International Orthopaedics (SICOT) (2013) 37:145–151
DOI 10.1007/s00264-012-1724-1

ORIGINAL PAPER

Osteoarthritis synovial fluid activates pro-inflammatory cytokines in primary human chondrocytes

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Received: 21 August 2012 / Accepted: 17 November 2012 / Published online: 5 December 2012

Abstract
Purpose Two of the most common joint diseases are rheumatoid arthritis (RA) and osteoarthritis (OA). Cartilage degradation and erosions are important pathogenetic mechanisms in both joint diseases and have presently gained increasing interest. The aim of the present study was to investigate the effects of the synovial fluid environment of OA patients in comparison with synovial fluids of RA patients on human chondrocytes in vitro.
Methods Primary human chondrocytes were incubated in synovial fluids gained from patients with OA or RA. The detection of vital cell numbers was determined by histology and by using the Casy Cell Counter System. Cytokine and chemokine secretion was determined by a multiplex suspension array.
Results Microscopic analysis showed altered cell morphology and cell shrinkage following incubation with synovial fluid of RA patients. Detection of vital cells showed a highly significant decrease of vital chondrocyte when treated with RA synovial fluids in comparison with OA synovial fluids. An active secretion of cytokines such as vascular endothelial growth factor (VEGF) of chondrocytes treated with OA synovial fluids was observed.
Conclusions Significantly increased levels of various cytokines in synovial fluids of RA, and surprisingly of OA, patients were shown. Activation of pro-inflammatory cytokines of human chondrocytes by synovial fluids of OA patient supports a pro-inflammatory process in the pathogenesis of OA.

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Introduction

Two of the most common joint diseases are rheumatoid arthritis (RA) and osteoarthritis (OA). It is known that RA is a systemic disorder resulting in synovial inflammation, hyperplasia within the inflammatory pannus and bone erosions. Cartilage degradation and erosions are important pathogenetic mechanisms in both joint diseases, and have become more interesting recently [1]. It is known that chemokines and cytokines play a key role in the pathogenesis of RA. Cytokines and their receptors are responsible for regulating normal homeostasis, (patho)physiological cell removal and inflammation [2]. At the present time, more than 65 cytokines and cytokine receptors with complex functions are known. It has been suggested that an imbalance among pro-inflammatory and anti-inflammatory cytokines could result in an accelerated inflammation in joints of patients with RA [3, 4]. Therefore, the blocking of pro-inflammatory cytokines as a therapeutic strategy in inflammatory joint diseases has become more and more important. This is also reflected in the rising number of new biologic therapeutics, such as TNF or IL-6 antagonists [5, 6].

Next to the rise of cytokines, the active part of chondrocytes in the inflammatory process of cartilage degradation is a largely unknown field. In a recent study, we could demonstrate that inflammatory synovial fluids of patients with

reactive arthritis, psoriasis arthritis or RA mediate apoptosis and cell death of chondrocytes. Furthermore, inflammatory cytokine secretion in human chondrocytes was shown [7].

In contrast, in the opinion of most physicians, osteoarthritis is considered as a long-term process of degenerative cartilage degradation resulting in cartilage loss and bone damage, especially as OA is normally not accompanied by signs of systemic inflammation. But this does not fit the scientific facts. Various studies could show a close relationship between cytokine expression and OA [8, 9]. It was shown that interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) can induce the production of interleukin-6 (IL-6) and interleukin-8 (IL-8) by synovial cells and chondrocytes [8, 9]. These studies focused on synovial tissue, but not on chondrocytes. Therefore, an interaction between the synovial fluid microenvironment and chondrocytes and its role in cartilage degradation in OA remains unknown. This interaction could be of interest in the development of new therapeutic strategies in the treatment of OA. With the increasing age of the population, degenerative joint diseases represent a major economic burden for humanity [10]. New and faster therapeutic treatment of degenerative disorders could improve economic work and quality of life and reduce healthcare costs.

The aim of the present study was to investigate the effects of the synovial fluid environment of OA patients on human chondrocytes in vitro. In order to classify the role of inflammatory processes in OA, the examined effects were compared to those found in the known inflammatory disease RA.

Materials and methods

Plastic tissue culture ware was obtained from TPP (Trasadingen, Switzerland). Culture medium Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), trypsin and phosphate buffer saline (PBS) were purchased from Biochrom (Berlin, Germany).

Synovial fluids

Synovial fluids were obtained from four patients with knee osteoarthritis (OA) not presenting with any clinical or laboratory signs of infections and four patients with active rheumatoid arthritis (RA). Diagnoses of RA were ensured by ACR/EULAR classification. Patients gave their informed consent, and the study was approved by the local ethics committee.

Chondrocyte isolation and culture

Cartilage was obtained from three human donors with knee osteoarthritis without any kind of infectious signals (normal C-reactive protein (CrP) levels, normal leucocyte counts). Chondrocyte isolation was performed as described previously [11]. Cartilage tissue was minced and digested in medium containing 1 mg/ml pronase (Sigma-Aldrich, Deisenhofen, Germany) for 30 minutes at 37 °C. Then the, digestion medium was discarded and the tissue was treated with medium containing 1 mg/ml clostridial collagenase (Sigma-Aldrich, Deisenhofen, Germany) at 37 °C overnight. In the next step, digested solution was filtered (70 μm Nylon, BD Falcon, Bedford, Germany) and centrifuged at 1200 rpm for 8 minutes. After discarding the supernatant, the cell pellet was washed three times with PBS. Finally, chondrocytes were suspended in DMEM Hams-F12 with 10 % FBS, 1 % penicillin/ streptomycin and cultured at 37 °C, 95 % air with 5 % CO_2.

Chondrocytes treatment

Culture medium was removed from sub-confluent (80 % confluence) human chondrocyte cultures. 200 ml of aspirated synovial fluid from patients with OA or RA was added to the cultures for 24 and 48 hours. Culture medium was added to control chondrocytes.

Analysis of cell morphology by light microscopy

Human chondrocytes, grown on 24-well plates to a density of 80 % confluence, were treated with synovial fluids of patients with OA or RA for 24 and 48 hours. Two percent Triton X 100 as an inductor of cell necrosis was used as positive control. Chondrocytes treated with PBS were used as negative control (CTR). The results were interpreted using light microscopy analysis (Axiovert 40 C Light Microscope, lens 10×0.25, ocular 10×18 Zeiss, Göttingen, Germany). The view fields were then digitized by a digital camera (Canon EOS 500D, 15.1 Megapixels).

Determination of vital cell number

Human chondrocytes, grown on a 24-well to 80 % subconfluence, were incubated with synovial fluids of patients with OA or RA for 24 and 48 hours. Chondrocytes treated with 2 % Triton X 100 and chondrocytes incubated in culture medium were used as control. The Casy Cell Counter and Analyzer System (Schärfe-System, Reutlingen, Germany) was used for the detection of living cells, according to the manufacturer's instructions.

Multiplex suspension array

Synovial fluids as obtained from RA and OA patients were analyzed ex vivo (0 hours). In addition, the supernatants of chondrocytes treated with synovial fluids were collected after 24 and 48 hours. The samples were kept at −80 °C.

The concentrations of Interleukin (IL)-6, IL-8, interferon-gamma (IFNg), monocyte chemotactic protein-1 (MCP-1), granulocyte-colony stimulating factor (G-CSF) and vascular endothelial growth factor (VEGF) were measured by Bio-Plex suspension array (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions. Values under the detection limit were replaced by the detection limit for analysis (IFNg 15 pg/ml, MCP-1 20 pg/ml), values exceeding the upper range were replaced by the highest measureable value (IL-6: 63,000 pg/ml)

Statistical methods

The statistical analysis was performed using Graph Pad Prism Software (Graph Pad Software, San Diego, USA). Differences between groups were analyzed using the Mann–Whitney U test. Probability values of $p<0.05$ were considered to be statistically significant; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. Data are shown as mean ± standard error of the mean (SEM).

Results

Synovial fluids of RA and OA patients (RA > OA) reduce cell numbers of human chondrocytes and exhibit a toxic effect

Human cultured chondrocytes were treated with synovial fluids of RA and OA patients. Their morphology after treatment was analyzed by light microscopy (Fig. 1). As a control, chondrocytes were incubated in the medium. These control chondrocytes showed a normal cell structure and density (Fig. 1a). As a further control, chondrocytes were incubated with 2 % Triton X100, which is a known mediator of cell necrosis. These chondrocytes showed a necrotic morphology, they were shrunken and lost cell contacts, and the cell number was enormously reduced (Fig. 1b). The treatment of chondrocytes with synovial fluid of OA patients led to clearly reduced cell numbers when compared to chondrocytes treated with the medium, but the cell morphology remained widely normal (Fig. 1c). In contrast, the treatment of chondrocytes with synovial fluid of RA patients led to further reduced cell numbers when compared to chondrocytes treated with OA synovial fluid, and the cell morphology pointed to cell damage with globular and shrunken cells and loss of cell contact (Fig. 1c).

Additionally, the numbers of vital chondrocytes were analyzed. The numbers were significantly ($p<0.05$, respectively) reduced when chondrocytes were treated with synovial fluids of RA and OA patients when compared to CTR after 24 and after 48 hours (Fig. 2). As expected, very few vital chondrocytes were left after 24 hours and almost none were left after 48 hours when treated with Triton X100. Interestingly and reflecting the results of the histological analysis, the treatment with RA synovial fluids reduced the cell number at significantly higher levels than the treatment with OA synovial fluids after 24 and 48 hours ($p<0.001$, respectively).

Treatment of chondrocytes with synovial fluids of RA but also OA patients led to the induction of inflammatory cytokines

Human chondrocytes were treated with synovial fluids of RA and OA patients for 24 and 48 hours. The synovial fluids were examined ex vivo and after incubation for the concentration of inflammatory factors (Fig. 3). Considerably higher amounts of IL-6, IL-8 ($p<0.05$) and IFNg ($p<0.05$) were found in ex vivo synovial fluids of RA patients when compared to OA patients. Interestingly, the pro-angiogenic factor VEGF was found at higher concentration in OA synovial fluids ex vivo. The concentrations of IL-6, IFNg, MCP-1, G-CSF and VEGF increased significantly when chondrocytes were incubated with RA synovial fluids. But it was not only the synovial fluids of the inflammatory disease RA that increased the concentrations of inflammatory and angiogenic factors. The treatment of chondrocytes with OA synovial fluids also led to the significant increase of the concentrations of IL-6, IL-8 and MCP-1, although the concentrations remained at much lower levels than found for incubation with RA synovial fluids at all time points investigated. Interestingly, the concentration of VEGF also increased in the OA synovial fluids after incubation, and reached significantly higher values when compared to RA synovial fluids ($p<0.001$ after 24 and 48 hours, respectively).

Discussion

The present study shows a significant cell number reduction and activation of pro-inflammatory cytokines induced by osteoarthritis synovial fluids in human chondrocytes. The pathogenesis of osteoarthritis is considered to be a long-term degenerative process [1, 12]. At the present time, short-term impacts of the pathogenesis of osteoarthritis on human chondrocytes are still poorly understood. Therefore, we examined the short-term effects of OA synovial fluids on human chondrocytes. We compared the influence of synovial fluids from OA as a known degenerative disease with those from RA as an inflammatory joint disease with sometimes rapid joint destruction [13]. First, we demonstrated a significant loss of cells after treatment with RA synovial fluids. Cells were shrunken, lost cell contacts and showed a largely destroyed cell structure. Interestingly, chondrocytes treated with OA synovial fluid also exhibited a reduction in cell numbers. However, cell-structure remained widely

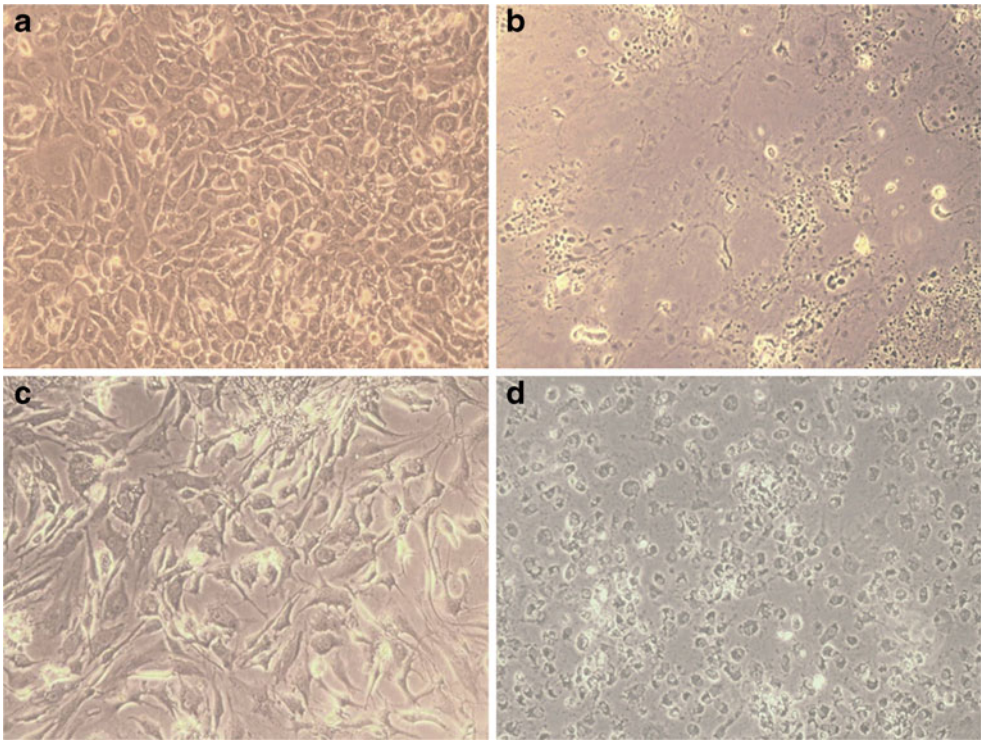

Fig. 1 Chondrocyte morphology after treatment with synovial fluids. Chondrocytes were analyzed by light microscopy after treatment with synovial fluids of RA and OA patients after 24 hours. **a** Control chondrocytes incubated in medium. **b** Chondrocytes treated with 2 % Triton X100 in order to induce necrosis. Chondrocytes incubated with OA (**c**) or RA (**d**) synovial fluids. One representative result from three independently performed experiments is shown, magnification: lens 10× 0.25, ocular 10×18

normal in comparison to RA synovial fluid-treated chondrocytes. The analysis of vital chondrocyte numbers after treatment with both synovial fluids confirmed the optical results. In both groups, vital cell numbers were clearly reduced. This seems surprising: although joint degradation in OA is a long-term development, short-term effects on human chondrocytes were clearly demonstrated.

In a recent study, we were able to show an active participation of human chondrocytes in inflammatory processes

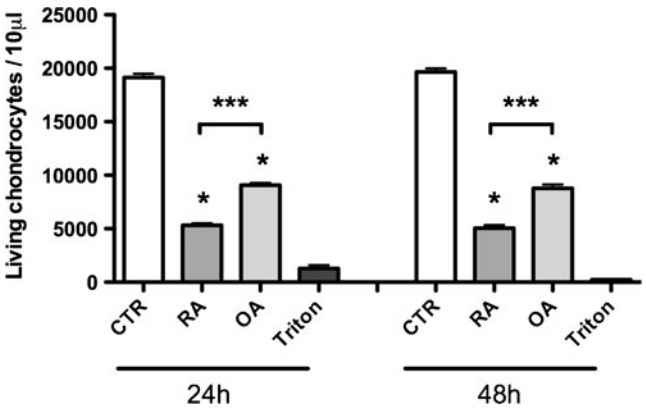

Fig. 2 Treatment of chondrocytes with RA but also OA synovial fluids led to reduction of living cells. Chondrocytes were incubated with medium (CTR, $n=3$), synovial fluids of patients with RA or OA (four RA/OA patients, treatment of chondrocytes from three donors, total sample number shown $n=12$), or the necrosis inductor Triton X100 at 2 % ($n=3$) for 24 and 48 hours. The number of vital chondrocytes after incubation is shown, Mann–Whitney U test; the asterisks above the bars refer to the statistical comparison with CTR, * $p<0.05$, and *** $p<0.001$

[7]. Here, we intended to investigate the behavior of human chondrocytes in a non-inflammatory environment, as is supposed to be found in the degenerative disease osteoarthritis. The results were compared to the effects induced by the strong inflammatory milieu found in the synovial fluid of rheumatoid arthritis patients. We found similarities in the effects of both entities on human chondrocytes, promoting a role of inflammation not only in RA but—to a lesser extent—also in OA. Surprisingly, VEGF was already found in ex vivo synovial fluids from OA patients at higher concentrations when compared to RA patients. Furthermore, the concentration of VEGF increased significantly during incubation with both synovial fluids, but the levels of VEGF even stayed at higher levels in the OA samples when compared to RA samples. It is known that growth factors play a pivotal role in the initiation and development of rheumatic joint diseases [14, 15]. VEGF modulates angiogenesis, the proliferation and formation of new capillaries from an existing vascular network, and is highly involved in inflammatory processes. Inflammatory joint diseases like RA are closely associated with increased angiogenesis at the site of inflammation [16, 17]. We could demonstrate higher levels of VEGF in synovial fluid of OA patients when compared to RA patients. We conclude that processes of angiogenesis also play a role in degenerative joint diseases seen in OA. In an animal study by Bauters et al., the effects of treatment of hindlimb ischemia in a rabbit model with VEGF were examined. A significant enhancement of anatomic and physiologic vessel formation after intravenous administration of VEGF was shown [18]. In an in vitro

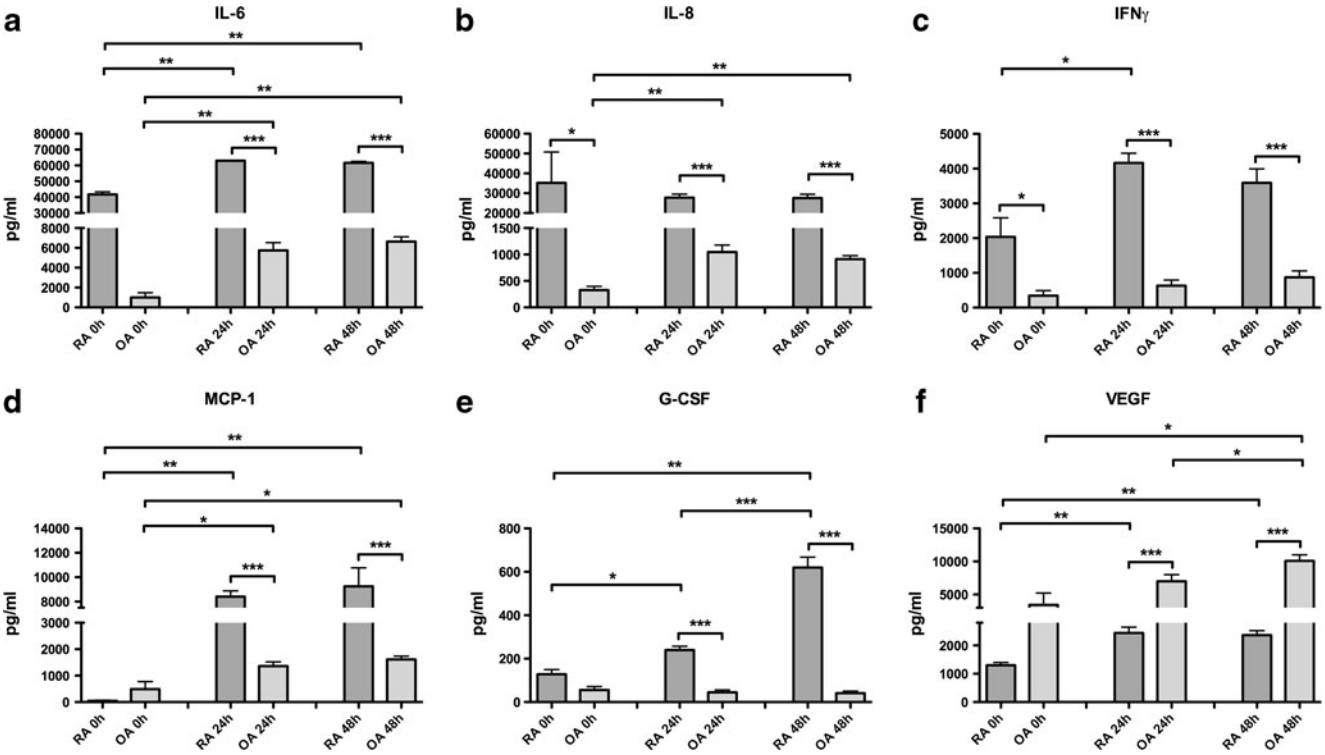

Fig. 3 Increase of pro-inflammatory cytokines after treatment of chondrocytes with RA but also OA synovial fluids. Chondrocytes were incubated with synovial fluids of patients with RA or OA (four RA/OA patients, treatment of chondrocytes from three donors, total sample number shown $n=12$). The concentrations of IL-6 (A), IL-8 (B), IFNg (C), MCP-1 (D), G-CSF (E) and VEGF (F) were analyzed in synovial fluids ex vivo and after 24/48 hours of incubation, Mann–Whitney U test * $p<0.05$, ** $p<0.01$, and *** $p<0.001$

study, Lambert et al. demonstrated a significant increase of VEGF in inflammatory areas of osteoarthritic synovial membrane [19]. Additionally, we demonstrated an increased expression of VEGF in chondrocytes treated with synovial fluids of osteoarthritis patients that have had no signs of systemic inflammation (normal CrP levels and normal leukocyte counts, no fever). From our results, we hypothesize a potential role of VEGF in cartilage destruction and in the pathogenesis of OA, perhaps a long time before clinical signs of inflammation appear [20]. Thus, our findings support the therapeutic relevance of anti-angiogenic therapies based on the inhibition of VEGF/VEGFR signaling, not only in RA, but also in OA [21].

Furthermore, we found clearly higher levels of pro-inflammatory cytokines like IL-6, IL-8 ($p<0.05$), IFN-y ($p<0.05$) and MCP-1 in ex vivo synovial fluids from RA patients when compared to OA patients. This result was expected, as RA is a known inflammatory disease. But interestingly, the concentrations of all these cytokines also increased during incubation of chondrocytes in the synovial fluids of OA patients. This was surprising, as we would not have expected an induction of inflammatory cytokines in chondrocytes during incubation with synovial fluids of OA patients, a degenerative disease. Interleukins-6 and 8 (IL-6, IL-8) are pro-inflammatory and angiogenic cytokines, and are also potent chemoattractants for neutrophils. Various studies showed increased levels of IL-6 and IL-8 in peripheral blood mononuclear cells or bone marrow of RA patients [15]. In the pathogenesis of OA, pro-inflammatory cytokines like IL-6 and IL-8 also play an important role. It is known that IL-6 promotes joint inflammation [22]. Livishits et al. showed a close relationship between serum levels of IL-6 and knee osteoarthritis. They were able to show that IL-6 levels in serum significantly increased in correlation with radiographic knee osteoarthritis. The main point of this study was that IL-6 levels in serum are a significant predictor of radiographic knee OA. Our results support an involvement of IL-6 in OA.

IL-8 is a chemokine produced by several inflammatory cells like neutrophils, basophils, macrophages and T-cells. Merz et al. showed that IL-8 expression is associated with chondrocyte hypertrophy in OA, which possibly promotes non-physiological calcification and matrix repair processes [23]. Matsukawa et al. was able to show that IL-8 induces destruction of cartilage [24]. Our study confirms a role of IL-8 in the pathogenesis of OA before conventional systemic inflammatory signs like CRP increase or leukocytosis can be detected in the patients. Thus, we hypothesize IL-8 levels to be—beside IL-6 levels—a further predictor for the level of cartilage degradation.

The next cytokine of interest was monocyte chemoattractant protein-1 (MCP-1), as increased MCP levels have been reported in the process of inflammation [25]. MCP is a member of the beta chemokine family; it regulates immune

processes, triggers chemotaxis and activates macrophages and is involved in mast cells and leukotriens activation [25]. This highly pro-inflammatory cytokine was induced at high levels when chondrocytes were treated with RA-synovial fluids, and thus is highly involved in the inflammatory processes in RA. But—at much lower levels—MCP-1 was induced during incubation of chondrocytes with OA-synovial fluids. This result also points towards an inflammatory pathogenesis of OA. In contrast, the concentration of G-CSF only increased during incubation of chondrocytes with synovial fluids of RA, but not OA patients, supporting the differences between the both diseases.

Clinical signs of inflammation of OA, like joint stiffness or painful mobility, are partly similar to RA. Significantly increased levels of various cytokines in synovial fluids of RA, and surprisingly of OA, patients were shown. Activation of pro-inflammatory cytokines of human chondrocytes by synovial fluids of OA patient supports a pro-inflammatory process in the pathogenesis of OA.

Nonetheless, future experiments in which we will block the cytokines found to be involved in OA will help us to define possible key players steering the inflammatory process. Furthermore, in future experiments, we will not only evaluate the effects of the local microenvironment in OA on chondrocytes, but we will also consider the crosstalk of immune cells and chondrocytes in co-culture experiments. Next, anti-inflammatory agents like anti-IL-6 or anti-IL-8 will be tested in animal models of OA.

In summary, based on our results, we hypothesize that anti-inflammatory therapy—which takes place via therapy with nonsteroidal anti-inflammatory drugs (NSAIDs)—could not only treat the symptoms of OA, but the patients could also benefit via the interference with the pathogenesis. Perhaps OA patients should not only be treated for relief of symptoms, but should be treated more aggressively to interfere early on with the pathogenic pathways. Furthermore, our results could also explain the successful application of the mildly immunomodulatory therapy with hydroxychloroquine, and this well-tolerated drug should be used more frequently in the treatment of OA.

Disclosure statement The authors have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the results and views presented in this article.

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