Response of human rheumatoid arthritis osteoblasts and osteoclasts to adiponectin

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Abstract

Objective

Adiponectin is an effector molecule in the pathophysiology of rheumatoid arthritis, e.g. by inducing cytokines and matrix degrading enzymes in synovial fibroblasts. There is growing evidence that adiponectin affects osteoblasts and osteoclasts although the contribution to the aberrant bone metabolism in rheumatoid arthritis is unclear. Therefore, the adiponectin effects on rheumatoid arthritis-derived osteoblasts and osteoclasts were evaluated.

Methods

Adiponectin and its receptors were examined in bone tissue. Primary human osteoblasts and osteoclasts were stimulated with adiponectin and analysed using realtime polymerase chain-reaction and immunoassays. Effects on matrix-production by osteoblasts and differentiation and resorptive activity of osteoclasts were examined.

Results

Immunohistochemistry of rheumatoid arthritis bone tissue showed adiponectin expression in key cells of bone remodelling. Adiponectin altered gene expression and cytokine release in osteoblasts and increased IL-8 secretion by osteoclasts. Adiponectin inhibited osterix and induced osteoprotegerin mRNA in osteoblasts. In osteoclasts, MMP-9 and tartrate resistant acid phosphatase expression was increased. Accordingly, mineralisation capacity of osteoblasts decreased whereas resorptive activity of osteoclasts increased.

Conclusion

The results confirm the proinflammatory potential of adiponectin and support the idea that adiponectin influences rheumatoid arthritis bone remodelling through alterations in osteoblast and osteoclast.

Key words

osteoblasts, osteoclasts, rheumatoid arthritis, adiponectin

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Introduction

Excessive bone loss is a result of imbalanced bone formation and bone resorption. Chronic inflammatory diseases such as inflammatory joint diseases or inflammatory bowel disease are frequently associated with systemic bone loss (1, 2). Rheumatoid arthritis (RA) is a chronic inflammatory disorder leading to bone erosion that results from the interaction between genetic and environmental factors leading to innate and adaptive immune response and to systemic inflammation (3-6). Focal bone loss in RA is mediated by osteoclasts (OC), the central cell type responsible for bone resorption under physiological and pathophysiological conditions (2, 4, 7). In RA, proinflammatory cytokines mediate an imbalance in bone remodelling, for example by promoting Receptor Activator of NF-κB Ligand (RANK-L) expression in cells other than osteoblasts (OB) such as RA synovial fibroblasts (RASF) but also by directly inhibiting OB activity (8, 9). Thus, these proinflammatory cytokines are able to decrease bone formation and increase bone resorption, leading to irreversible bone erosions in RA. Inflammatory cytokines (*e.g.* TNF, IL-6 and IL-1β) stimulate osteoclastogenesis (8, 10, 11), while others like transforming growth factor-β (TGF-β) show inhibitory effects (12). Besides classical cytokines, adipokines attracted interest as mediators of inflammation and bone erosion. Specifically, adiponectin had immunomodulatory properties in RA. Adiponectin appears to act mainly proinflammatory on central RA effector cells (13-15). For example, adiponectin induces proinflammatory cytokines and matrix-metalloproteinases (MMP) in RASF (13, 15). Adiponectin mediates altered gene and protein expression in RASF including chemokines as well as proinflammatory factors and MMP (15, 16). Moreover, several reports have demonstrated a correlation of adiponectin with the severity of RA (17, 18). The idea that adiponectin may affect the process of bone remodelling by modulating OB and OC function originated from studies revealing an impact of adiponectin on human OB influencing osteoprotegerin (OPG) and RANK-L expression or the expression of the essential OB transcription factors RunX2 and Osterix (19, 20). Additionally, an increased bone mass was observed in adiponectin KO mice (20, 21). However, these studies were based on experiments with adiponectin in a non-inflammatory background using an adiponectin KO mouse model. Some studies focused on the evaluation of adiponectin specifically in the context of bone erosion in RA examining radiographic progression of joint damage and assessing adipokine levels in serum and synovial fluid of RA patients. For example, Alkady *et al*. identified a positive correlation between serum and synovial adiponectin and the disease activity (22). Giles *et al*. examined serum adiponectin levels in relation to radiographic progression in RA patients and discovered a temporal and concentration-dependent relationship between circulating adiponectin and erosive joint destruction (23). Interestingly, Xibillé-Friedmann *et al*. showed that higher baseline adiponectin was associated to higher disease activity only at baseline but adiponectin level were not associated with clinical response to treatment over time at 1 and 2 years of follow-up (24). To elucidate the effect of adiponectin on cellular level in the context of RA we investigated the effects of adiponectin using OB, OC and articular tissues derived from RA patients. To further clarify the role of adiponectin in human RA, changes in gene and protein expression, as well as alterations in differentiation and functional activity of these cells were evaluated.

Methods

Tissues and cell culture

RA OB were isolated from trabecular bone explants of RA patients undergoing joint replacement surgery (Dept. of Orthopaedics and Trauma Surgery, Markus-Hospital, Frankfurt; Dept. of Orthopedics and Orthopaedic Surgery, University Hospital Giessen). All specimens were obtained with the approval of the Ethics Committee of the Justus-Liebig-University Giessen. Patients gave written informed consent and ful-

filled the criteria of the American College of Rheumatology.

Primary OB were cultured in alphaMEM Glutamax (Gibco, Carlsbad, USA) supplemented with 10%FCS (Sigma Aldrich, St Louis, USA), 100U/ml penicillin (PAA, Coelbe, Germany), and 10µg/ml streptomycin (PAA) and were cultured for a maximum of 4 passages at 37°C and 5% CO_2. OC were cultured in alphaMEM Glutamax containing 10% FCS, 100U/ml penicillin, 10µg/ml streptomycin, 10ng/mL macrophage colony-stimulating factor (M-CSF) and 1ng/ml RANK-L (Peprotech, Rocky Hill, USA).

Osteoclast differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-blood of RA patients (male: 2, mean age: 62±7.07; female: 10, mean age: 63.3±11.01) and healthy donors (male: 1, age: 28; female: 7, mean age: 27.18±4.51) using Ficoll gradient centrifugation (Biochrom, Berlin, Germany). $1.5x10^6$ cells/ml were cultured in alphaMEM Glutamax (Gibco) supplemented with 10% FCS (Sigma Aldrich), 100U/ml penicillin (PAA), 10µg/ml streptomycin (PAA), 10ng/ml M-CSF, and 1ng/ml RANK-L (Peprotech) at 37°C and 5% CO_2.

Osteoblast isolation and culture

Bone of RA patients (male: 1, age: 27; female: 8, mean age: 62±13.78) was minced to approximately 2 mm fragments and washed. Bone fragments were placed in culture flasks containing alphaMEM Glutamax supplemented with 10% FCS, 100U/ml penicillin, and 10 µg/ml streptomycin and cultured at 37°C, 5% CO_2. After cells grew out and reached confluency, they were passaged using trypsin. Experiments were performed with OB cultured up to passage 4.

Stimulation experiments

Primary OB were stimulated with adiponectin (10 µg/ml, Biovendor, Brno, Czech Republic) for 24-72h compared to untreated controls. Cells were harvested for RNA isolation and subsequently quantified by realtime PCR. Supernatants were collected to determine cytokine levels. In a second set of experiments, RA OB were treated with 2.5 to 100 µg/ml of adiponectin. After 17 hours, supernatants were collected.

PBMCs were cultured for 7 or 14 days in alphaMEM Glutamax containing 10ng/ml M-CSF and 1ng/ml RANK-L to obtain (pre)OC. Pre-OC were defined as TRAP positive mononuclear cells. Cells were treated with adiponectin (10 µg/ml). RNA was isolated after 7 or 14 days of differentiation for subsequent gene expression analysis by realtime PCR. Supernatants were collected at day 7 and 14.

LPS contamination found in recombinantly produced proteins may contribute to the effects observed for adiponectin but was excluded as published previously (15).

Mineralisation assay

Cells were cultured in alphaMEM Glutamax supplemented with glycerol phosphate (5 mM, Calbiochem) and ascorbic acid (100 µg/ml, Sigma Aldrich). Cells were stimulated with adiponectin (10 µg/ml). After 14 days, the OB secreted matrix was stained with Alizarin Red S (Sigma Aldrich). Matrix production was documented by visualisation. Colorimetric detection and semiquantification of Alizarin Red S was implemented by acetic acid extraction as described by Gregory *et al*. (25).

TRAP staining

To assess the role of adiponectin on the differentiation of OC, cells were cultured in alphaMEM Glutamax with adiponectin (1 µg/ml). After 14 days, cells were fixed and stained using the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich). TRAP positive, multinucleated cells (≥3 nuclei) were quantified.

Resorption assay

PBMCs ($1.5x10^6$ cells/ml) were cultured in alphaMEM Glutamax containing 10ng/ml M-CSF and 1ng/ml RANK-L and 10 µg/ml adiponectin. After 7 days, cells were detached and seeded into OsteoAssay surface plates (Corning, Corning, USA) using $2–3x10^4$ cells/well. Cells were cultured for 7 days with/without adiponectin. Subsequently, OC were fixed and wells were stained by von Kossa staining. Resorption areas were quantified using Image J 1.47 (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Decalcified RA bone tissue sections (5µm) were deparaffinised followed by antigen retrieval (10 mM sodium citrate; 30-60min 65°C). Endogenous peroxidase was blocked (0.3% H_2O_2 in methanol). Immunohistochemistry was performed for adiponectin, ADIPOR1, -R2, PAQR3 (R&D systems, Wiesbaden, Germany; epitomics, Burlingame, USA; Abgent, San Diego, USA). The antibodies were incubated in a moist chamber at RT. Sections were incubated with Histofine Simple Stain MAX PO (multi)anti-mouse/rabbit (Nichirei Biosciences, Tokyo, Japan) for 30 minutes. Colour development with AEC-substrate (Vector Laboratories, Burlingame, USA) was stopped after microscopic examination followed by nuclei staining with haematoxylin. Serial sections were stained for TRAP using the Leukocyte Acid Phosphatase Kit (Sigma Aldrich).

Immunocytochemistry was performed on cultured primary OB and OC. Cells were seeded on chamber slides and cultured overnight (OB) or 14 days (OC). Cells were fixed with 4% formaldehyde for 20 min. Immunostaining was performed according to the immunohistochemistry procedure.

Cytokine measurement

Cytokines in supernatants were measured using ELISA kits (R&Dsystems). Absorption was measured at 450 nm and data were analysed using the Magellan software (Tecan).

Determination of cell proliferation

To determine the proliferative response to adiponectin, OB ($8×10^3$ cells/well) were cultured at 37°C and stimulated with adiponectin (10 µg/ml) for 48hours. Proliferation was analysed by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenylterazolium bromide, Sigma Aldrich) assays (26).

Realtime PCR

Total RNA was extracted using the RNeasy™ Mini Kit (Qiagen, Venlo,

Netherlands). Reverse transcription was performed using AMV Reverse Transcriptase (Promega, Fitchburg, WI, USA) and random hexamer primers (Roche, Rotkreuz, Schweiz). After denaturation (2 minutes at 70°C) and immediate cooling on ice, reverse transcription was performed for 15 minutes 25°C, 30 minutes 42°C, 30 minutes 55°C, and 10 minutes 70°C. Complementary DNA samples were analysed by realtime PCR in a LightCycler (Roche) using Absolute qPCR SYBR® Green Capillary Mix (Thermo Fisher Scientific, Waltham, USA). Realtime PCR primers for OB and OC marker genes are listed in Table I. Realtime PCR cycling conditions were 15 minutes at 95°C, 50 cycles of 15 seconds at 95°C, 35 seconds at 50–57°C, and 35seconds at 72°C, and were finished using a melting curve analysis programme. The reference gene for normalisation was 18S ribosomal RNA or GAPDH RNA. Fold change in gene expression was determined by using the comparative ΔΔCt method (15). Expression levels of ADIPOR1, -R2 and PAQR3 were determined using the ΔCt ($2^{-(\Delta Ct)}$) method.

Table I. Summary of primers used for real-time PCR.

Gene product	Sequence (5'-3')	Tm (°C)
Alkaline phosphatase forward	caagcactcccacttcatct	57
Alkaline phosphatase reverse	gttgttcctgttcagctcgt	57
RunX forward	tcacaaatcctccccaagta	57
RunX reverse	ggcggtcagagaacaaacta	57
Osteocalcin forward	cttctttcctcttccccttg	55
Osteocalcin reverse	ccacagattcctcttctgga	55
Osterix forward	tgcttgaggaggaagttcac	57
Osterix reverse	aggtcactgcccacagagta	57
OPG forward	agatgtccagatgggttctt	57
OPG reverse	tgagttagcaggagaccaaa	57
Collagen type 1 forward	caagatgtgccactctgact	53
Collagen type 1 reverse	agtctccatgttgcagaaga	53
NFATC1 forward	ataaccagtttttccacgat	54
NFATC1 reverser	cacgaggggtctctgtag	54
Oscar forward	cttggagatttggacttttca	54
Oscar reverse	gtcacctcctccagaaagaa	54
RANK forward	acttctctgatgccttttcc	55
RANK reverse	ttctctgtcccatgatgttc	55
TRAP forward	cacttcaagatcccacagac	54
TRAP reverse	agtcatctgagttgccacat	54
Cathepsin k forward	taacaacaaggtggatgaaa	53
Cathepsin k reverse	tggttcatagccagttcata	53
MMP-9 forward	gtaaggagtactcgacctgtaccac	53
MMP-9 reverse	ccacttcttgtcgctgtcaaagttcg	53
Chloride channel 7 forward	gcaatatcgacaagttcaca	53
Chloride channel 7 reverse	tatgaaagccacaatcacag	53
Subunit of a V-type proton ATPase forward	gatctggcagactttcttca	55
Subunit of a V-type proton ATPase reverse	tgaagcactcgttgtagatg	55
ADIPOR1 forward	agccagatgtcttcccacaaagga	55
ADIPOR1reverse	tgggcatgtttgctcttcttcagc	55
ADIPOR2 forward	ctatcctgaaggtccattctcc	50
ADIPOR2 reverse	atcacctcttcgtgtaccatcc	50
PAQR3 forward	tgttaccttcagcaagtgcg	55
PAQR3 reverse	cccacagagcaaagcataca	55

Summary of primers used for realtime polymerase chain reaction.

Statistics

Statistical evaluation was performed with Microsoft Excel and Graphpad Prism. Biological replicates were used to calculate arithmetic means. Standard deviation of the mean (SD) was calculated. Student's *t*-test was performed and *p*-values of <0.05 were considered statistically significant.

Results

Adiponectin in RA bone remodelling

Immunohistochemistry on RA bone revealed widely distributed adiponectin expression. Besides the known expression in the RA synovium within the perivascular areas (Fig. 1A-B), adiponectin was detectable in bone local ised with OB and OC at sites of bone remodelling (Fig. 1C-D). Adiponectin receptors (ADIPOR1, PAQR3) were expressed by OB and OC (Fig. 1E-H).

In addition, the adiponectin expression was detectable in cultured primary human RA OB and OC on protein level (Fig. 2A). The expression of adiponectin receptors (ADIPOR1, -R2, PAQR3) was confirmed on RNA level (Fig. 2B). While ADIPOR2 and PAQR3 showed equal expression in osteblasts and OC, ADIPOR1 was significantly higher in OC compared to OB (OC:$2^{-(\Delta Ct)}$=0.18±0.1, n=12; OB:$2^{-(\Delta Ct)}$=0.05±0.02, n=7; p=0.003).

Dose- and time-dependent response to adiponectin with IL-6 secretion in osteoblasts

To determine whether adiponectin concentration or incubation period play a role in the production of proinflammatory markers known to influence OC formation and bone resorption, dose-response experiments were performed. Different incubation periods were tested as well. Adiponectin induced IL-6 in RA OB in a dose-dependent manner (Fig. 3A). The basal IL-6 secretion (986±1394 pg/ml) was increased by 5µg/ml adiponectin (1623±1797pg/ml). Higher adiponectin doses resulted in an additional IL-6 increase reaching peak levels at 100 µg/ml adiponectin (10567±5844 pg/ml).

OB responded in a time-dependent way to adiponectin. The basal IL-6 concentration (338 pg/ml) was increased after 12 hours (572 pg/ml). Extending the incubation period up to 72 hours resulted in a further IL-6 increase (882 pg/ml; Fig. 3B). For subsequent experiments, 10 µg/ml adiponectin were used. This concentration showed a significant effect inducing proinflammatory cytokines. Of note, this adiponectin concentration is within the concentration range measured in synovial fluid of active RA patients (22, 27). For the following stimulations, durations of 48 hours was selected as a reliable effect of adiponectin was present at this time point.

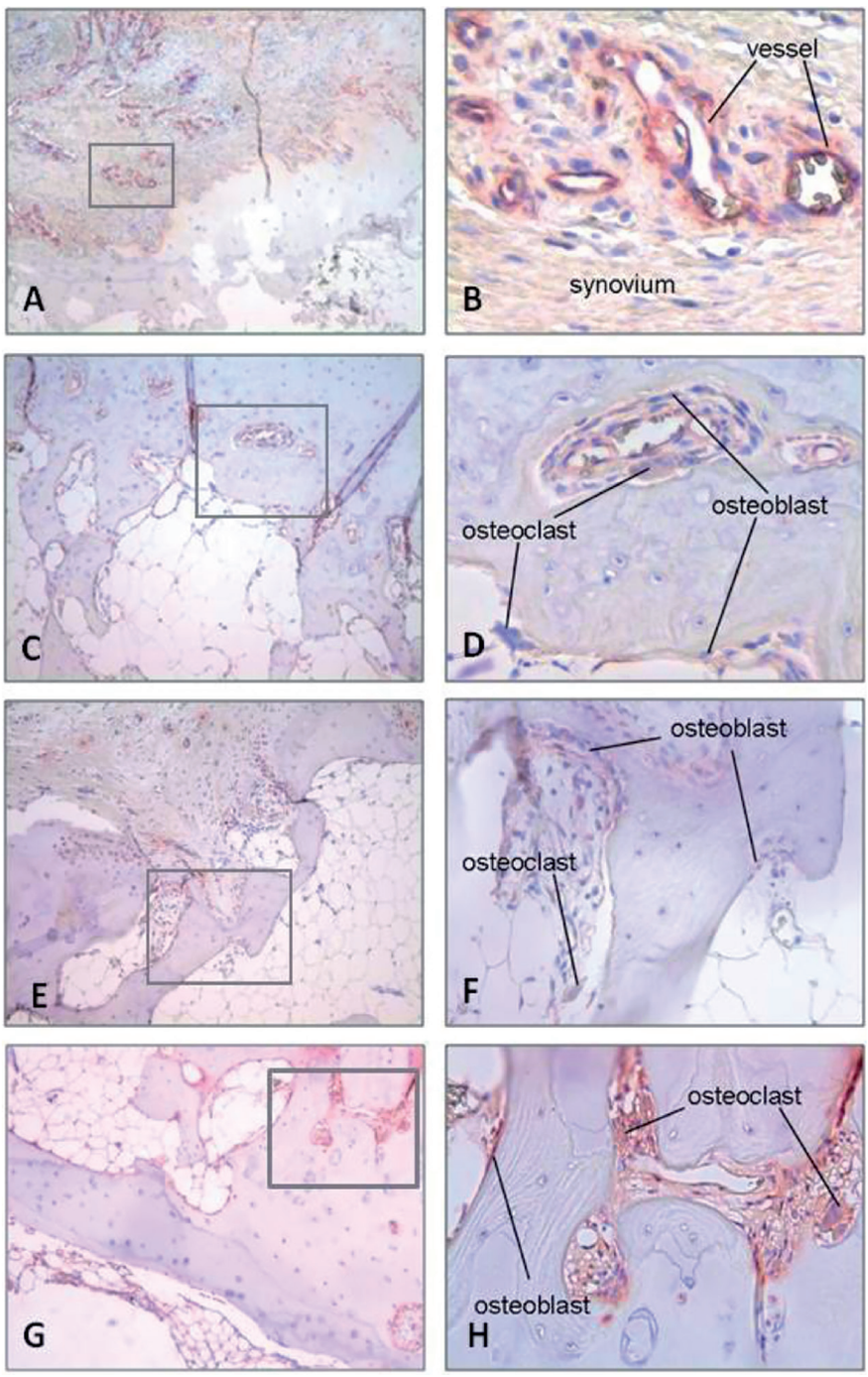

Fig. 1. Expression of adiponectin in rheumatoid arthritis bone tissue. Immunohistochemical staining showed an expression of adiponectin at the border of newly formed, non mineralised bone and in the adjacent synovium. **A**, **B)** In the cartilage invading synovium an expression of adiponectin was found especially around blood vessels. **C**, **D)** Additionally, an expression of adiponectin in localisation with osteoblasts and osteoclasts was detectable. Further examination revealed an expression of the adiponectin receptors **E**, **F)** ADIPOR1 and **G, H)** PAQR3 by osteoblasts and osteoclasts. Adiponectin and adiponectin receptors are stained in red, nuclei are stained in blue (original magnification: **A**, **C** and **E** x50; **D**, **F** and **H** x200).

Cytokine response of osteoblasts and (pre-) osteoclasts to adiponectin

To examine the cytokine-modulating effect of adiponectin in OB and OC, secretion of OB/OC specific cytokines/chemokines with known effects on bone remodelling was analysed. Adiponectin treatment of RA OB resulted in increased production of IL-6, -8 and Gro-α (IL-6 from 1409±284pg/ml to 8068±2806 pg/ml, n=2; IL-8 from 72.28±77.7 pg/ml to 841.23±1151pg/ml, p=0.07, n=7; Gro-α from 20.91±25pg/ml to 75.46±68.79pg/ml; p=0.15, n=4, Fig. 3C). Production of MCP-1 and VEGF in OB was not altered by adiponectin.

OC responded to an adiponectin with an increased production of IL-8 (Fig. 3D). In pre-OC from healthy donors, IL-8 increased from 4897±4957pg/ml to 16765±4911pg/ml (9.57-fold, n=6, p=0.001). Adiponectin also induced IL-8 in RA pre-OC, but to a higher extent ranging from 10229±13359pg/ml to 29243±22107pg/ml (12.9 fold, n=8; p=0.03). In mature (RA) OC the secretion of IL-8 was also increased (RA: 9438±5549 pg/ml to 18194±13646 pg/ml, 2.6-fold, p=0.16, n=8; healthy: 7914±5969 pg/ml to 19500±14877pg/ml, 9.4-fold, p=0.144, n=5). MCP-1 was not altered in (pre-)OC.

Adiponectin-mediated alteration of gene expression in osteoblasts

To examine the functional effects of adiponectin on OB and OC, changes in gene expression of differentiation and activity markers were analysed by realtime PCR. Treatment of RA OB with adiponectin (10 μg/ml) led to a significant down-regulation of osterix (-1.51-fold; p=0.019; n=4, Fig. 4A). OPG and ALP were up-regulated but not significantly altered (Fig. 4A; OPG: 2.15-fold; p=0.051; n=4; ALP: 1.58-fold; p=0.112; n=4).

Adiponectin-mediated alteration of gene expression in osteoclasts

Likewise, OC responded to adiponectin with alterations on transcriptional level (Fig. 4B). Mature OC from healthy donors showed a significant increase of TRAP (1.42-fold, p=0.02, n=5) and MMP-9 expression (1.63-fold, p=0.006, n=5). Similarly, mature RA OC showed an up-regulation of MMP-9 mRNA (1.94-fold, p=0.033, n=10) but not for TRAP (1.5-fold, p=0.052, n=10). (Pre)-OC from healthy donors and RA patients were not sensitive to adiponectin. Other parameters *e.g.* NFATC1, OSCAR, Cathepsin K, or RANK did not show significant differences.

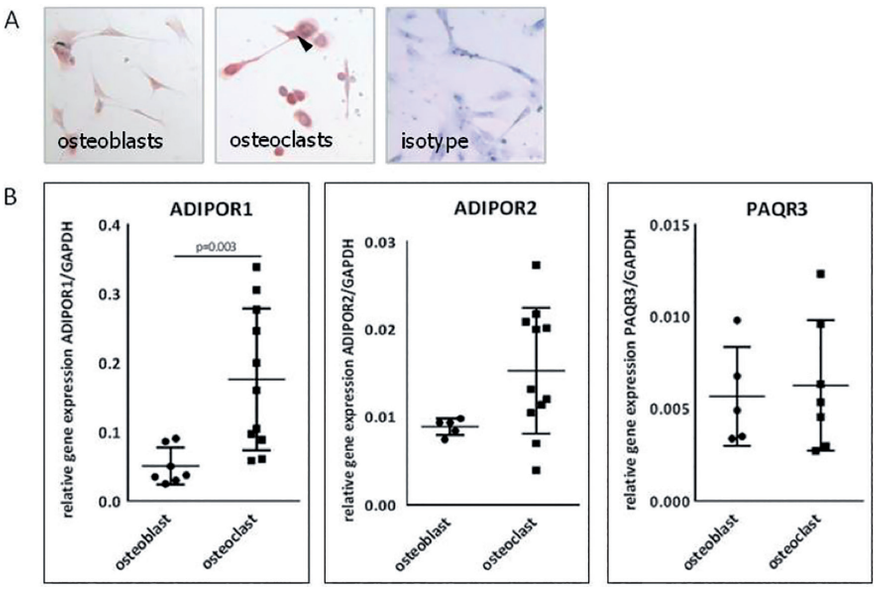

Fig. 2. Expression of adiponectin and adiponectin receptors in cultured primary human osteoblasts and osteoclasts.
A: Immunocytochemistry revealed an expression of adiponectin in osteoblasts and osteoclasts (adiponectin is stained red, nuclei are stained blue, magnification x200).
B: Realtime polymerase chain reaction revealed an expression of ADIPOR1, ADIPOR2 and PAQR3 in rheumatoid arthritis osteoblasts and osteoclasts. Expression levels were standard ised to GAPDH by calculating ΔCt.

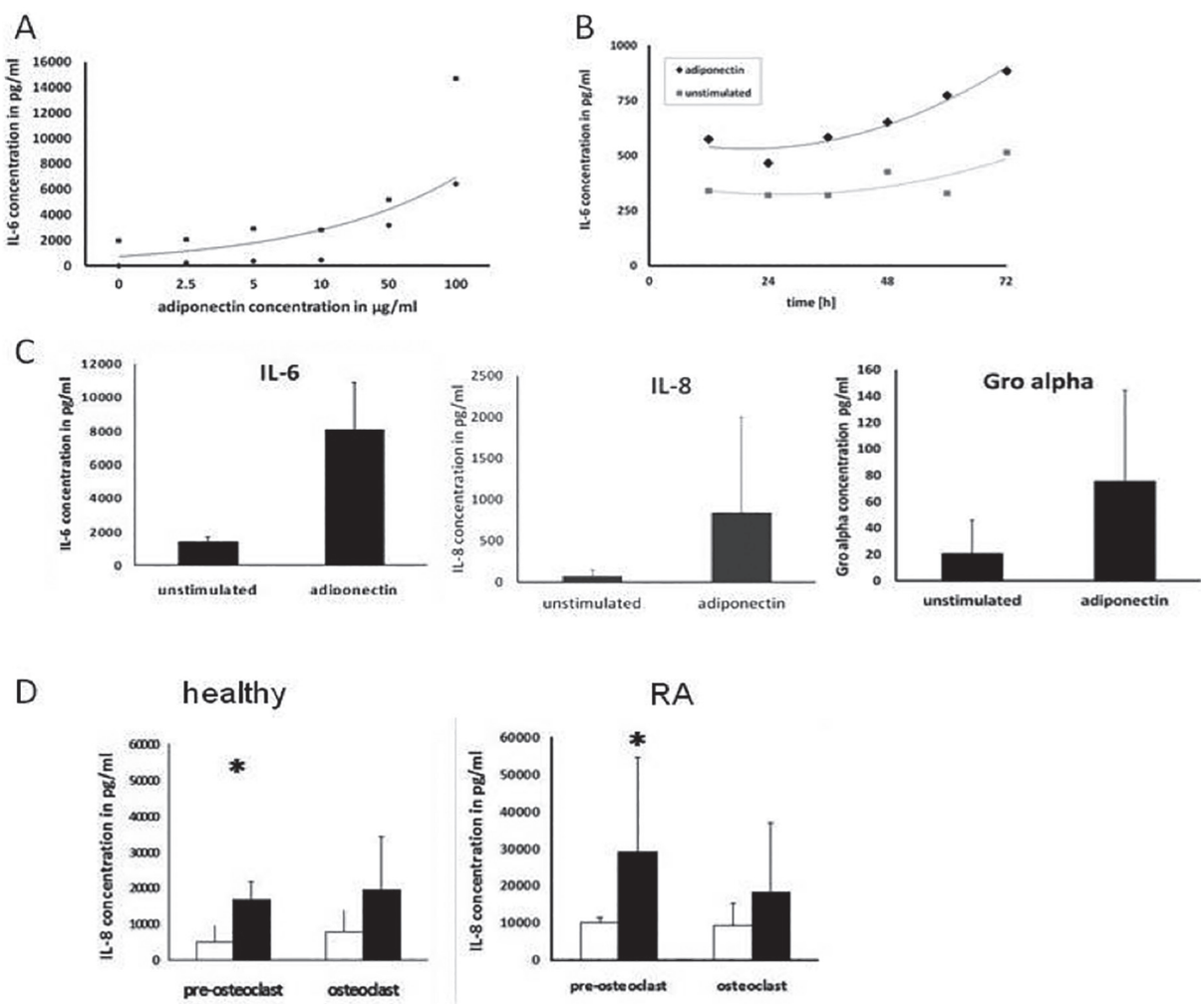

Fig. 3. Proinflammatory response of rheumatoid arthritis osteoblasts upon adiponectin treatment is dose- and time-dependent in terms of IL-6 release.
A: Osteoblasts were stimulated with different concentrations of adiponectin (range: 2.5 to 100 µg/ml) for 17 hours or **B)** were incubated with adiponectin (10 µg/ml) from 12 up to 72 hours. IL-6 was measured by enzyme-linked immunosorbent assay. Data is expressed as concentration of IL-6 (pg/ml) to adiponectin concentrations or stimulation time. **C**: In response to adiponectin, osteoblasts secreted the proinflammatory factors and chemokines IL-6, IL-8, Gro-α.
D: Pre-osteoclasts from healthy donors and rheumatoid arthritis patients responded to adiponectin with an increased IL-8 secretion. Cytokines were measured by enzyme-linked immunosorbent assay. Results are expressed as mean ± SD.

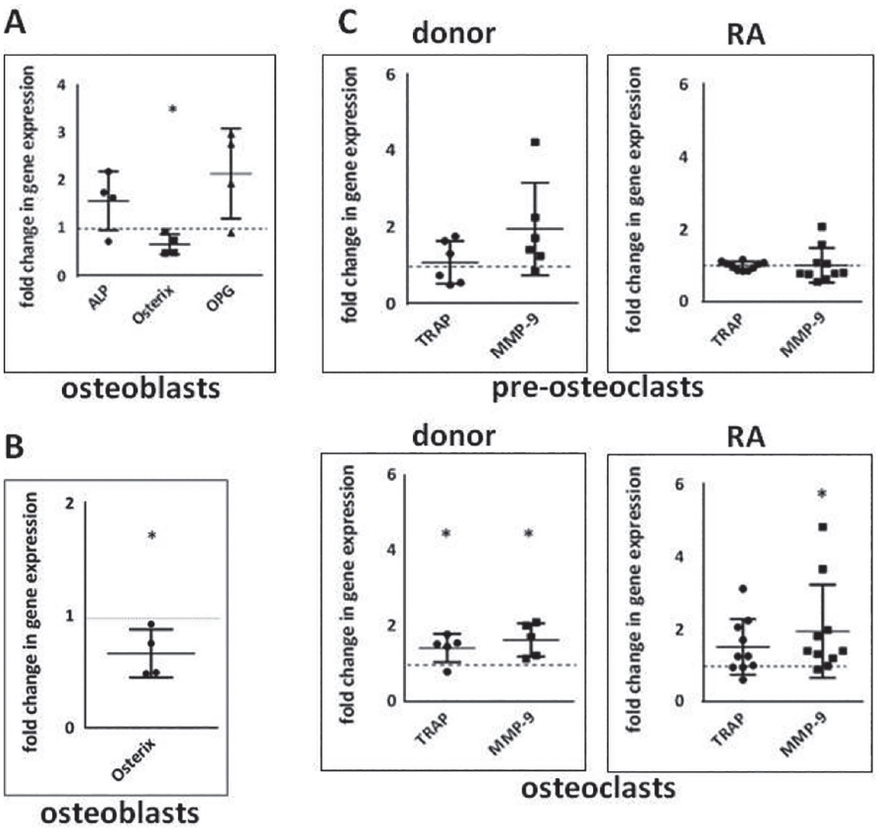

Fig. 4. Adiponectin mediated alteration in gene expression of rheumatoid arthritis osteoblasts and osteoclasts. **A**, **B**: rheumatoid arthritis osteoblasts **C**: and (pre-) osteoclasts were stimulated with adiponectin (10 µg/ml). Expression levels were standardised to 18S ribosomal RNA by calculating ΔΔCt. Changes in mRNA level are indicated as fold changes relative to unstimulated control. Bars show the mean ± SD.

Mineralisation activity of RA osteoblasts and resorption activity of RA osteoclasts with adiponectin

To verify the results of gene expression analysis, functional assays were performed investigating the mineralisation activity of RA OB. In accordance with the adiponectin-mediated downregulation of osterix, OB showed a decrease of proliferation (87.1%±15.3%, p=0.144, Fig. 5A) and mineralised nodule formation by adiponectin (Fig. 5A). Semi-quantitative analysis (n=6) revealed a decrease of Alizarin Red S stained matrix to 85.2%±14.7% (p=0.035) compared to the mineralisation capacity of unstimulated controls (set to 100%; Fig. 5A).

Functional assays regarding the differentiation capacity of PBMCs in presence of adiponectin displayed an increase of TRAP-positive multinucleated cells in comparison to non-treated cells (RA: 131.2%±60.7%, p=0.32, n=5; healthy: 114.9%±23.4%, p=0.19, n=5). In accordance to increased osteoclastogenesis, the resorptive activity of RA OC was significantly increased (134%±16.3, p=0.016; n=5). Likewise, OC from healthy donors showed increased resorptive activity after adiponectin stimulation (120%±35; p=0.22; n=5) (Fig. 5B). However, the increase was considerably lower compared to RA OC and did not reach statistical significance.

Discussion

Adiponectin is a known effector molecule in RA (15, 28). Especially in RA synovium, adiponectin contributes to joint inflammation. Adiponectin induces significant changes in cell types involved in RA pathogenesis, *e.g.* synovial fibroblasts (SF), promoting matrix destruction at sites of cartilage invasion (15). RA bone loss results from imbalanced bone formation and resorption. The present study demonstrates the immunomodulatory properties of adiponectin as well as its impact on central cells of bone remodelling specifically in RA.

In RA, activated cells of the bone marrow and the synovium create a proinflammatory microenvironment that disturbs the balance between bone resorption and formation (5, 29, 30). Recent studies showed an expression of adiponectin and its receptors in RA synovium and the impact of this adipokine on central RA effector cells (31). In this study, we observed an adiponectin expression at sites of RA bone remodelling. Adiponectin receptors were detectable on RA OB and OC. The expression of ADIPOR1 and -R2 in OB, PBMCs and OC has already been reported (19, 32, 33). So far, these reports showed the adiponectin expression under *in vitro* condition in bone cells. We show the expression of adiponectin and its receptors *in vivo* in bone samples of RA patients. The expression of all three receptors by OB and OC as well as the presence of adiponectin in the bone compartment of RA indicates a direct influence of adiponectin on bone metabolism. Therefore, we investigated the impact of adiponectin on the cells of bone remodelling in RA.

Adiponectin is known for its immunomodulatory action, able to induce factors including PGE_2, IL-6 or IL-8 in chondrocytes, RASF and lymphocytes, respectively (15, 16). In our study, we similarly observed that adiponectin enhanced the production of cytokines and chemokines specifically in OB and pre-OC obtained from RA patients, including the central proinflammatory cytokines IL-6 and -8. These cytokines modulate differentiation and recruitment of OC and negatively regulate OB differentiation (34-36). IL-8, which was significantly increased in RA OB and pre-OC by adiponectin, directly stimulates differentiation of human PBMCs into OC (34). IL-6, which was induced in RA OB, could contribute to OC-mediated bone resorption since IL-6 promotes osteoclastogenesis. Furthermore, IL-6 is supposed to have regulatory influence on T lymphocyte-mediated OC recruitment (11, 35). This cytokine-inducing effect of adiponectin could therefore contribute to the pro-

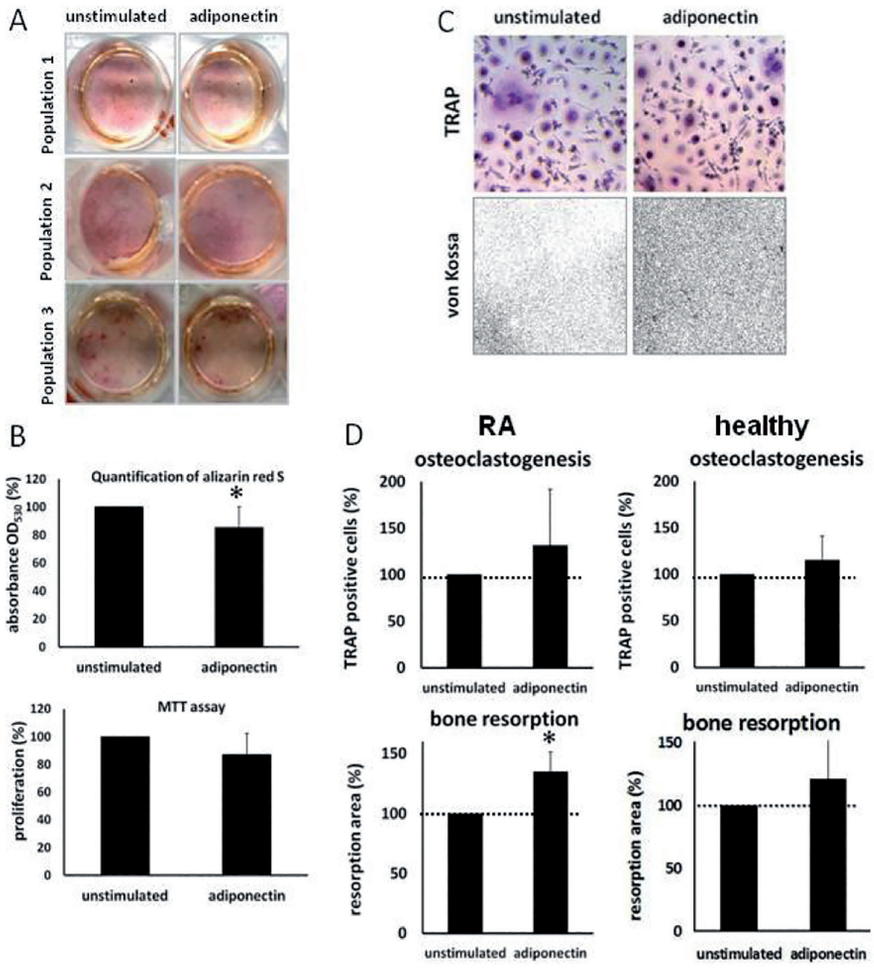

Fig. 5. Functional response of rheumatoid arthritis osteoblasts and osteoclasts to adiponectin. Rheumatoid arthritis osteoblasts were stimulated with **A)** adiponectin (10 µg/ml) over 14 days. Mineralisation was visualised by Alizarin Red S. The calcified areas of the matrix are stained red. After visualisation of the matrix, Alizarin Red S was **B)** quantified by acetic acid exctraction (n=6). Population 1-3 represent osteoblastic cells from 3 different donors. The effect of adiponectin on osteoblast (n=4) proliferation was measured by MTT assay. Data are shown as mean ± SD. **C**: rheumatoid arthritis and healthy osteoclast precursors were treated with adiponectin. Osteoclastogenesis was confirmed after 14 days by tartrate resistant acid phosphatase staining, resorption activity of osteoclasts by von Kossa staining. **D**: tartrate resistant acid phosphatase positive, multinucleated osteoclasts (≥3 nuclei) were quantified (n=5 each) and bone resorption activity was visualised and quantified after 14 days of differentiation by von Kossa staining (white areas: remaining mineralised surface; black areas: resorption area).

cess of bone resorption. The observed up-regulation of MMP-9 and TRAP in OC by adiponectin supports this idea. Both are important enzymes in bone resorption. TRAP is released into the resorptive lacunae of OC, where it is supposed to produce highly destructive reactive oxygen species that facilitate collagen fragmentation (37, 38). MMP-9 is a highly efficient collagenase cleaving both, type I and II collagen (39). The observed increase of TRAP positive cells and resorption activity by adiponectin favour the hypothesis that adiponectin negatively influences bone metabolism in RA.

In our study, RA OB showed a decreased mineralised nodule formation in the presence of adiponectin. This finding is in accordance with the decrease in viability and the down-regulation of osterix, an essential transcription factor of OB required for OB differentiation and bone formation (40, 41). Wang *et al*. observed equal characteristics of bone marrow derived mesenchymal stem cells (MSCs) derived from adiponectin deficient mice(APN KO mice) (20). Studies using MSCs from APN KO mice demonstrated enhanced osteogenic differentiation and extracellular matrix calcification and it was assumed that higher gene expression of osterix might induce acceleration of osteogenesis in an adiponectin-negative background (20). The importance of osterix for bone formation was also demonstrated in osterix null mice that completely lack mineralised skeleton (40). OPG, being the decoy receptor of RANK-L, is a modulator of osteoclastogenesis (42). However, OPG and ALP in RA OB were not significantly changed by adiponectin.

The increased OC and inhibited OB differentiation may result to some extent from increased IL-8 or -6 production by these cells upon adiponectin stimulation (34, 36). Therefore, further studies will determine whether adiponectin acts directly or indirectly by promoting the cytokine production in OB and OC.

Of note, primary OB and OC from different patients showed a high variability regarding the baseline levels as well as induction of cytokines after stimulation with adiponectin. Besides individual variation, the expression levels of adiponectin receptors most likely contribute to the observed variability. Nevertheless, the results of our study indicate that the action of adiponectin is not restricted to synovium in RA, but influences bone remodelling by modulating the inflammatory environment in the bone compartment and thereby affecting OB and OC activity. As the effects of adiponectin show an activation of OC function as well as a reduction of OB function, the results support the idea of a joint destructive function of adiponectin in RA.

Conclusion

For the first time, the expression of adiponectin and its receptors was shown in bone samples of RA patients as well as the influence of adiponectin on cells of bone remodelling isolated from RA patients. Adiponectin induces proinflammatory factors, alters gene expression and mineralisation activity of OB and affects gene expression and resorption activity of OC. Therefore, the presence of adiponectin within the bone remodelling zone directly contributes to the inflammatory matrix destructive milieu in the RA bone compartment.

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