OA, i.e. cartilage oligomeric matrix protein (COMP) and matrix metalloproteinase 3 (MMP-3), were measured. Cartilage samples obtained at the surgery were cultured *ex vivo* and the levels of adiponectin, nitric oxide (NO), interleukin-6 (IL-6) and metalloproteinases MMP-1 and MMP-3 were determined in the culture media. In addition, the effects of adiponectin on the production of NO, IL-6, MMP-1 and MMP-3 were studied in cartilage and in primary chondrocyte cultures.

Results: Plasma adiponectin levels and adiponectin released from OA cartilage were higher in patients with radiologically most severe OA (Ahlbäck grades 4-5) than in patients with less severe disease (grades 1-3). Plasma adiponectin concentrations correlated positively with biomarkers of OA, i.e. COMP ($r = 0.55$, $p = 0.001$) and MMP-3 ($r = 0.34$, $p = 0.046$). Adiponectin was released by OA cartilage *ex vivo* and it correlated positively with NO ($r = 0.43$, $p = 0.012$), IL-6 ($r = 0.42$, $p = 0.018$), and MMP-3 ($r = 0.34$, $p = 0.051$) production. Further, adiponectin enhanced production of NO, IL-6, MMP-1 and MMP-3 in OA cartilage and in primary chondrocytes *in vitro* by a mitogen-activated protein kinase (MAPK) dependent manner.

Conclusions: These findings show that adiponectin is associated with and possibly mediates cartilage destruction in OA.

Introduction

Adiponectin belongs to the adipokine hormones, which were initially found to be synthesized by white adipose tissue and to control appetite and metabolism. Adiponectin was discovered in 1995 by Scherer et al. and it was first named as Acrp30, adipocyte complement-related protein of 30 kDa [1]. Adiponectin has been found to improve insulin sensitivity [2,3] and to have antiatherogenic properties [4]. Interestingly, adiponectin has also been identified as a regulatory factor in inflammation and arthritis [5-8].

Adiponectin can be found in synovial fluid from OA patients [9,10]. Tissues in the joint, including synovium, meniscus, osteophytes, cartilage, bone and fat have been reported to produce adiponectin [10-12]. The biological effects of adiponectin are mediated through two adiponectin receptor subtypes, adiponectin receptor type 1 (AdipoR1) and type 2 (Adipo R2), which have been shown to be expressed in articular cartilage, bone and synovial tissue [13,14].

In arthritis models and in joint tissues, adiponectin has been postulated to have both pro- and anti-inflammatory effects. Adiponectin has been reported to increase the production of cartilage degrading MMP enzymes, cytokines and prostaglandin E_2 in chondrocytes and in synovial fibroblasts [11,14-19]. By contrast, intra-articularly injected adiponectin was reported to mitigate the severity of collagen-induced arthritis in the mouse, and to decrease immunohistochemically detected expression of tumor necrosis factor, interleukin-1 and MMP-3 [20]. Recently, high circulating adiponectin was found to correlate with cartilage degradation in patients with rheumatoid arthritis (RA) [21-23] although partly contradictory results have also been published [24,25].

Adiponectin has emerged as a regulator of immune responses and inflammatory arthritis [5-7], but its role in OA and cartilage degradation is controversial and, in many aspects, poorly known. The purpose of the present study was to investigate if adiponectin is associated with radiographic severity or biomarkers of OA, or with inflammatory / destructive factors released by cartilage samples obtained from OA patients. Since MAPK pathways have been proposed as therapeutic targets in osteoarthritis [26,27], we decided also to study the possible involvement of these pathways in adiponectin-induced responses in OA cartilage.

Materials and methods

Patients and clinical studies

Patients fulfilled the American College of Rheumatology classification criteria for OA [28]. Preoperative radiographs, blood samples and cartilage tissue were collected from 35 male patients with OA (age 69.5 ± 1.6 years, BMI 29.3 ± 0.8 kg/m²; mean ± SEM) undergoing total knee replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. Radiographs were evaluated according to the Ahlbäck criteria, from grade I to V, grade V representing the most severe findings [29]. Plasma and serum samples were stored at -80ºC until analyzed for COMP, MMP-3 and adiponectin. Cartilage samples were processed as described below and the amounts of adiponectin, NO, IL-6, MMP-1 and MMP-3 released by the cartilage *ex vivo* during 42 h incubation were measured as described below. The study was approved by the Ethics Committee of Tampere University Hospital and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients.

Cartilage cultures

Leftover pieces of OA cartilage from knee joint replacement surgery were used. Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces showing macroscopical features of early OA were removed aseptically from subchondral bone with a scalpel and cut into small pieces and cultured in Dulbecco´s modified Eagle´s medium (DMEM) with glutamax-I (from Lonza, Basel, Switzerland) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) (all from Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified 5% carbon dioxide atmosphere.

Cartilage samples were incubated for 42 h with or without adiponectin (Recombinant Human Adiponectin produced in human embryonic kidney (HEK) cells, Biovendor, Modrice, Czech Republic) and MAP-kinase inhibitors PD98059 (Erk1/2 inhibitor, 10 μM; from Promega, Madison, WI, USA), SB220025 (p38 inhibitor, 0.5 μM; from Calbiochem, Merck, Darmstadt, Germany) and SP600125 (JNK inhibitor, 10 μM; from Calbiochem, Merck, Darmstadt, Germany). The concentrations of adiponectin and MAPK inhibitors used in the experiments were based on preliminary experiments and studies previously carried out in our laboratory [30-32]. After the experiments, the cartilage explants were weighed and the results were expressed per mg of cartilage. The culture media were kept at -20°C until analyzed.

Primary chondrocyte experiments

Leftover pieces of OA cartilage from knee replacement surgery were used. 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 fine pieces. The pieces were washed with phosphate buffered saline (PBS) and chondrocytes were isolated by enzymatic digestion for 16 h at 37°C in a shaker by using collagenase enzyme blend (1 mg/ml; Liberase TM Research Grade from Roche, Mannheim, Germany). Isolated chodrocytes were washed and plated on 24-well plates (1.5 x 10^5 cells/ml) in culture medium (DMEM with supplements, see above) containing 10% of fetal bovine serum. Cells were treated with increasing concentrations of adiponectin (0.1-3 μg/ml) for 24 h. The culture media were kept at -20°C until analyzed. Concentrations of NO, IL-6, MMP-1 and MMP-3 were determined in culture media as described below. To investigate MAP kinase activation (phosphorylation), cells were treated with adiponectin for 30 or 60 min and processed for Western blotting.

NO production

Concentrations of nitrite, a stable metabolite of NO in aqueous solutions, were measured by the Griess reaction [33].

Measurement of adiponectin, COMP, MMP-1, MMP-3 and IL-6

Concentrations of adiponectin, COMP, MMP-1, MMP-3 and IL-6 in plasma, serum and/or medium samples were determined by enzyme-linked immunosorbent assay (ELISA) by using commercial reagents (adiponectin, MMP-1 and MMP-3: R&D Systems, Europe Ltd, Abindgon, UK; COMP: Biovendor, Modrice, Czech Republic; IL-6: Sanquin, Amsterdam, Netherlands).

Western blot analysis

Western blot analysis was performed as previously described [34] using following antibodies: rabbit polyclonal anti-human iNOS, actin and JNK antibodies and HRP-conjugated goat anti-rabbit IgG antibody from Santa Cruz Biotechnology, Inc (Santa Cruz, California, USA); rabbit polyclonal anti-human p38, phospho-p38, phospho-JNK, Erk1/2 and phospho-Erk1/2 antibodies from Cell Signaling Technology, Inc (Beverly, Mass, USA).

Statistical analysis

Data were analyzed using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Instat version 3.00 (GraphPad software, San Diego, CA, USA). The results are presented as mean ± standard error of mean (SEM) or as otherwise indicated. Pearson´s correlation analysis was carried out and r values over 0.3 and under -0.3 were considered to indicate a correlation. Differences between groups were tested by one-way ANOVA or by repeated measures ANOVA followed by LSD or Bonferroni multiple comparisons test when appropriate. P-values less than 0.05 were considered significant. Standard multiple regression

analysis was used to predict circulating biomarker levels (COMP and MMP-3) when adiponectin, age and BMI were set as independent variables. Unstandardized regression coefficients (B) and coefficient of determination squared (R^2) with the related p-values were calculated. Binary logistic regression was used to compute (BMI or age adjusted) odds ratios (OR) for plasma adiponectin and adiponectin released by cartilage to predict the most severe radiographic findings (Ahlbäck grades 4-5 vs. grades 1-3). Statistician was consulted regarding the statistical analysis.

Results

Correlation between plasma adiponectin and biomarkers of OA

Thirty-five male OA patients were included in the study. Mean adiponectin concentration in plasma was 2.5 ± 0.2 µg/ml and no correlation between plasma adiponectin and BMI was found ($r = -0.15$, $p = 0.379$). Interestingly, adiponectin correlated positively with the biomarkers of OA, i.e. COMP ($r = 0.55$, $p = 0.001$; Figure 1A) and MMP-3 ($r = 0.34$, $p = 0.046$; Figure 1B) pointing to a possible connection between adiponectin and cartilage matrix degradation.

In multiple regression analysis, where serum COMP was set as a dependent variable and plasma adiponectin, age and BMI as predictive variables, adiponectin (B (i.e. expected change in COMP with one unit change in adiponectin) = 1.7, $p = 0.010$), but not BMI ($B = -0.10$, $p = 0.566$) or age ($B = 0.14$, $p = 0.160$), was a significant determinant of COMP ($R^2 = 0.39$, $p = 0.001$ for model). Also, adiponectin was a significant determinant of MMP-3 when it was set alone as an independent variable ($B = 0.85$, $p = 0.046$; $R^2 = 0.12$, $p= 0.046$ for model). Addition of BMI or age as independent variables did not improve the model ($R^2 = 0.15$, $p = 0.070$ or $R^2 = 0.14$, $p = 0.089$, respectively).

Plasma adiponectin levels and radiographic severity of OA

Preoperative radiographs of the knees were evaluated by Ahlbäck classification from grade 1 to 5, grade 5 presenting the most severe findings [29]. Grades 1-2 and 4-5 were combined in order to have more equally distributed subgroups. Mean plasma adiponectin concentrations were higher in grades 4-5 group as compared to grade 3 and grades 1-2 groups (Figure 2A) while there was no difference between grades 1-2 group and grade 3 group. There were no

significant differences in age or BMI between the radiographic subgroups, while serum COMP was higher in grades 4-5 group as compared to grade 3 and grades 1-2 group ($p = 0.012$ and $p = 0.006$, respectively) (Table 1). Binary logistic regression analysis was used to further evaluate if adiponectin is associated with the radiographic severity of OA (Ahlbäck grades 4-5 group vs. 1-3 group). When set alone in the model, plasma adiponectin and cartilage culture medium adiponectin, but not BMI or age, were significant explanatory factors of radiographic severity (Table 2). After adjusting for BMI, plasma adiponectin was a significant predictor of disease severity and almost significant after adjusting for age (Table 2). Adiponectin measured in the cartilage culture media was a significant predictor of OA severity after controlling for age and BMI (Table 2).

Production of adiponectin and inflammatory / degrading factors by OA cartilage *ex vivo*

Cartilage samples were obtained during joint replacement surgery from the same patients from whom the preoperative radiographs and the blood samples had been collected (see above), and tissue culture experiments were carried out. The amounts of adiponectin, NO, IL-6, MMP-1 and MMP-3 released from the cartilage into the culture medium during 42 h incubation were measured. Adiponectin release was increased in patients with radiographically most severe OA (Ahlbäck grades 4-5) as compared to grades 1-2 and grade 3 patients ($p = 0.004$ and $p < 0.001$, respectively; Figure 2B). Interestingly, adiponectin levels in the cartilage culture media correlated positively with those of NO ($r = 0.43$, $p= 0.012$; Figure 3A), IL-6 ($r = 0.42$, $p = 0.018$; Figure 3B) and MMP-3 ($r = 0.34$, $p = 0.051$; Figure 3C), whereas no correlation between adiponectin and MMP-1 production was found ($r = 0.17$, $p = 0.31$).

Effect of adiponectin on OA cartilage and primary chondrocytes *in vitro*

To further evaluate the role of adiponectin in OA, we studied the effect of this adipokine on MAP-kinase phosphorylation (i.e. activation) and on NO, IL-6, MMP-1 and MMP-3 production in primary chondrocytes from OA patients. Adiponectin treatment resulted in time-dependent phosphorylation of p38, Erk1/2 and JNK in primary OA chondrocytes which was obvious in 30 min and decreased towards baseline in 60 min (Figure 4). Adiponectin also enhanced NO, IL-6, MMP-1 and MMP-3 production in primary OA chondrocytes in a dose-dependent manner (Figure 5). Because cartilage matrix is an important regulator of chondrocyte metabolism, we wanted to investigate the effects of adiponectin also on OA cartilage in tissue culture. Because of the limited amount of tissue available for the experiments, one concentration of adiponectin (1 μg/ml) was selected based on the cell culture studies (Figure 5) and previously published data on adiponectin levels in OA synovial fluid [9,10,12,35]. Adiponectin enhanced NO, IL-6, MMP-1 and MMP-3 production and iNOS expression in OA cartilage culture and their production was suppressed by p38 MAPK inhibitor SB220025 (0.5 μM) (Figure 6). In addition, Erk1/2 inhibitor PD98059 (10 μM) and JNK inhibitor SP600125 (10 μM) inhibited adiponectin-induced production of IL-6 and NO, and expression of iNOS in a statistically significant manner while their effect on MMP-1 and MMP-3 was smaller and did not reach statistical significance (Figure 6).

Discussion

Adiponectin is found in OA joints and proinflammatory and catabolic effects have been reported [9,10,13-19]. In the present study, we show for the first time that the circulating adiponectin concentrations correlate positively with the levels of the widely used biomarkers of OA, i.e. COMP and MMP-3, and that plasma adiponectin levels, and adiponectin levels released by cultured cartilage, are associated with the radiographic severity of OA. Interestingly, the amount of adiponectin released by OA cartilage *ex vivo* also correlated positively with the production of inflammatory mediators NO and IL-6, and with the matrix degrading enzyme MMP-3. Further, adiponectin, when added at physiological concentrations to cultures of intact human OA cartilage or primary OA chondrodytes, enhanced the production of inflammatory / destructive factors NO, IL-6, MMP-1 and MMP-3. These findings suggest that adiponectin is associated with cartilage matrix degradation and has a role in the pathogenesis or as a biomarker in OA.

In the present study, we measured circulating levels of COMP (cartilage oligomeric matrix protein) and MMP-3 to evaluate the degree of on-going cartilage destruction in OA [36]. The level of serum COMP has been shown to correlate with the grade of OA assessed by the radiological score [37], which was also seen in the current study. Also, the concentrations of MMP-3 have been reported to associate with joint space narrowing [38]. The present results demonstrate for the first time that plasma adiponectin levels correlate with COMP and MMP-3 suggesting an association between adiponectin and the degree of on-going cartilage matrix degradation.

In the present study, we also found that plasma adiponectin levels and adiponectin amounts released by cultured OA cartilage *ex vivo* were higher in patients with radiographically

advanced OA (grade 4-5 according to the Ahlbäck classification) as compared to patients with less severe disease (grades 1-3). This further suggests that adiponectin is associated with cartilage degradation in patients with OA. Our results are supported by the recent studies by Giles et al. [22], Ebina et al. [21] and Klein-Wieringa et al. [23] showing that circulating adiponectin correlates with joint erosions in RA patients; and also by the study of Laurberg et al. [39] reporting elevated plasma adiponectin concentrations in OA patients as compared to healthy controls. In our study, adiponectin released by cultured cartilage also correlated positively with NO, IL-6 and MMP-3 production in the cartilage.

Two recent studies have reported somewhat different findings on the association between plasma adiponectin levels and radiographic findings. In the study by Honsawek et al. [24] adiponectin concentrations in plasma and synovial fluid were lower in patients with more severe knee OA measured by Kellgren and Lawrence scaling. However, after adjustment for gender, age and BMI the plasma finding became non-significant but the differences between adiponectin levels in synovial fluid within the radiographic groups remained significant. Most of their patients were female, which may, at least partly, explain the differences between their and our results. Also, the two different radiographic scaling systems emphasize different findings. We chose to use Ahlbäck grading since it tends to divide the end-stage OA patients less roughly than Kellgren and Lawrence scaling as reported e.g. by Petersson et al [40]. Accordingly, most of our patients (80%) were scaled into the most severe Kellgren and Lawrence grade (grade 4). We included only male patients in the present study as gender is likely to be a confounding factor. A study by Yusuf et al. [25] revealed that higher levels of plasma adiponectin decreased risk for hand OA progression in a six-years follow up, measured by radiographic changes. The findings of that study appear to be somewhat contradictory to our results and to those by the other groups reported above. The differences may be explained by many factors, including different methodology to measure adiponectin,

differences in patient characteristics and study protocol, gender differences (most of the patients in the study of Yusuf et al. were women), and even by possible differences in the pathophysiologies of hand and knee OA. It is also possible that the significance of adiponectin varies according to the phase/severity of the OA process. It is noteworthy that all our patients had advanced OA and were undergoing joint replacement surgery. This made it possible to obtain simultaneous cartilage and blood samples. Lack of patients with less severe OA, however, limits us to generalize the results to milder cases.

An inverse relationship between adiponectin levels and BMI, and especially with visceral fat, has been reported in studies with endocrinological approach [41]. However, no correlation between adiponectin and BMI was found in several recent clinical studies where patients with OA or RA were investigated [35,39,42]. This was also the case within our group of OA patients. This may be explained by the fact that circulating concentrations of adiponectin can be regulated by various hormonal, nutritional or pharmacological factors and that adiponectin is produced not only by white adipose tissue, but by other tissues as well [41]. The question remains open whether there is such a systemic factor that affects the adiponectin levels in patients with arthritis, or might the joint disease itself be actually a greater explanator than BMI to define adiponectin levels in these patients.

As the clinical data suggested that the amount of adiponectin released by cartilage is related to the severity of cartilage degradation, we decided to study its possible mechanisms in OA cartilage. In agreement with recent findings [11,14-16,18] adiponectin was found to stimulate human OA cartilage and primary OA chondrocytes to produce NO, IL-6, MMP-1 and MMP-3, which are proinflammatory / catabolic mediators in OA [43-50]. Accordingly, adiponectin was very recently reported to increase also the production of chemokine IL-8 in human chondrocytes [19]. MAP kinase inhibitors are under development for treatment of OA

[26,27], and MAP kinase pathways have been reported to be activated by adiponectin [51,52]. Therefore we studied if MAP-kinase signaling pathways are activated by adiponectin also in articular chondrocytes and might mediate its effects on NO, IL-6, MMP-1 and MMP-3 production. Adiponectin was found to activate p38, JNK and Erk1/2 kinases at physiologically relevant concentrations. P38 inhibitor decreased the production of all factors studied in a statistically significant manner while Erk1/2 was involved in adiponectin induced iNOS expression and NO production, and JNK in NO, iNOS and IL-6 production. These results together with recently published findings [14,18] show that MAP kinases, especially p38, are significant pathways in adiponectin signaling in chondrocytes. Also, MAP kinase inhibitors are likely to attenuate adiponectin-induced gene expression in OA cartilage.

Adipokines, i.e., hormones secreted by adipose tissue have emerged to be important modulating agents not only in energy metabolism and appetite, but also in immune system and inflammation [53], and they are likely to have a role in mediating the connection between obesity and chronic inflammatory diseases. Actions of adiponectin, leptin, resistin and other, less studied adipokines in OA and other rheumatic diseases have recently been reviewed by Gómez et al and by Neumann et al [7,8]. The most studied adipokine in the pathophysiology of arthritis is leptin, which has been proven to have proinflammatory and catabolic role in OA [8,19,54-58]. Knowledge about adiponectin in joint diseases has accumulated only lately. The present results together with the other recent reports strongly suggest a proinflammatory and catabolic role also for adiponectin in OA and RA cartilage.

Conclusions

Adiponectin was associated with markers and signs of cartilage degradation, i.e. with circulating concentrations of COMP and MMP-3 and with radiographic severity of OA. Adiponectin was released by OA cartilage *ex vivo*, and it correlated with production of NO, IL-6 and MMP-3, which are important mediators in the pathogenesis of OA. Subsequent *in vitro* studies demonstrated that adiponectin when added into the culture enhanced the production of NO, IL-6, MMP-1 and MMP-3 in OA cartilage and primary OA chondrocytes. Adiponectin also activated p38, Erk1/2 and JNK in chondrocytes, and the adiponectin-induced production of NO, IL-6, MMP-1 and MMP-3 were mediated by MAP kinases, especially by p38. These findings strongly suggest that adiponectin is involved in the pathogenesis of joint inflammation and cartilage destruction in osteoarthritis and may be a target for disease-modifying drug development.

Abbreviations

OA: osteoarthritis; COMP: cartilage oligomeric matrix protein; MMP: matrix metalloproteinase; NO: nitric oxide; IL-6: interleukin-6; MAPK: mitogen-activated protein kinase; BMI: body mass index; RA: rheumatoid arthritis; DMEM: Dulbecco´s modified Eagle´s medium; HEK cells: human embryonic kidney cells; Erk1/2: extracellular-signal-regulated kinases ½; JNK: c-Jun N-terminal kinase; PBS: phosphate buffered saline; iNOS: inducible nitric oxide synthase; ELISA: enzyme-linked immunosorbent assay

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AK was involved in the conception and design of the study, in the laboratory analyses, in calculating the results, in interpretation of the data, and she also drafted the manuscript. SJ was involved in the conception and design of the study, in the laboratory analyses, in calculating the results, in interpretation of the data and in drafting the manuscript. KV was involved in the conception and design of the study, in the laboratory analyses, in calculating the results, in interpretation of the data, and in writing the manuscript. RN was involved in conception and design of the study, in the laboratory analyses, in the interpretation of the data, and in revising the manuscript. TM was involved in the conception and design of the study, in selecting the patients and in acquiring the patient samples, in interpretation of the data, and in revising the manuscript. EM was involved in the conception and design of the study, in interpretation of the data and in writing the manuscript. All authors approved the final version of the manuscript.

Acknowledgments

The excellent technical assistance of Meiju Kukkonen, Marja-Leena Lampén, Marja Jousimies, Elina Jaakkola, Petra Miikkulainen and Ella Lehto, and the skilful secretarial help of Heli Määttä are greatly acknowledged. Statistician Heini Huhtala is warmly thanked for her advice in the statistical analysis.

The study was financially supported by The Academy of Finland, the Competitive Research Funding of the Pirkanmaa Hospital District, Päivikki ja Sakari Sohlberg Foundation, Orion-

Farmos Research Foundation and by Tampere Graduate Program in Biomedicine and Biotechnology.

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Figure legends

Figure 1 Adiponectin correlates with biomarkers of OA. Scatter plots showing positive correlations between plasma adiponectin and biomarkers of OA, serum COMP (A) and plasma MMP-3 (B). N = 35 OA patients.

Figure 2 Adiponectin levels in plasma and those released by cultured cartilage were higher in patients with radiologically more severe OA. Adiponectin levels in plasma (A) and the amount of adiponectin released by OA cartilage samples into culture media (B) in OA patients classified according to the severity of their knee OA by the Ahlbäck grading scale. Horizontal solid and dashed bars within the boxes represent the median and mean, respectively; boxes represent the interquartile range; lines outside boxes represent minimum and maximum. Outliers are indicated.

Figure 3 *Ex vivo* cartilage releases adiponectin and its concentration correlates with NO, IL-6 and MMP-3 production. Scatter plots showing positive correlations between adiponectin and NO (A), IL-6 (B) and MMP-3 (C) released by OA cartilage into the culture medium. Cartilage samples were collected from 35 OA patients undergoing knee replacement surgery.

Figure 4 Adiponectin induces activation of MAP kinases in human primary chondrocytes. The effect of adiponectin (3 µg/ml) on MAP kinase phosphorylation in human primary chondrocytes obtained from patients with OA. The figure shows the results of a representative experiment, which was repeated three times (i.e. with cells from three donors)

with similar results. MAP kinases were determined by Western blot analysis at baseline and at 30 min and 60 min after addition of adiponectin.

Figure 5 Adiponectin enhances NO, IL-6, MMP-1 and MMP-3 production in human primary chondrocytes in a dose dependent manner. Chondrocytes obtained from patients with OA were treated with increasing concentrations of adiponectin (0.1-3 µg/ml). Concentrations of NO (A), IL-6 (B), MMP-1 (C) and MMP-3 (D) were measured by the Griess reaction and ELISA in the culture medium after 24 h incubation. The figure shows the results of a representative experiment which was repeated three times (i.e. with cells from three donors) with similar results. Results are expressed as mean ± SEM, n = 4. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ as compared to the control sample.

Figure 6 MAP kinase pathways are involved in adiponectin induced NO, IL-6, MMP-1 and MMP-3 production in OA cartilage. The effects of MAP kinase inhibitors on adiponectin-induced NO production (A), iNOS expression (B), IL-6 production (C), MMP-1 production (D) and MMP-3 production (E) in human OA cartilage. Cartilage explants were incubated for 42 h with adiponectin (1µg/ml) and the inhibitor indicated. Samples were collected from 6 patients in A and B and from 5 patients in C, D and E. Results are expressed as mean ± SEM. # = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ as compared to explants treated with adiponectin alone. PD98059 – Erk1/2 inhibitor; SB 220025 – p38 inhibitor; SP600125 – JNK inhibitor; inhibitor concentrations used were based on previous studies in our laboratory [30-32].

Table 1 Plasma adiponectin levels and clinical characteristics of patients in radiographic subgroups

	radiograhic severity of OA by Ahlbäck classification				
	Grades 1-2 (n = 11)	Grade 3 (n = 16)	Grades 4-5 (n = 8)	Total (n = 35)	p
Adiponectin (µg/ml)	2.4 (0.4)	2.1 (0.2)	3.5 (0.5)	2.5 (0.2)	0.03
Age (years)	68.7 (3.0)	67.3 (2.6)	75.0 (2.0)	69.5 (1.6)	0.17
BMI (kg/m^2)	28.7 (1.1)	29.8 (1.4)	29.0 (1.6)	29.3 (0.8)	0.84
COMP (U/l)	10.6 (1.1)	11.7 (1.0)	17.0 (2.4)	12.6 (0.9)	0.01
MMP-3 (ng/ml)	9.6 (1.1)	9.2 (0.6)	10.9 (1.5)	9.7 (0.6)	0.52

Values are mean (SEM)

p value refers to the significance of the comparison of the three groups calculated by ANOVA

Table 2 Association of adiponectin and radiographic severity of OA. Odds ratios for beloging to Ahlbäck grades 4-5 group vs. grades 1-3 group.

	Crude OR	95% CI	p	Adjusted OR	95% CI	p
Plasma adiponectin (µg/ml)	2.2	1.1 - 4.3	0.022	2.2[a]	1.1 - 4.4	0.022
				1.9[b]	0.9 - 3.8	0.090
Culture media adiponectin (pg/mg cartilage)	1.1	1.0 - 1.2	0.007	1.1[a]	1.0 - 1.2	0.007
				1.1[b]	1.0 - 1.2	0.016
BMI (kg/m^2)	1.0	0.8 - 1.2	0.852			
Age (years)	1.1	1.0 - 1.3	0.078			

[a]adjusted for BMI [b]adjusted for age

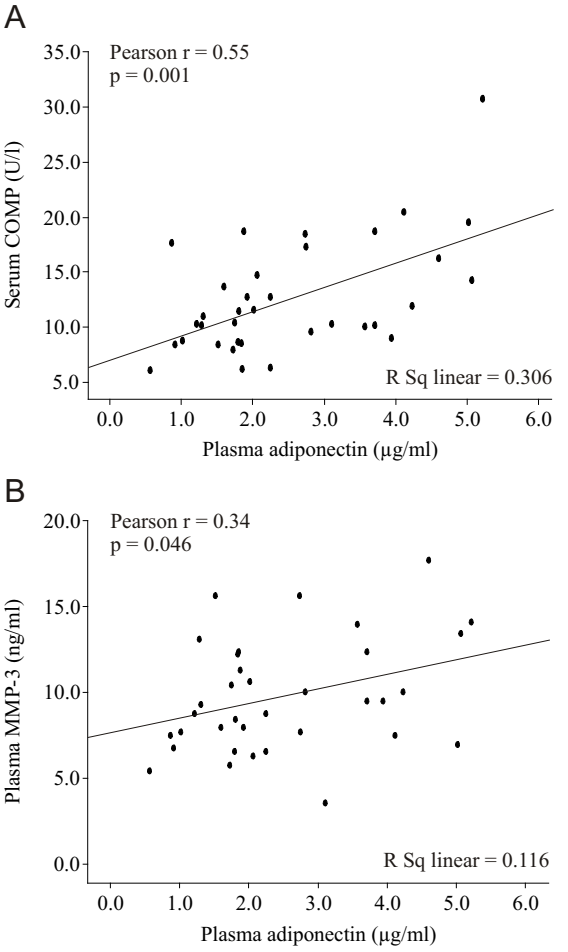
A
Pearson r = 0.55
p = 0.001
Serum COMP (U/l)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
R Sq linear = 0.306
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Plasma adiponectin (µg/ml)
B
Pearson r = 0.34
p = 0.046
Plasma MMP-3 (ng/ml)
20.0
15.0
10.0
5.0
0.0
R Sq linear = 0.116
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Plasma adiponectin (µg/ml)

Figure 1

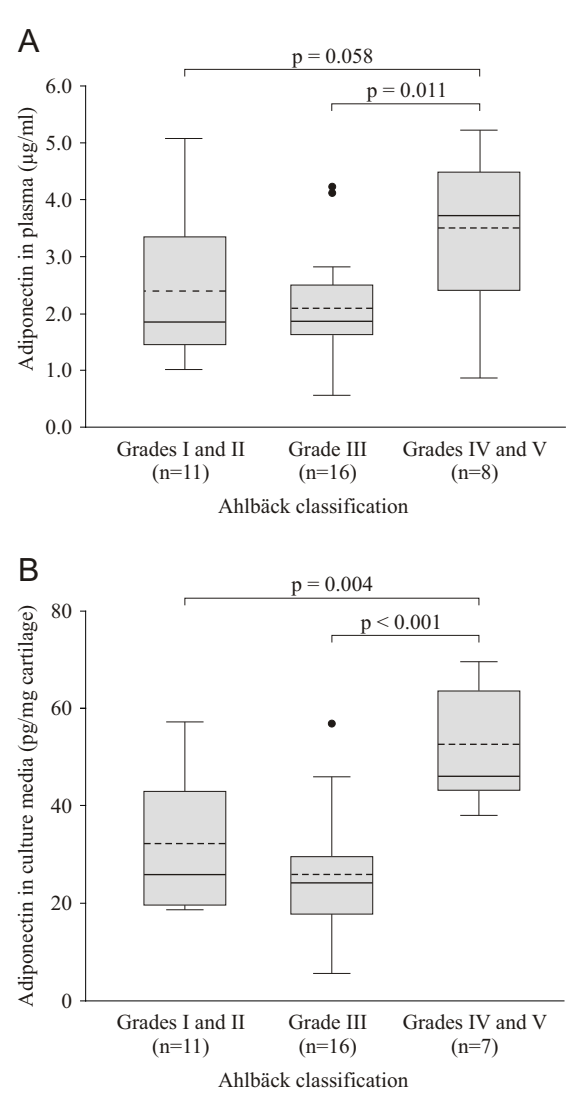
A
p = 0.058
p = 0.011
Adiponectin in plasma (µg/ml)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Grades I and II
(n=11)
Grade III
(n=16)
Grades IV and V
(n=8)
Ahlbäck classification
B
p = 0.004
p < 0.001
Adiponectin in culture media (pg/mg cartilage)
80
60
40
20
0
Grades I and II
(n=11)
Grade III
(n=16)
Grades IV and V
(n=7)
Ahlbäck classification

Figure 2

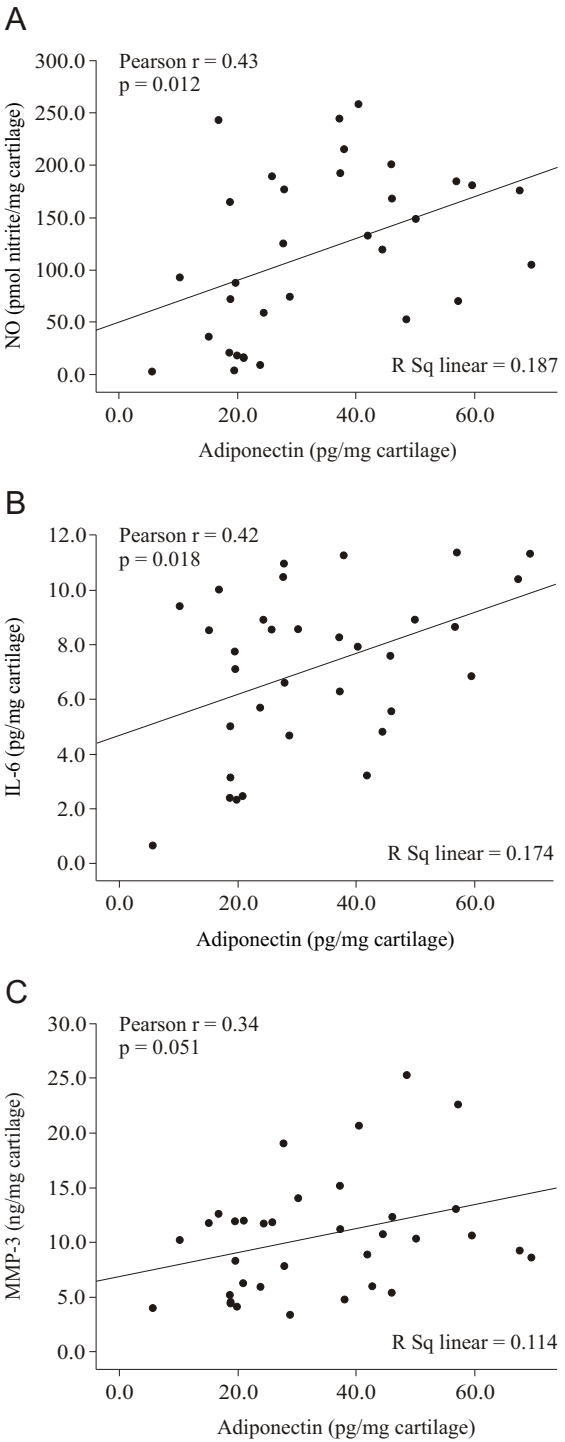
A
Pearson r = 0.43
p = 0.012
NO (pmol nitrite/mg cartilage)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
R Sq linear = 0.187
0.0
20.0
40.0
60.0
Adiponectin (pg/mg cartilage)
B
Pearson r = 0.42
p = 0.018
IL-6 (pg/mg cartilage)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
R Sq linear = 0.174
0.0
20.0
40.0
60.0
Adiponectin (pg/mg cartilage)
C
Pearson r = 0.34
p = 0.051
MMP-3 (ng/mg cartilage)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
R Sq linear = 0.114
0.0
20.0
40.0
60.0
Adiponectin (pg/mg cartilage)

Figure 3

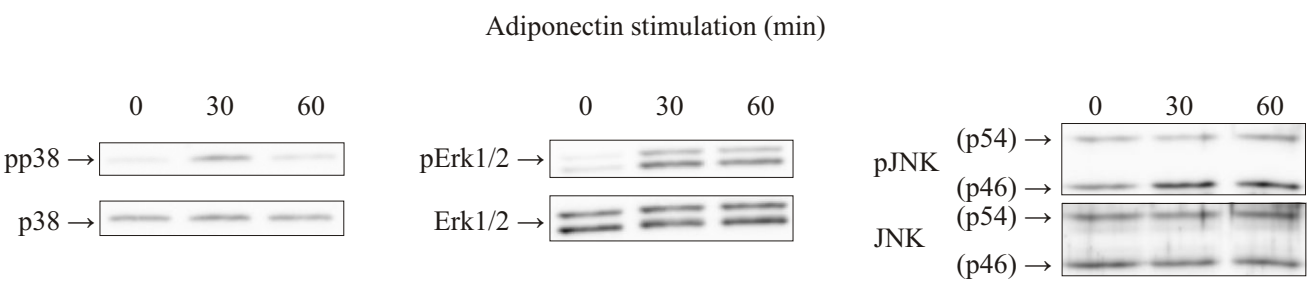
Adiponectin stimulation (min)
0
30
60
pp38 →
p38 →
pErk1/2 →
Erk1/2 →
pJNK
(p54) →
(p46) →
JNK
(p54) →
(p46) →

Figure 4

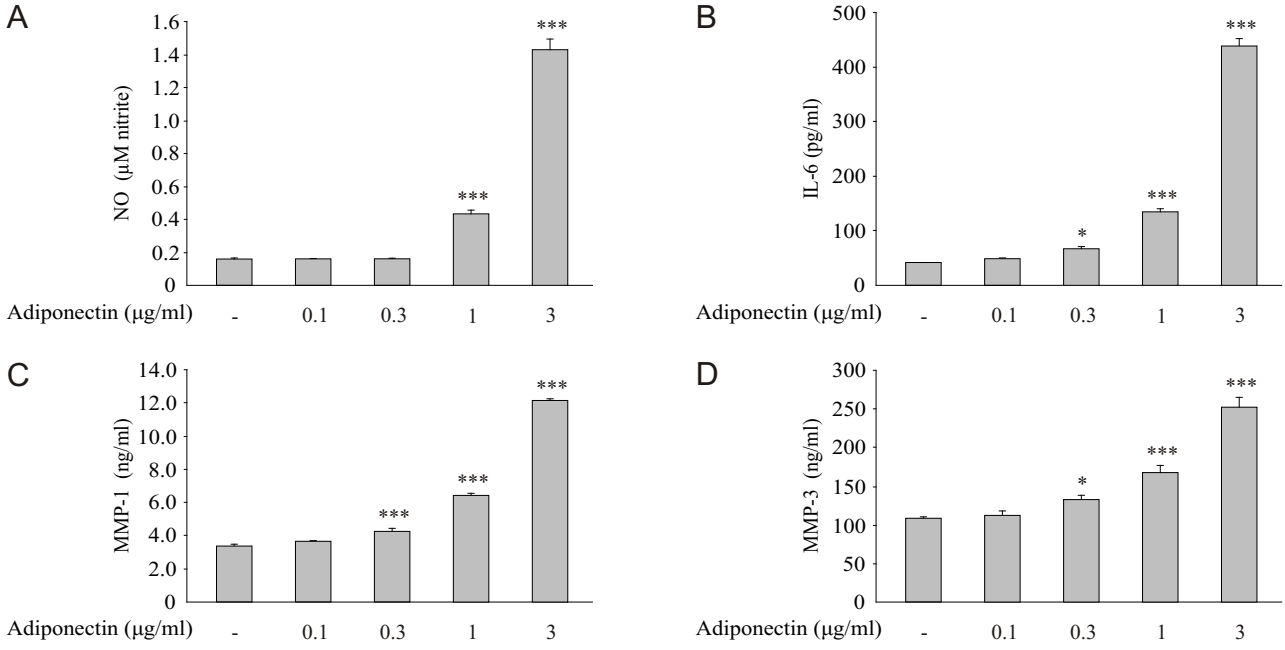
A
NO (μM nitrite)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
***
Adiponectin (μg/ml)
-
0.1
0.3
1
3
B
IL-6 (pg/ml)
500
400
300
200
100
0
***
*
***
Adiponectin (μg/ml)
-
0.1
0.3
1
3
C
MMP-1 (ng/ml)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
***
***
***
Adiponectin (μg/ml)
-
0.1
0.3
1
3
D
MMP-3 (ng/ml)
300
250
200
150
100
50
0
*
***
***
Adiponectin (μg/ml)
-
0.1
0.3
1
3

Figure 5

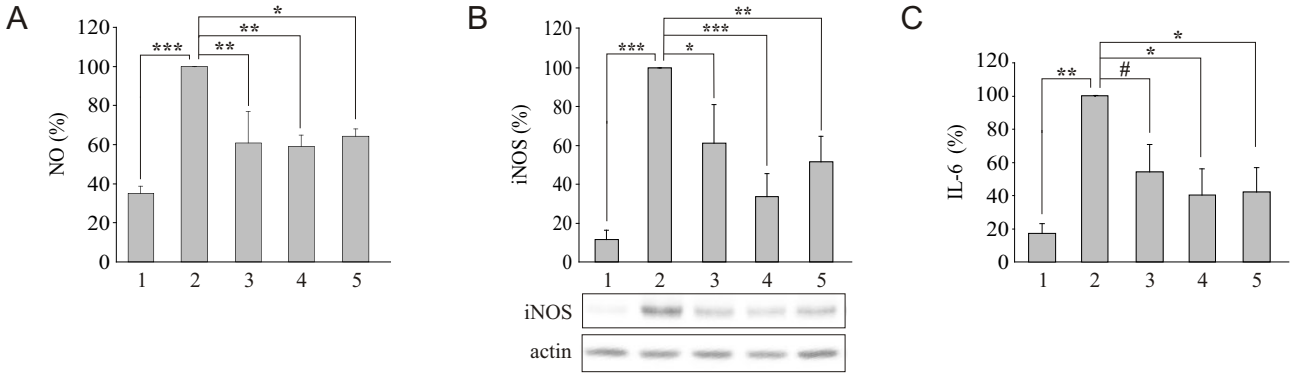
A
NO (%)
B
iNOS (%)
iNOS
actin
C
IL-6 (%)
D
MMP-1 (%)
E
MMP-3 (%)
1 control
2 adiponectin 1 μg/ml
3 adiponectin 1 μg/ml + PD98059 10 μM
4 adiponectin 1 μg/ml + SB220025 0.5 μM
5 adiponectin 1 μg/ml + SP600125 10 μM

Figure 6