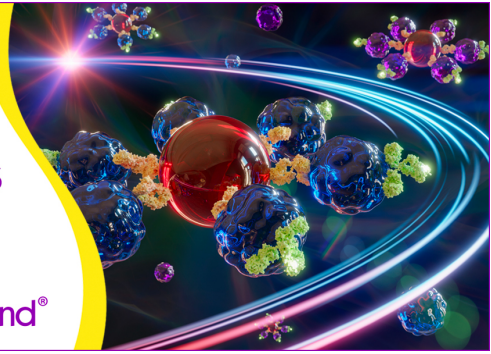


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J Immunol (2006) 176 (7): 4468–4478.

<https://doi.org/10.4049/jimmunol.176.7.4468>

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The Potential of Adiponectin in Driving Arthritis¹

Angela Ehling,^{3*†} Andreas Schäffler,^{3*} Hans Herfarth,^{*} Ingo H. Tarner,^{†¶} Sven Anders,[‡] Oliver Distler,[§] Gisela Paul,^{*} Jörg Distler,[§] Steffen Gay,[§] Jürgen Schölmerich,^{*} Elena Neumann,[¶] and Ulf Müller-Ladner^{2¶}

Articular adipose tissue is a ubiquitous component of human joints, but its local functions are largely unknown. Because recent studies revealed several links between adipose tissue, adipocytokines, and arthritis, we investigated the expression of the adipocytokine adiponectin and its functional role in articular adipose tissue and synovium of patients with different arthritides. In contrast to its protective role in endocrinological and vascular diseases, adiponectin was found to be involved in key pathways of inflammation and matrix degradation in the human joint. The effects of adiponectin in human synovial fibroblasts appear to be highly selective by inducing only two of the main mediators of rheumatoid arthritis pathophysiology, IL-6 and matrix metalloproteinase-1, via the p38 MAPK pathway. Owing to the observation that these effects could be inhibited by different TNF- α inhibitors, adipocytokines such as adiponectin may also be key targets for therapeutic strategies in inflammatory joint diseases. In summary, articular adipose tissue and adipocytokines cannot be regarded as innocent bystanders any more in chronic inflammatory diseases such as arthritis. *The Journal of Immunology*, 2006, 176: 4468–4478.

Adipose tissue is a ubiquitous tissue, which can be found as a structural component of many organs of the human body, including the skin, gut, heart, and joints, and frequently serves the purpose of smoothing out gaps or incongruities between different tissues. In terms of biological function, adipose tissue in general has been regarded for decades as a specialized type of connective tissue that only plays a role in energy metabolism by storing energy as triglycerides or releasing energy as fatty acids. Specifically, the adipose tissue of diarthrodial joints has not been regarded as having any biological function other than padding gaps between the articulating bones.

Recently, however, there has been growing evidence that the dominant cell type of adipose tissue, the adipocyte, has the ability to synthesize and release proinflammatory molecules, complement factors, signaling molecules, growth factors, and adhesion molecules (1, 2), suggesting an integrated function of adipocytes in tissue inflammation. Among these molecules are IL-6, macrophage migration inhibitory factor, M-CSF, TNF- α , complement factor 3a, C1-inhibitor, C1r, complement factor B, adipsin (complement factor D), leptin, resistin, and adiponectin (3–15). For these molecules, the term “adipocytokines” was introduced (1), which re-

fects the novel function of adipose tissue as an immunological, endocrine, and paracrine organ.

Given these findings, articular and synovial adipose tissue can be suspected of playing a role in joint inflammation, but thus far, the local biological function of articular adipose tissue has remained largely unknown. Therefore, it is of special interest that recent studies have revealed numerous novel links between adipose tissue, adipocytokines, and inflammatory joint disease (16–21). For instance, Ushiyama et al. (19) described the synthesis of proinflammatory cytokines and growth factors in the infrapatellar fat pad from patients with osteoarthritis (OA),⁴ and Yamasaki et al. (20) demonstrated that fibroblasts have the potential to transform into adipocytes under the influence of cytokines. Moreover, when analyzing adipocytokine levels in the synovial fluid of patients with rheumatoid arthritis (RA) and OA, we detected high levels of resistin and adiponectin (21).

Adiponectin was originally described as an adipocytokine exclusively expressed by adipose tissue, but meanwhile, it was found to be expressed by osteoblasts as well (22). Interestingly, adiponectin shares strong homologies with the complement factor C1q and the proinflammatory cytokine TNF- α . Thus, it belongs to the C1q-TNF-superfamily, the members of which are thought to be derived from a common progenitor molecule and to share common (proinflammatory) functions (23).

Thus far, adiponectin has been investigated primarily in the context of lipid and glucose metabolism as well as the pathogenesis of atherosclerosis. For instance, adiponectin has been found to prevent the transformation of macrophages into foam cells by inhibiting lipoprotein lipase and class-A-scavenger receptor, thus reducing intracellular cholesterol levels (24). In addition, adiponectin down-regulates TNF-dependent expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin in human aortic endothelial cells, thus inhibiting endothelial adhesion of monocytes (3, 25).

*Department of Internal Medicine I, University Hospital Regensburg, Germany; [†]Stanford University School of Medicine, Stanford, CA 94305; [‡]Department of Orthopedics, University Hospital Regensburg, Regensburg, Germany; [§]Center for Experimental Rheumatology, University Hospital Zürich, Zürich, Switzerland; and [¶]University Hospital Giessen, Department of Rheumatology and Clinical Immunology, Kerckhoff-Klinik Bad Nauheim, Germany

Received for publication July 28, 2004. Accepted for publication January 14, 2006.

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¹ This work was supported by an European League Against Rheumatism Young Investigator Award and by grants of the German Research Society (Deutsche Forschungsgemeinschaft Scha 789/2-3, Mu 1383/1-3 and 1383/3-3, Ta 297/2-1, SFB585).

² Address correspondence and reprint requests to Dr. Ulf Müller-Ladner, Justus-Liebig-University Giessen, Department of Rheumatology and Clinical Immunology, Kerckhoff-Klinik, Benkestrasse 2-8, D-61231 Bad Nauheim, Germany. E-mail address: u.mueller-ladner@kerckhoff-klinik.de

³ A.E. and A.S. contributed equally to this work.

⁴ Abbreviations used in this paper: OA, osteoarthritis; RA, rheumatoid arthritis; RASF, RA synovial fibroblasts; OASF, OA synovial fibroblasts; MMP-1, matrix metalloproteinase-1; SSc, systemic sclerosis; Nf, normal fibroblast; siRNA, small interfering RNA; PKC, protein kinase C; PKA, protein kinase A; cPKA, cAMP-dependent PKA.

Taken together, these findings suggest that adiponectin has anti-inflammatory properties. In contrast, our finding of elevated adiponectin levels in RA synovial fluid in comparison to OA indicates a proinflammatory role in arthritis (21). Given the fact that various other members of the C1q-TNF-superfamily are also involved in synovial pathophysiology, we hypothesized that adipocytokines, and particularly adiponectin, play a distinct pathogenic role in inflammatory joint disease. Therefore, in this study we investigated the following: 1) adiponectin expression in RA and OA synovial tissue; 2) adiponectin receptor expression in RA synovial fibroblasts (RASf) and OA synovial fibroblasts (OASf); 3) the functional role of adiponectin in proinflammatory and matrix-degrading pathways; 4) the intracellular regulation of adiponectin-induced signaling mechanisms; and 5) the effects of anti-adiponectin Abs and different anti-TNF biologics (etanercept and adalimumab) on adiponectin-dependent synthesis of IL-6 and pro-matrix metalloproteinase-1 (MMP-1).

Materials and Methods

Tissue specimens

Synovial tissue and articular adipose tissue samples were obtained from patients with RA and OA who all met the respective criteria of the American College of Rheumatology (ACR). The tissue samples were obtained during routine surgery at the Department of Orthopedics of the University of Regensburg (Regensburg, Germany), approved by the local ethics committee.

Two types of control tissues and control fibroblasts were used for the experiments: 1) systemic sclerosis (SSc) as a different type of inflammatory rheumatic disease; and 2) normal skin as a healthy control (normal fibroblasts; Nf). To this end, we obtained 1) skin biopsies from involved skin of SSc patients ($n = 5$), who met the ACR criteria for SSc; and 2) specimens of normal skin from dermatological biopsies.

Immediately after joint surgery, arthroscopy, or skin biopsy, one portion of the respective tissue samples was used for fibroblast cultures, whereas the remaining samples were either snap frozen in OCT Tissue Tek-embedding medium (Sakura) and stored at -80°C for histology, or stored in RLT lysis buffer/0.5% 2-ME (Qiagen) for RT-PCR analysis. All experiments were performed with permission from the local ethic committees and after informed written consent from the patients.

Cell culture

Culture of synovial fibroblasts was performed as described recently (26, 27). Briefly, following enzymatic digestion, fibroblasts were grown in DMEM (PAN Biotech) containing 10% heat-inactivated FCS (Invitrogen Life Technologies), 100 U/ml penicillin and streptomycin (PAA Laboratories), and cultured for up to five passages at 37°C in 10% CO_2 . The synovial fibroblasts were stained for fibroblast markers by immunohistochemistry. More than 95% could be stained positively for the fibroblast enzyme prolyl 4-hydroxylase, and none were positive for the macrophage marker CD68 or the neutrophil marker cathepsin G (data not shown). In addition, testing for mycoplasma contamination was performed routinely.

SSc fibroblast cultures were performed after enzymatic digestion of the skin biopsies in Dispase II (Roche). Cells were grown at 37°C and 10% CO_2 in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin, and 0.5 $\mu\text{g}/\text{ml}$

amphotericin B (all obtained from Invitrogen Life Technologies). Fibroblasts from passages four to nine were used for the experiments.

RT-PCR for adiponectin and adiponectin receptors I and II from fibroblasts

Total RNA from cultured synovial fibroblasts and normal skin fibroblasts was isolated using the RNeasy Spin Column Purification Kit (Qiagen) according to the manufacturer's protocol. Two hundred nanograms of total RNA of RASf and OASf were transcribed with standard RT-PCR using human adiponectin-specific primers. Similarly, RT-PCR was performed for adiponectin receptor types I and II. Primer sequences and fragment lengths are shown in Table I.

As a positive control, commercially available adipocyte cDNA (BD Biosciences) was used, and the results were normalized for 18S as an endogenous control. Each of the experiments was repeated three times.

RT-PCR for adiponectin from SSc skin biopsies

Tissue RNA was isolated from whole biopsies in one part of the samples. In another part of the samples, the epidermis was selectively removed from the dermis by microdissection, and total RNA was isolated from both compartments separately to differentiate between expression of adiponectin in the dermis and epidermis.

The respective tissue samples were homogenized in RLT lysis buffer (Qiagen) on ice for 3–5 min using a standard rotor-stator homogenizer (Dispergierstation T8.10; IKA Labortechnik). Isolation of total RNA was performed using the RNeasy Spin Column Purification Kit (Qiagen) according to the manufacturer's protocol.

Total RNA was reverse transcribed into cDNA using 300 ng of total RNA, 2.5 U/ μl murine leukemia virus reverse transcriptase, 2.5 μM random hexamers, 2 mM dNTPs, and 1 U/ μl RNase inhibitor (all obtained from PE Applied Biosystems). Samples with the same reagents and equal amounts of RNA, but without enzyme in the reverse transcription reaction, were used as negative controls (nonreverse transcription controls) to exclude genomic contamination. The results were normalized for 18S as an endogenous control. Each of the experiments was repeated three times.

Preparation of riboprobes

In vitro transcription of human adiponectin cDNA plasmid was performed using a RNA transcription kit (Ambion) and T7 or SP6 RNA polymerases (Ambion) to generate antisense and sense riboprobes. All experiments were performed according to protocols described previously (26).

In situ hybridization

In situ hybridization, using digoxigenin-labeled cRNA, was performed on 8- μm sections from RA ($n = 3$) and OA ($n = 3$) synovial samples as described previously (26). Briefly, sections were fixed for 1 h in 3% paraformaldehyde (pH 7.4) and hybridized for 16 h at 50°C with riboprobes diluted 1/5 in hybridization buffer: 50% formamide (Fluka), 40% dextrane sulfate (Fluka) in $20\times$ SSC, 0.5 mg of herring sperm DNA (Invitrogen Life Technologies), and 0.25 mg of yeast tRNA (Sigma-Aldrich).

After hybridization, three consecutive washes at increasing stringency (final wash in $0.1\times$ SSC containing 0.1% SDS for 5 min) were performed at 50°C . Immunological detection was performed by incubating slides with anti-digoxigenin alkaline phosphatase-conjugated F(ab')₂ (Roche Diagnostics) followed by 5-bromo-4-chloro-3-indolyl-phosphate/4-NBT chloride-color substrate solution (Roche Diagnostics).

Table I. Primer sequences and fragment lengths

Gene	Primer	Sequence	T α ($^{\circ}\text{C}$)/Cycles	Fragment Length (bp)
Adiponectin	Forward	5'-TTTCCGGGAATCCAAGGCAG-3'	57/35	390
	Reverse	5'-TCCATTACGCTCTCCTTCCC-3'		
Adiponectin receptor type I	Forward	5'-CCTTCCCAAGCTGAAGCTGC-3'	57/35	924
	Reverse	5'-CCTTGACAAAGCCCTCAGCGAT-3'		
Adiponectin receptor type II	Forward	5'-AACGAGCCAACAGAAAACCGATTG-3'	59/35	496
	Reverse	5'-ATACACACAGAAACAGGCAACATTTG-3'		
18S RNA	Forward	5'-TCAAGAACGAAAGTCGGAG-3'	54-60/35	488
	Reverse	5'-GGACATCTAAGGGCATACA-3'		

Immunological detection

For detection of adiponectin protein, synovial tissue sections from two patients with RA and two patients with OA were analyzed by immunohistochemistry (26). Briefly, sections were fixed in acetone and then blocked in 2% normal goat serum to block nonspecific binding. Primary mouse anti-human adiponectin Abs (Chemicon) were diluted in binding buffer (1% fish skin, 0.07% glycine, 0.05 M Tris buffer (pH 7.6)) at 12 $\mu\text{g}/\text{ml}$ (for tissue) and 60 $\mu\text{g}/\text{ml}$ (for cells) and applied onto the sections for 60 min. The slides were rinsed and incubated for 60 min at room temperature with a secondary biotinylated goat anti-mouse IgG Ab (DakoCytomation), in a 1/600 dilution in binding buffer. Incubation with streptavidin-HRP (1/600; Dianova) for 60 min followed by color development using the 3-amino-9-ethylcarbazole substrate (Alexis) for 30 min was performed at room temperature. The color development was stopped and the slides mounted immediately (Aquatex; Merck).

For double-labeling, alkaline phosphatase-anti-alkaline phosphatase method was performed using a commercially available kit (Vector Laboratories). For all immunohistochemistry experiments, primary Abs against fibroblasts (anti-vimentin; DakoCytomation) and macrophages (anti-CD68; DakoCytomation) were used.

Fibroblast stimulation by adiponectin

Cultured RASF and OASF were distributed in 48-well plates, cultured overnight, and stimulated with increasing concentrations of 5 $\mu\text{g}/\text{ml}$, 12 $\mu\text{g}/\text{ml}$, 40 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 75 $\mu\text{g}/\text{ml}$, and 100 $\mu\text{g}/\text{ml}$ human full-length adiponectin (R&D Systems) in a total volume of 250 $\mu\text{l}/\text{well}$ for 15 h. Stimulation time was chosen based on preliminary experiments demonstrating optimal response after 15 h (data not shown). Unstimulated RASF and OASF served as controls. Supernatants were collected and frozen immediately at -20°C until further evaluation. Each of the experiments was repeated twice.

Inhibition experiments of adiponectin with anti-adiponectin Abs

Cultured RASF and OASF were distributed equally in a 48-well plate and cultured for 24 h. Adiponectin (25 $\mu\text{g}/\text{ml}$) (R&D Systems) was incubated with mouse anti-human adiponectin Abs (1/10 dilution; Chemicon) for 2 h. Then, cells were stimulated with the adiponectin/anti-adiponectin solution for 15 h in a total volume of 250 μl each. Unstimulated fibroblasts, adiponectin-stimulated fibroblasts, and unstimulated fibroblasts that were incubated with the anti-adiponectin Abs served as controls. Supernatants were collected and frozen immediately at -20°C until further evaluation. Each of the experiments was repeated twice.

Evaluation of synthesis of proinflammatory cytokines and matrix-degrading enzymes induced by adiponectin

To further investigate the effects of adiponectin stimulation, synthesis of those proinflammatory cytokines, growth factors and matrix degrading enzymes, which are known to mediate key pathways in arthritis pathophysiology, were examined by commercially available ELISA (R&D Systems) according to the manufacturer's protocol, specifically IL-1 β , IL-4, IL-6, IL-10, pro-MMP-1, pro-MMP-13, TNF- α , VEGF, and TGF- β . An additional control consisted of fresh culture medium (DMEM + 10% FCS), because VEGF and TGF- β are part of FCS. Each of the experiments was repeated twice.

After measuring the absolute concentration of the cytokines and MMPs in the supernatants, the effects of the stimulation and inhibition experiments were calculated as the ratio of the absolute concentrations in the supernatant to which adiponectin and/or inhibitors had been added, divided by the concentrations in the supernatants without stimulation. Results were expressed as x-fold increase in synthesis (= stimulation index) when compared with unstimulated cells to allow better comparability.

Analysis of adiponectin-induced signaling pathways using intracellular signaling inhibitors and small interfering RNA (siRNA)

Cultured RASF and OASF were distributed equally in a 48-well plate. After 24 h, cells were preincubated for 2 h with each of the following intracellular signaling inhibitors: 1) p38 MAPK-inhibitor RWJ67657 (Johnson and Johnson) and SB203580 (Sigma-Aldrich) at a final concentration of 20 μM ; 2) cell-permeable myristoylated protein kinase C (PKC) inhibitor 20-28 (Calbiochem) at a final concentration of 40 μM ; 3) cell-permeable myristoylated protein kinase A (PKA)-inhibitor 14-22 (Calbiochem) at a final concentration of 1 μM ; and 4) cAMP-dependent PKA (cPKA) inhibitor Rp-cAMPS, TEA (Calbiochem) at a final concentration of 100 μM . Following the preincubation period, cells were stimulated with 250

$\mu\text{g}/\text{ml}$ human adiponectin (R&D Systems) for 15 h in a total volume of 250 $\mu\text{l}/\text{well}$. Okadaic acid served as a control as an unspecific inducer of MAPK, including p38. Adiponectin-stimulated fibroblasts without the inhibitors and unstimulated fibroblasts that were incubated with or without the inhibitors served as controls. Supernatants were collected and frozen immediately at -20°C until further evaluation. Each of the experiments was repeated five times.

siRNAs against MAPK14 and MAPK11 (p38 α and - β) corresponding to the inhibitory capacity of the SB203580 inhibitor and GAPDH (Ambion) as control were used to confirm the results using the inhibitors mentioned above. Transduction of cells was performed by nucleofection using the Amaxa system (Amaxa) as described by the manufacturer. For transfection, 2×10^5 fibroblasts were resuspended in 100 μl of Nucleofector Solution for human dermal fibroblasts (Amaxa), which provides the conditions required for transfer of DNA into the nucleus. Thereafter, 1.5 μg of siRNA against MAPK14 and MAPK11 were added, then the mixture was transferred into an electroporation cuvette and placed in the Nucleofector device (Amaxa) for electroporation. Eight hours after transfection, the medium was changed and the fibroblasts were stimulated with recombinant adiponectin as described above. After 15 h, supernatants and RNA lysates were collected and stored by -20°C .

Potential of TNF inhibitors adalimumab and etanercept to inhibit adiponectin-mediated effects on synovial fibroblasts

In addition to the inhibition experiments with the signaling inhibitors, another set of inhibition experiments was conducted using two agents approved for the therapy of active RA (28), the TNF inhibitors etanercept (a soluble TNFR) and adalimumab (a fully human anti-TNF-Ab).

Part I. Cultured RASF and OASF were distributed equally in a 48-well plate. After 24 h of culture, cells were preincubated for 2 h with either 2.5 mg/ml commercially available etanercept (Wyeth) or 2.5 mg/ml adalimumab (Abbott Immunology), respectively. After preincubation, cells were stimulated with 25 $\mu\text{g}/\text{ml}$ human adiponectin (R&D Systems) for 15 h in a total volume of 250 $\mu\text{l}/\text{well}$.

The doses of etanercept and adalimumab were chosen arbitrarily because it is difficult to extrapolate the effective in vitro doses of these drugs from the therapeutic dosages that are used in clinical practice, due to different pharmacokinetics. Thus, differences in biologic effectiveness cannot be fully ruled out.

Part II. To clarify the question, whether the inhibitory effects of etanercept and/or adalimumab were due to a direct effect on the synovial fibroblasts or due to binding to the TNF-like structure of adiponectin, the following experiment was performed. Adiponectin (25 $\mu\text{g}/\text{ml}$) was preincubated for 2 h with etanercept (2.5 mg/ml) and adalimumab (2.5 mg/ml), respectively, to allow the TNF inhibitors to "capture" adiponectin before stimulation of the cells for 15 h. Unstimulated fibroblasts, adiponectin-stimulated fibroblasts, and unstimulated fibroblasts that were incubated with or without the inhibitors served as controls. Supernatants were collected and frozen immediately at -20°C until further evaluation. Each of the experiments was repeated five times.

Western blot

To confirm the presumed ability of etanercept and adalimumab to bind to adiponectin, Western blotting experiments were performed using adiponectin as a substrate, recombinant TNF- α as a positive control substrate, and vimentin as a negative control substrate. Briefly, 2.5 μg of recombinant adiponectin (R&D Systems), 2 μg of recombinant TNF- α (PeproTech), and 1.5 μg of vimentin (Research Diagnostics) were mixed with reducing Laemmli Sample Buffer (Bio-Rad)/0.5% 2-ME (Bio-Rad), boiled at 95°C for 10 min, loaded onto a Tris/Glycine/SDS gel (Bio-Rad), run at 200 V for 1 h, and transferred onto a nitrocellulose membrane at 100 V for 1 h. Membranes were blocked with 3% dry milk (Bio-Rad), and immunoblotted with etanercept (1:50), adalimumab (1:50), or specific Abs directed against TNF- α (1:2000) or against vimentin (1:10000), respectively. Detection of enhanced chemiluminescence was done following the manufacturer's protocol (Pierce).

Statistics

For statistical evaluation, Mann-Whitney *U* test for non-Gaussian parameters and Student's *t* test for Gaussian parameters (including Bonferroni correction if required) was performed using Sigmaplot 2.03 for Windows software.

Results

Expression of adiponectin mRNA and adiponectin protein in synovium and in articular adipose tissue

Examination of synovium and articular adipose tissue of all patients with RA and OA revealed strong expression of adiponectin mRNA, in particular in cells in the synovial lining layer, in the perivascular area and in the synovial sublining (Fig. 1A). Articular adipose tissue also showed a strong expression of adiponectin mRNA, which was predominantly located in articular adipose tissue adipocytes (Fig. 1B). A similar expression pattern could be seen using immunohistochemistry for adiponectin protein detection. Numerous synoviocytes (Fig. 1C) as well as articular adipocytes (Fig. 1D) strongly expressed adiponectin protein.

Double-labeling using anti-fibroblast (vimentin) and anti-macrophage (CD68) Abs revealed that a number of adiponectin mRNA-expressing cells were synovial fibroblasts, whereas macrophages did not express adiponectin (Fig. 1, E and F). In contrast, in the skin samples of patients with SSc, adiponectin mRNA could not be detected (data not shown).

Expression of adiponectin and adiponectin receptors type 1 and 2 mRNA in cultured fibroblasts

To confirm the expression of adiponectin and adiponectin receptor by synovial fibroblasts, RT-PCR was performed using mRNA of cultured RASF and OASF and normal skin fibroblasts (Nf). Both adiponectin (Fig. 2A) and adiponectin receptors type 1 (Fig. 2B) and type 2 mRNA (Fig. 2C) could be detected in all fibroblast populations, whereas, similar to the histological findings in scleroderma skin, none of the cultured scleroderma fibroblasts expressed adiponectin mRNA (data not shown).

Stimulation of proinflammatory cytokines and MMPs by adiponectin

With regards to the potential pathogenic role of adiponectin in joint inflammation, we investigated the effects of adiponectin on the release of cytokines and matrix degrading enzymes, which are known to play important roles in the pathophysiology of inflammatory joint disease. To this end, we incubated RASF and OASF with increasing amounts of adiponectin *in vitro*. Fibroblasts isolated from normal skin (Nf) served as controls.

Following adiponectin stimulation, we observed a highly selective increase of proinflammatory IL-6 and pro-MMP-1, the precursor of the matrix-degrading enzyme MMP-1 (Fig. 3). In contrast, none of the cytokines IL-1 β , IL-4, IL-10, and TNF- α , nor pro-MMP-13 could be detected by ELISA. Expression of the growth factors VEGF and TGF- β was also not altered by adiponectin (data not shown). Interestingly, the selectivity for IL-6 and pro-MMP-1 induction appears not to be restricted to RASF and OASF, because up-regulation of these molecules could also be observed in Nf. However, spontaneous expression of these inflammation-related molecules in normal skin fibroblasts is minimal, whereas RASF and OASF exhibit elevated spontaneous expression levels of IL-6 (up to 14 times higher than in Nf) and pro-MMP-1 (up to 2.4 times higher than in Nf), which is consistent with an activated, inflammatory phenotype of RASF and OASF.

Furthermore, the effect of adiponectin on IL-6 and pro-MMP-1 was dose-dependent in all three types of fibroblasts (Fig. 3). In RASF, basal IL-6 concentration was 2.8 ng/ml, which was increased up to 23.1 ng/ml (8.3-fold increase) when using 100 μ g/ml adiponectin. Similarly, pro-MMP-1 synthesis could be increased from 1.7 to 13.9 ng/ml (8.2-fold increase). In OASF, basal IL-6

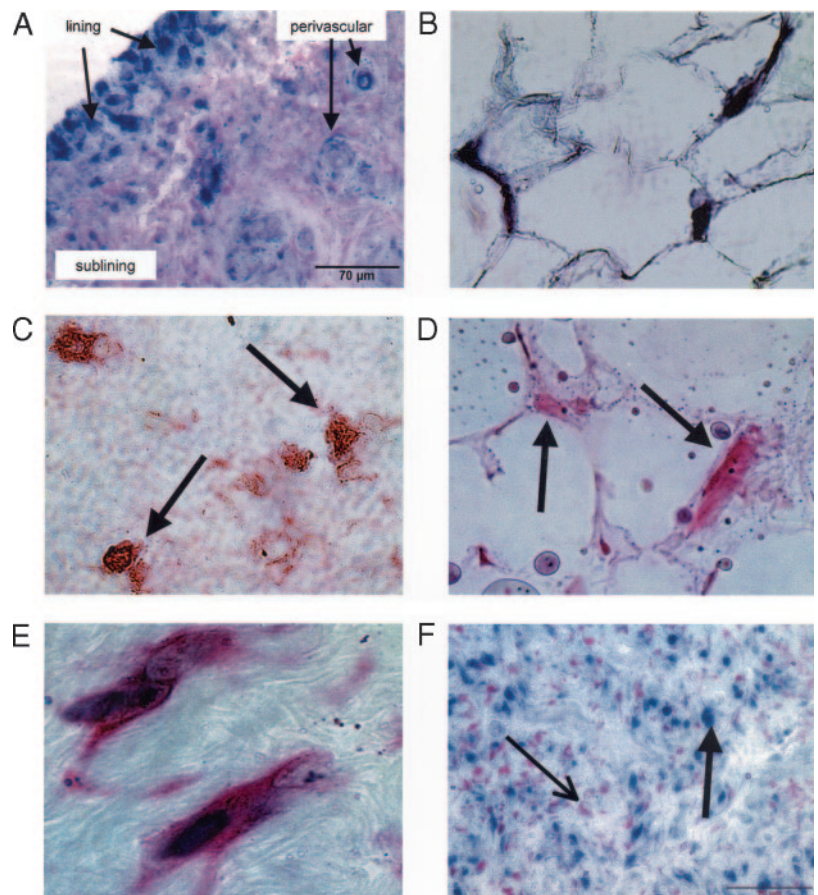


FIGURE 1. Demonstration of adiponectin mRNA and protein in synovial tissue and articular adipose tissue. *A*, Intensive expression of adiponectin mRNA in the synovial lining layer, the perivascular areas, and to a lesser extent in the sublining of a patient with RA. In situ hybridization, antisense probe (black staining), double-labeling with anti-vimentin Abs (red staining) (original magnification, $\times 200$). *B*, Articular adipose tissue showed also a strong expression of adiponectin mRNA (black staining), which was predominantly located in articular adipose tissue adipocytes. In situ hybridization, antisense probe (original magnification, $\times 400$). *C* and *D*, Expression pattern of adiponectin protein (arrows, red staining) in synovium and articular adipose tissue. Numerous synoviocytes (*C*) as well as articular adipocytes (*D*) express strongly adiponectin protein (red staining) (original magnification, $\times 640$). *E*, Double-labeling using in situ hybridization and anti-fibroblast (anti-vimentin) Abs showed that the majority of adiponectin mRNA (black staining)-expressing cells are synovial fibroblasts (red staining). (original magnification, $\times 640$). *F*, Double-labeling using in situ hybridization and anti-macrophage (anti-CD68) Abs showed that the majority of adiponectin mRNA (black staining, bold arrow)-expressing cells are synovial fibroblasts and not macrophages (red staining, thin arrow) (original magnification, $\times 200$).

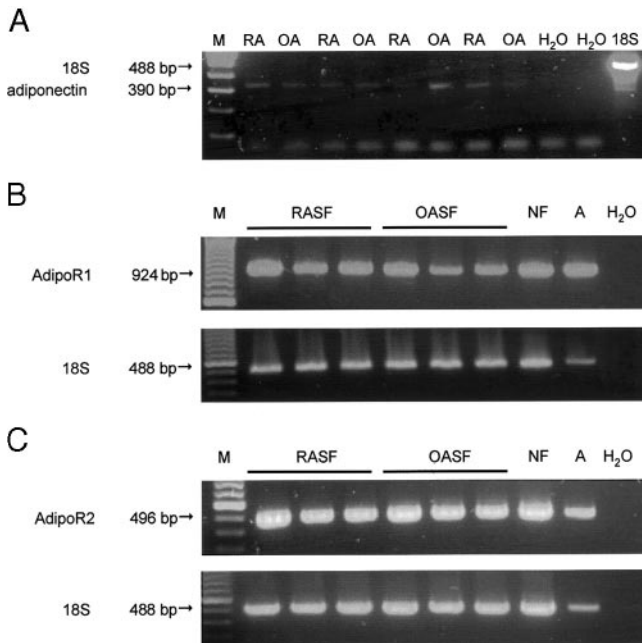


FIGURE 2. A, RT-PCR: expression of adiponectin mRNA in cultured synovial fibroblasts. Expression of the adiponectin receptor mRNA AdipoR1 (B) and AdipoR2 (C) in cultured RA and OA fibroblasts, normal skin fibroblasts (Nf), and in adipocyte DNA (A).

concentration was 2.5 ng/ml, which was increased up to 23.1 ng/ml (9.2-fold increase) when using 100 $\mu\text{g/ml}$ adiponectin, and pro-MMP-1 synthesis was increased from 0.9 to 5 ng/ml (5.6-fold increase). The basal IL-6 concentration in Nf was 0.2 ng/ml, which was increased up to 8.4 ng/ml (42-fold increase) when using 100 $\mu\text{g/ml}$ adiponectin. The basal pro-MMP-1 concentration was 0.7

ng/ml and could be increased to 3.4 ng/ml (4.9-fold increase) when using 100 $\mu\text{g/ml}$ adiponectin.

As suggested by the asymptotic nature of the stimulation curve, the maximal stimulation concentration of 100 $\mu\text{g/ml}$ adiponectin used in the experiments appears to be close to the concentration resulting in the maximal stimulatory effect (Fig. 3). Based on this result, 25 $\mu\text{g/ml}$ adiponectin was used for the inhibition experiments described below.

In addition, preincubation of adiponectin with anti-adiponectin Abs before stimulation prevented the increase of IL-6 and pro-MMP-1 production, which suggests that these effects were adiponectin-dependent (data not shown).

Effect of intracellular signaling inhibitors on adiponectin-induced IL-6 and pro-MMP-1 synthesis in synovial fibroblasts

To investigate the possibility that intracellular signaling pathways, which are well known to regulate the expression of IL-6 and pro-MMP-1 in RASF, also mediate adiponectin-induced IL-6 and pro-MMP-1 synthesis, we selectively blocked these pathways using four different small molecule inhibitors, including the p38 MAPK inhibitor SB203580, a PKA inhibitor, a PKC inhibitor, and cPKA inhibitor.

IL-6. Of the four different signaling inhibitors, only incubation with the p38 MAPK inhibitor SB203580 resulted in a significant reduction of adiponectin-dependent IL-6 synthesis in synovial fibroblasts (Fig. 4A). This could be confirmed by the use of a second p38 MAPK inhibitor, RWJ67657 in RASF (Fig. 4B). Interestingly, the inhibitory effects of the MAPK inhibitor did not differ between RASF and OASF. In RA, stimulation indices were reduced from 22.7 ± 10.4 to 6.3 ± 3.6 (mean \pm SD; $p = 0.002$) and in OA from 28.1 ± 23.4 to 8.5 ± 5.2 (mean \pm SD; $p = 0.02$) (Fig. 4A).

MMP-1. Similar to the results of IL-6 inhibition, significant inhibitory effects on adiponectin-induced pro-MMP-1 synthesis

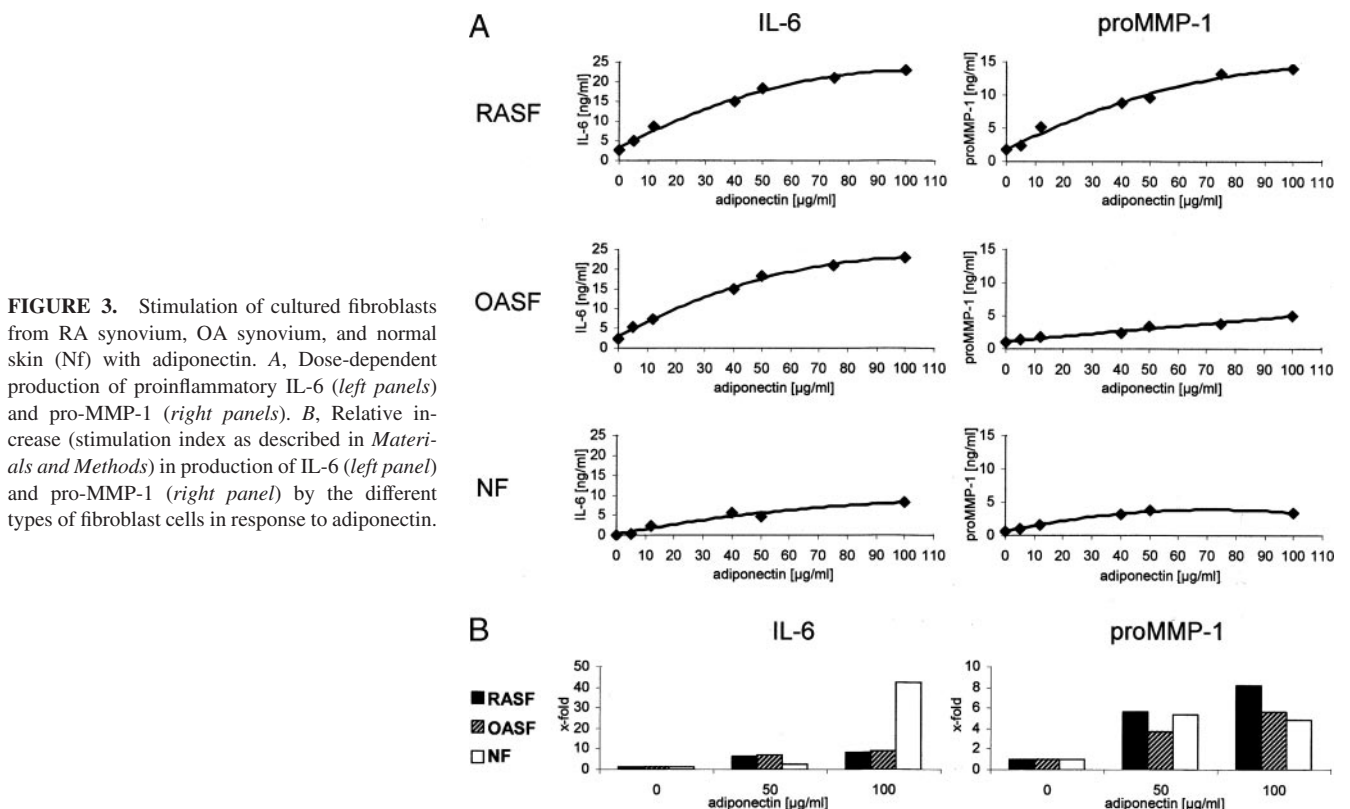


FIGURE 3. Stimulation of cultured fibroblasts from RA synovium, OA synovium, and normal skin (Nf) with adiponectin. A, Dose-dependent production of proinflammatory IL-6 (left panels) and pro-MMP-1 (right panels). B, Relative increase (stimulation index as described in Materials and Methods) in production of IL-6 (left panel) and pro-MMP-1 (right panel) by the different types of fibroblast cells in response to adiponectin.

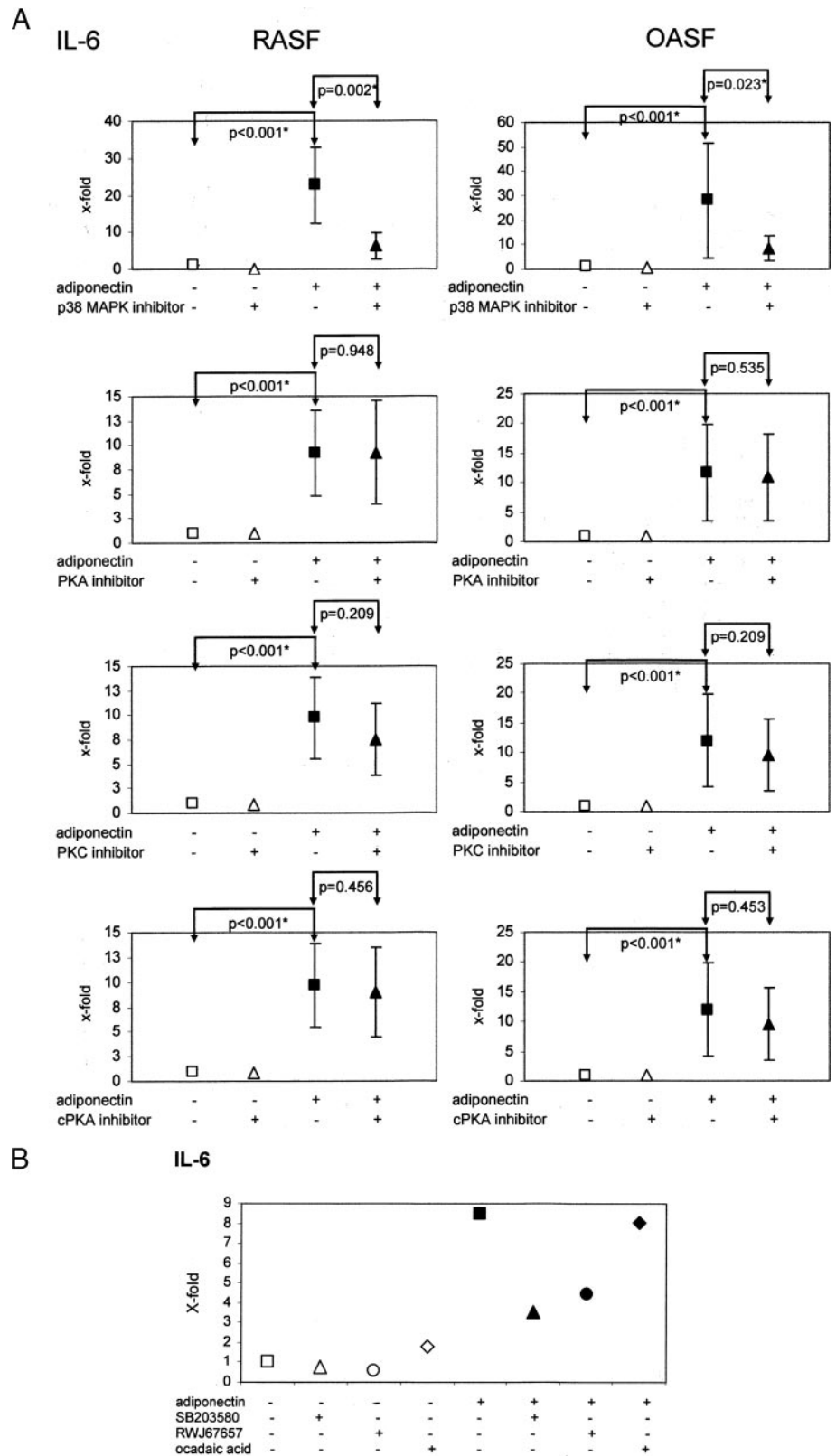


FIGURE 4. Effects of known inhibitors of adiponectin-dependent intracellular signaling on adiponectin-induced synthesis of IL-6 in RASF and OASF. A concentration of 25 $\mu\text{g/ml}$ adiponectin was used for stimulation, and values are given as stimulation indices ($x\text{-fold} \pm \text{SD}$) as compared with unstimulated fibroblasts. **A**, Of the four different signaling inhibitors, only incubation with the p38 MAPK inhibitor SB203580 resulted in a significant reduction of adiponectin-dependent IL-6 synthesis in RASF and OASF (*upper panels*), whereas neither the PKA inhibitor, PKC inhibitor, nor cPKA inhibitor resulted in a significant down-regulation of IL-6 synthesis (*lower three panels*). **B**, As control for the effectiveness of the p38 MAPK inhibitor, an additional inhibitor, RWJ67657, was used similar to the experiments with SB203580. Both inhibitors were able to reduce the adiponectin-dependent IL-6 synthesis in RASF. In addition, an unspecific p38 MAPK inducer, okadaic acid, was used to control the p38 MAPK-dependent IL-6 induction in RASF. The adiponectin-mediated IL-6 induction was more obvious than the okadaic acid-mediated IL-6 induction. Open symbols, unstimulated; closed symbols, stimulation with adiponectin; squares, without signaling inhibitor; triangles, preincubation with signaling inhibitor.

could only be obtained by incubation with the p38 MAPK inhibitor, whereas none of the other inhibitors had any significant effects on pro-MMP-1 synthesis (Fig. 5A). The inhibitory effects of the MAPK inhibitor SB203580 could be confirmed using a second inhibitor for p38 MAPK, RWJ67657 in RASF (Fig. 5B). In addition, siRNAs against p38 were used as additional control as described in *Materials and Methods* (Fig. 5C). However, in contrast

to IL-6 inhibition, the inhibitory effects of the MAPK inhibitor on pro-MMP-1 production differed between RASF and OASF. In RA, stimulation indices were reduced by 38% from 2.3 ± 0.6 to 1.5 ± 0.3 (mean \pm SD), but this reduction did not reach the required level of statistical significance ($p = 0.055$). In contrast, the stimulation indices in OA were reduced significantly ($p = 0.035$) by 45% from 4.0 ± 1.9 to 2.2 ± 0.6 (mean \pm SD) (Fig. 5A).

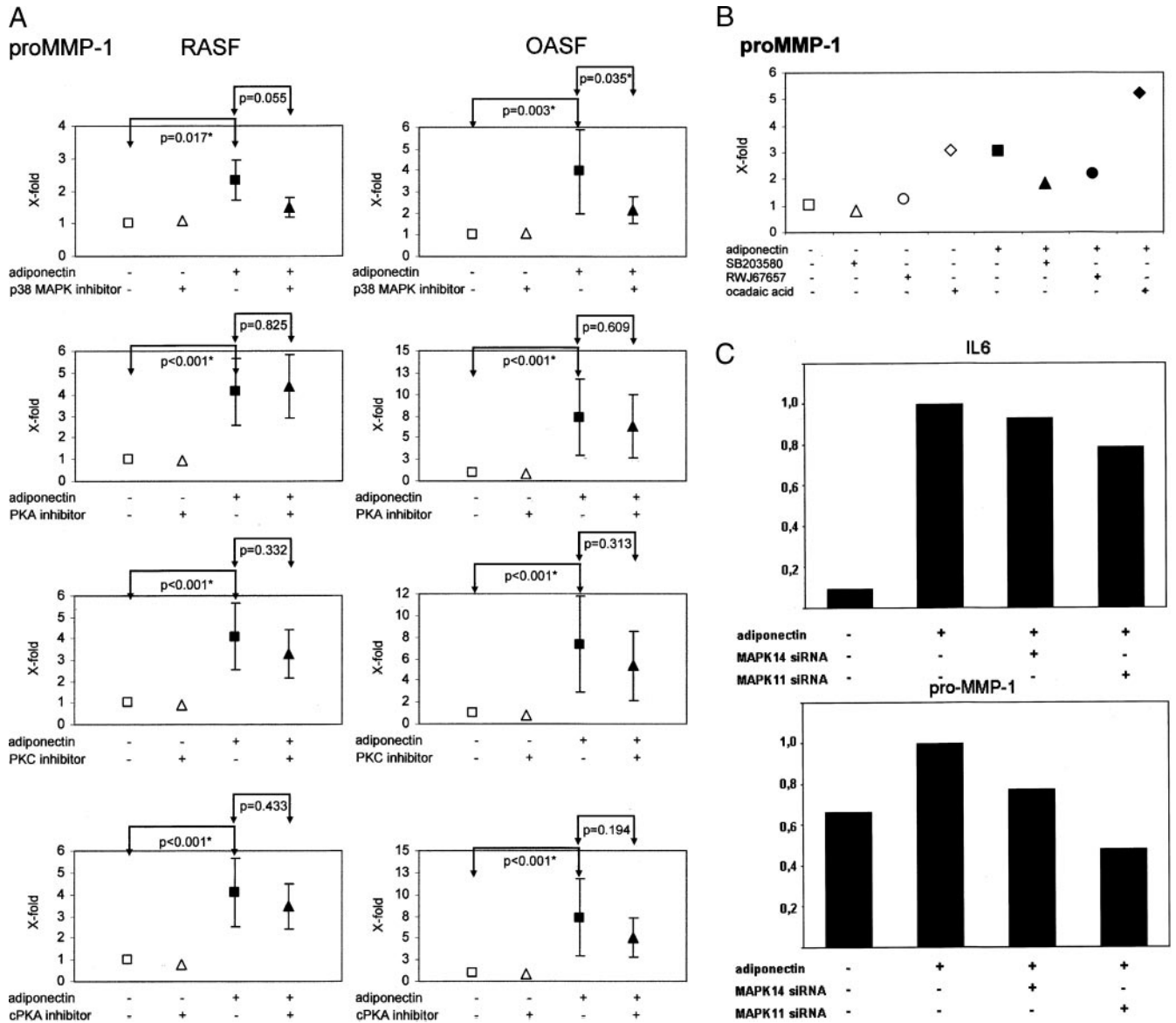


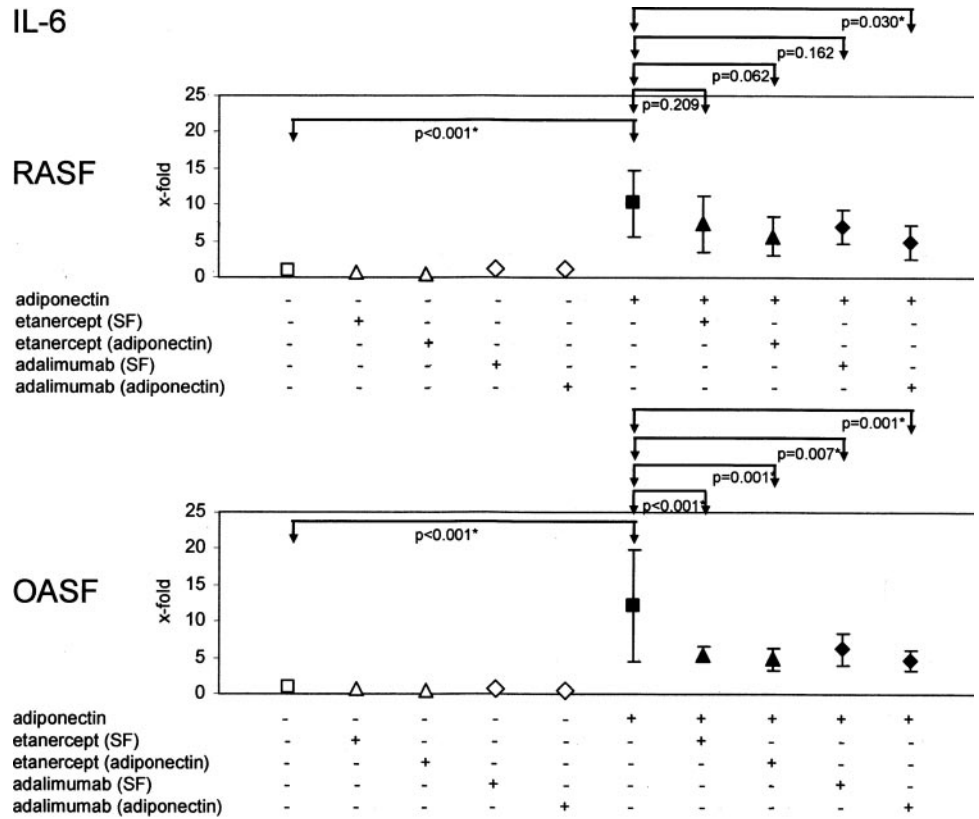
FIGURE 5. Effects of known inhibitors of adiponectin-dependent intracellular signaling on adiponectin-induced synthesis of pro-MMP-1 in RASF and OASF. A concentration of 25 $\mu\text{g}/\text{ml}$ adiponectin was used for stimulation, and values are given as stimulation indices ($x\text{-fold} \pm \text{SD}$) as compared with unstimulated fibroblasts. **A**, Of the four different signaling inhibitors, only incubation with the p38 MAPK inhibitor resulted in a significant reduction of adiponectin-dependent pro-MMP-1 synthesis of OASF (*upper panels*) and to a strong but not significant inhibitory effect on RASF, whereas neither the PKA inhibitor, PKC inhibitor, nor cPKA inhibitor resulted in a significant down-regulation of pro-MMP-1 synthesis (*lower three panels*). **B**, As control for the effectiveness of the p38 MAPK inhibitor, an additional inhibitor, RWJ67657, was used similar to the experiments with SB203580. Both inhibitors were able to reduce the adiponectin-dependent pro-MMP-1 synthesis in RASF. In addition, an unspecific p38 MAPK inducer, okadaic acid, was used to control the p38 MAPK-dependent pro-MMP-1 induction in RASF. Open symbols, unstimulated; closed symbols, stimulation with adiponectin; squares, without signaling inhibitor; triangles, preincubation with signaling inhibitor. **C**, siRNAs against p38 MAPK were used as described in *Materials and Methods* to confirm the results using the chemical inhibitors RWJ67657 and SB203580 (**B**). Experiments were performed in duplicate, the figure represents a representative analysis.

Effect of TNF inhibitors etanercept and adalimumab on adiponectin-induced IL-6 and pro-MMP-1 synthesis

The experiments were performed using four experimental settings: 1) preincubation of the cells with etanercept for 2 h and subsequent stimulation with adiponectin; 2) preincubation of the cells with adalimumab for 2 h and subsequent stimulation with adiponectin; 3) preincubation of adiponectin with etanercept for 2 h and subsequent fibroblast stimulation with this mixture; or 4) preincubation of adiponectin with adalimumab for 2 h and subsequent fibroblast stimulation with this mixture. For all experimental settings, 25 $\mu\text{g}/\text{ml}$ adiponectin and 2.5 mg/ml of either anti-TNF agent were used.

IL-6. In RASF, all four experimental settings resulted in a reduction of mean IL-6 synthesis by $\sim 50\%$. Yet, the observed reduction reached statistical significance only when adiponectin was preincubated with adalimumab (reduction of stimulation index from 10.2 ± 4.6 to 4.9 ± 2.4 ; mean \pm SD; $p = 0.03$) (Fig. 6). In contrast, both TNF inhibitors were able to significantly inhibit IL-6 synthesis in OA, regardless of which experimental setting was used, resulting in a decrease of stimulation indices from 12.2 ± 7.7 to 1) 4.9 ± 2.4 (preincubation of cells with etanercept; $p = 0.001$), 2) 4.8 ± 1.6 (preincubation of adiponectin with etanercept; $p = 0.001$), 3) 6.2 ± 2.2 (preincubation of cells with adalimumab; $p = 0.007$), and 4) 4.7 ± 1.5

FIGURE 6. Effects of TNF inhibitors adalimumab and etanercept on adiponectin-dependent IL-6 synthesis of synovial fibroblasts. Experiments were performed with 25 $\mu\text{g/ml}$ adiponectin, and all experiments were performed with and without preincubation of adiponectin with the TNF inhibitors before stimulation of the cells. Open symbols, unstimulated cells; closed symbols, with adiponectin-stimulated cells; squares, cells without TNF inhibitors; triangles, cells with etanercept; rhombs, cells with adalimumab; synovial fibroblasts (SF), preincubation of each TNF inhibitor with the cells 2 h before stimulation with adiponectin; (adiponectin), preincubation of each TNF inhibitor with adiponectin 2 h before stimulation of the cells. Of the four experimental settings (adiponectin stimulation \pm preincubation with adalimumab or etanercept), inhibition with TNF blockers resulted in reduction of IL-6 synthesis by RASF. Statistical significant reduction of adiponectin-dependent IL-6 synthesis was reached by preincubation of adalimumab with adiponectin. In contrast, in OA, both TNF blockers resulted in significant reduction of IL-6 synthesis (*lower panel*), regardless of the experimental setting.



(preincubation of adiponectin with adalimumab; $p = 0.001$), respectively (Fig. 6).

MMP-1. Pro-MMP-1 was regulated similar to IL-6. In RASF, all four experimental settings reduced the adiponectin-dependent synthesis of mean pro-MMP-1 by $\sim 40\%$. Yet, the observed reduction reached statistical significance only when adiponectin was incubated with adalimumab (from 4.1 ± 1.5 to 2.4 ± 1.1 mean \pm SD; $p = 0.045$) (Fig. 7). In OA, again both TNF inhibitors were able to significantly inhibit pro-MMP-1 synthesis, regardless of the experimental settings. Stimulation indices were decreased from 7.1 ± 4.7 to 1) 2.2 ± 1.8 ($p = 0.027$), 2) 3.2 ± 1.5 ($p = 0.032$), 3) 3.1 ± 1.5 ($p = 0.03$), and 4) 2.1 ± 1.0 ($p = 0.008$), respectively (Fig. 7).

Analysis of the binding capacity of adiponectin to TNF inhibitors

The binding of the TNF inhibitors adalimumab and to adiponectin was tested by Western blotting as described in *Materials and Methods*. The binding of adiponectin to the TNF inhibitors could not be confirmed. In contrast, the binding of etanercept and adalimumab to TNF- α , which served as a positive control, could be detected by Western blot (data not shown).

Discussion

In contrast to the ample data in the field of endocrinology and cardiovascular disease, little is known about the role of adipose tissue and adipocytokines, especially of adiponectin, in immunological and inflammatory diseases, such as arthritis (7, 8, 29–37). Therefore, the aim of this study was to investigate the expression and the functional role of adiponectin in arthritis.

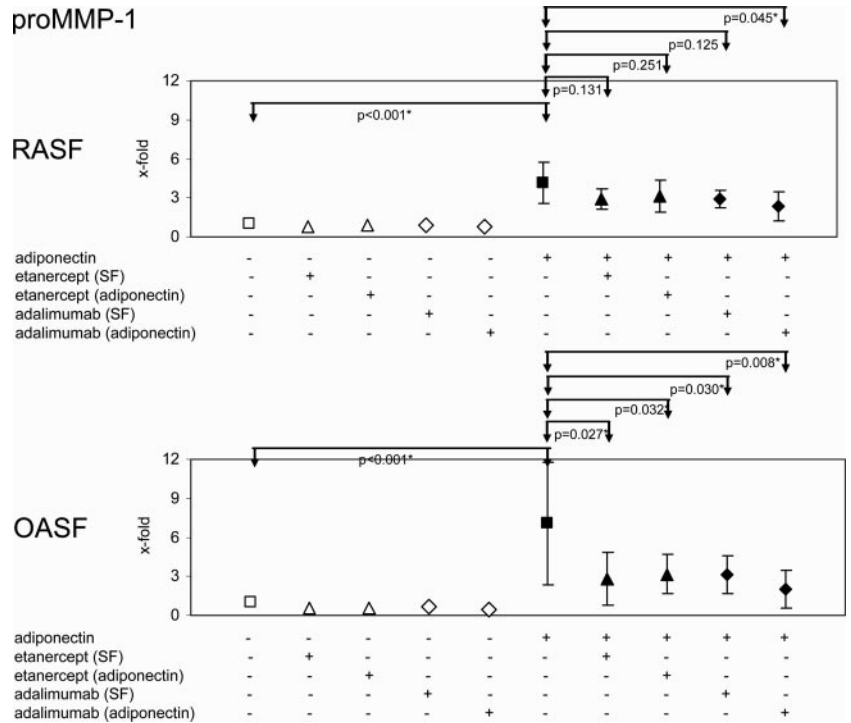
Until recently, the expression of adiponectin was considered to be restricted to adipose tissue. However, Berner et al. (22) have

demonstrated that adiponectin is also expressed and secreted by osteoblasts, and therefore suggested a role of adiponectin in bone homeostasis. In addition, hepatocytes, which normally do not express adiponectin, can synthesize adiponectin in response to IL-6 stimulation or tissue injury (38). Similarly, skeletal muscle cells are also able to express adiponectin when stimulated with inflammatory cytokines (39). These findings indicate that adiponectin expression can be induced in cells other than adipocytes in the context of an inflammatory process.

With regards to joints, the results of this study clearly demonstrate that articular adipose tissue and synovium of patients with inflammatory joint diseases are a significant source of adiponectin. This is also reflected by the recent finding of high levels of the adipocytokines adiponectin and resistin in synovial fluid from RA and OA patients (21). Of interest, our data for the first time show that not only adipocytes, which are known to synthesize these molecules, but especially activated synovial fibroblasts within the synovial lining, the perivascular area, and the inflamed sublining are primary producers of adiponectin. This could be confirmed on the mRNA and protein levels in tissue samples of inflamed synovium as well as in cultured fibroblasts.

In addition, expression of the respective adiponectin receptors, which are necessary to transfer the adiponectin-dependent signals, was demonstrated in RASF and OASF. Therefore, we investigated the molecular effects of adiponectin in synovial fibroblasts. Thus far, the majority of studies in endocrinology and vascular biology addressing the functional role of adiponectin have reported a protective rather than a proinflammatory or destructive role of these molecules (33). In contrast, this study demonstrates for the first time that adiponectin can also exert significant proinflammatory and matrix-degrading effects. Our results show that two of the

FIGURE 7. Effects of TNF inhibitors adalimumab and etanercept on adiponectin-dependent pro-MMP-1 synthesis of synovial fibroblasts. Experiments were performed with 25 $\mu\text{g/ml}$ adiponectin, and all experiments were performed with and without preincubation of adiponectin with the TNF inhibitors before stimulation of the cells. Open symbols, unstimulated cells; closed symbols, with adiponectin-stimulated cells; squares, cells without TNF inhibitors; triangles, cells with etanercept; rhombs, cells with adalimumab; (SF), preincubation of each TNF inhibitor with the cells 2 h before stimulation with adiponectin; (adiponectin), preincubation of each TNF inhibitor with adiponectin 2 h before stimulation of the cells. Of the four experimental settings (adiponectin stimulation \pm preincubation with adalimumab or etanercept), inhibition with TNF blockers resulted in reduction of pro-MMP-1 synthesis by RASF. Statistical significant reduction of adiponectin-dependent pro-MMP-1 synthesis was reached by preincubation of adalimumab with adiponectin. In contrast, in OA, both TNF blockers resulted in significant reduction of pro-MMP-1 synthesis (*lower panel*), regardless of the experimental setting.



main mediators of pathophysiology in RA, IL-6 and pro-MMP-1, are up-regulated by adiponectin.

IL-6 is overexpressed in blood, synovial fluid, and synovium in RA patients in comparison to other arthritides and healthy controls, and IL-6 levels correlate with the degree of inflammation (40–42). Of note, IL-6 induces proliferation of RASF in vitro (43). In vivo, Alonzi et al. (44) demonstrated that IL-6 deficient mice are resistant to collagen-induced arthritis. IL-6 production by RASF can be induced by TNF- α and IL-1, which also up-regulate secretion of matrix-degrading enzymes, including MMPs (45–49). In healthy tissue, MMPs are usually barely expressed, whereas tissues undergoing pathologic remodeling are high in MMPs (50). In RA, MMP-1, also known as collagenase, is highly expressed perivascularly and in the synovial lining layer (51) and plays a pivotal role in both cartilage and bone destruction (52).

The observed up-regulation of IL-6 and pro-MMP-1 by adiponectin in this study appears to be highly selective, because other proinflammatory cytokines, such as IL-1, TNF- α , VEGF, and TGF- β , as well as protective cytokines, such as IL-4 and IL-10, were not affected. In clinical RA in vivo, adipocytes might therefore be key interaction partners for the synovial fibroblasts, which are specifically active at sites of destruction (53–55).

Interestingly, the ability to respond to adiponectin is not restricted to synovial fibroblasts. As shown in Fig. 3, fibroblasts from normal skin also exhibit the potential to secrete IL-6 and pro-MMP-1 upon stimulation with adiponectin in vitro. It is known that fibroblast, adipocytes, and osteogenic cells are mesenchymal cells, which all derive from a common multipotential stem cell (56). Furthermore, it has been demonstrated that adipocytes can be dedifferentiated into fibroblast-like cells and that this process is reversible (56). Similarly, synovial fibroblasts can be differentiated into adipocytes (20). Considering this, our result suggests that the ability to mount a proinflammatory response when stimulated by adiponectin could be a common trait of mesenchymal cells. Thus, it can be hypothesized that, in vivo, mesenchymal cells might use their potential to express proinflammatory cytokines in the context of a pathologic condition, such as arthritis, whereas under physi-

ological conditions, such as in normal skin, this is unlikely to occur.

To address the question which molecular mechanisms might mediate the observed production of IL-6 and pro-MMP-1 in response to adiponectin, we examined key pathways of intracellular signaling. Previous studies addressing intracellular effects of adiponectin in lipid and glucose metabolism and in cardiovascular disease revealed that binding to its respective receptors AdipoR1 and -2 results predominantly in activation of 5'-AMP kinase, PKA, and p38 MAPK (57–59). Because up-regulation of IL-6 and pro-MMP-1 in RASF and other mesenchymal cells is known to be mediated by PKC and p38 MAPK (60–65), we chose to investigate the signaling molecules that are common to both processes.

Based on pharmacological blockade of these signaling pathways, we identified p38 MAPK as the common denominator that provides a link between adiponectin stimulation and IL-6/pro-MMP-1 production. Of note, p38 MAPK is a key regulator in stress-induced cellular responses and inflammatory joint disease. It plays a role in the production of the pivotal proinflammatory cytokines IL-1 and TNF- α (66, 67), and inhibition of p38 MAPK leads to amelioration of arthritis in animal models (68, 69). Thus, involvement of p38 MAPK in mediating the proinflammatory effects of adiponectin further supports the concept of a pathogenic role of this adipocytokine in RA.

To further assess the pathogenic relevance of adiponectin, we examined whether its effects could be influenced by anti-inflammatory drugs that are currently in use for the treatment of RA. Among these, the TNF-blocking biologics etanercept and adalimumab, belong to the most potent agents (28). Because adiponectin is a member of the recently described C1q/TNF superfamily it shares strong structural homology with TNF- α . Thus, we hypothesized that adiponectin, in analogy to TNF, could bind to TNF inhibitors, resulting in suppression of its effects on IL-6 and pro-MMP-1. Consistent with this hypothesis, the application of etanercept and adalimumab resulted in a marked reduction of IL-6 and pro-MMP-1 levels in vitro. To elucidate whether the inhibitory effects of the TNF inhibitors were indeed due to binding to the

TNF-like structure of adiponectin, we preincubated adiponectin with each TNF inhibitor to allow capturing of adiponectin before stimulation of the cells. Unexpectedly, preincubation of the cells with either TNF inhibitor was equally effective, suggesting that specific binding of adiponectin by etanercept or adalimumab did not occur. This notion was supported by the lack of specific binding between adiponectin and etanercept or adalimumab, respectively, on Western blotting.

Thus, TNF blockers clearly confer inhibitory effects on adiponectin-induced expression of IL-6 and pro-MMP-1; however, further work will be needed to clarify the exact mechanism. Nevertheless, it cannot be excluded that the ameliorating effects of these TNF inhibitors on inflammatory and joint-destructive mechanisms, particularly on IL-6 and MMP-1-dependent pathways (70–73), are partially based on an—as yet undefined—antiadipocytokine effect.

In RASF, the inhibitory effects of TNF blockers were less extensive, reaching statistical significance only in one of the four experimental settings, whereas in OASF, all four settings yielded statistically significant inhibition. A similar behavior of RASF was also observed in response to the p38 MAPK inhibitor. These observations support the hypothesis that RA fibroblasts are in a constitutive state of activation (53–55).

In summary, these data show for the first time that adiponectin is present in the synovium in inflammatory joint diseases and is expressed not only by articular adipocytes but also by synovial fibroblasts. Furthermore, we could show that adiponectin stimulates the production of key mediators of destructive arthritis, IL-6, and pro-MMP-1, by synovial fibroblasts. Most strikingly, the results of this study reveal that the effects of adiponectin on synovial fibroblasts are highly selective and appear to be regulated by a limited number of mechanisms. Therefore, our data support the hypothesis that articular adipose tissue and adipocytokines such as adiponectin cannot be regarded as innocent bystanders any more but have an active role in the pathogenesis of chronic inflammatory joint diseases.

Acknowledgments

We acknowledge the expert technical assistance of Birgit Riepl, Kerstin Winkler, Andreas Kampmann, and Dirk Schröder, as well as the excellent technical advice of Orr Sharpe.

Disclosures

The authors have no financial conflict of interest.

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