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Fibroblast-like synoviocytes from fluid and synovial membrane from primary osteoarthritis demonstrate similar production of interleukin 6, and metalloproteinases 1 and 3

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ABSTRACT

Objective. *The objective of the study was to compare the production of metalloproteinases (MMP)-1, -3 and interleukin (IL)-6 by fibroblast-like synoviocytes (FLS) derived from synovial fluid (FD-FLS), and FLS derived from synovial tissue (TD-FLS) of patients with primary osteoarthritis (OA). The more accessible FD-FLS could facilitate the study of the role of these cells in OA pathophysiology.*

Methods. *MMP-1, MMP-3, and IL-6 levels were measured in the supernatant culture at baseline and 22 hours after stimulation with TNF-α and IL-1 β.*

Results. *There was no difference at baseline between MMP-1, MMP-3 and IL-6 production by FD-FLS and TD-FLS. Analogous to baseline, stimulation of FD-FLS and TD-FLS with IL-1β and TNF-α did not result in difference on MMP-3 and IL-6 production. However, TD-FLS produced more MMP-1 than FD-FLS after stimulation with IL-1β (p=0.01). Additionally, there was a positive correlation for production of MMP-1, MMP-3 and IL-6 between FD-FLS and TD-FLS (r=0.40 and p<0.0008; r=0.66 and p<0.0001; r=0.76 and p<0.0001, respectively). Supporting this statistical significant positive correlation, the Bland-Altman plotting, showed a homogeneous distribution of the values and low mean disagreement rates between all results of FD-FLS and TD-FLS (23.1%, 56.8% and 48.1%, respectively).*

Conclusion. *Our data demonstrated functional similarity between FD-FLS and TD-FLS and support the use of a more accessible source of FLS for the study of the pathogenesis of joint destruction and therapeutic targets in primary OA.*

Introduction

Osteoarthritis (OA) is the most common joint disease, but its pathogenic process of cartilage destruction remains largely unknown (1). Biomechanical factors, pro-inflammatory mediators, and proteases determine the damage of cartilage and subchondral bone tissues in OA. The synovial membrane becomes inflamed and the cartilage is degraded by catabolic enzymes. Synovial membrane and the synovial fluid show an increase in inflammatory cells population and cytokines (2). Fibroblast-like synoviocytes (FLS) actively participate in the synovitis-structural damage cycle of OA through the production of inflammatory cytokines, such as interleukin-6 (IL-6), and cartilage-degrading enzymes, such as metalloproteinases (MMPs) (3). Of special interest, interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) have been shown to be involved in the initiation and progression of articular cartilage destruction. The capability of IL-1 β and TNF-α to promote cartilage degradation is associated to their ability to promote the synthesis and release of MMPs and to suppress proteoglycan and collagen synthesis by human synovial cells and chondrocytes (4, 5). *In vitro* studies using FLS cultures from patients with joint diseases represent a basic model for translational research but there is a substantial difficulty in obtaining synovial tissue-derived FLS (TD-FLS) in patients with primary OA. It is debatable if synovial fluid-derived FLS (FD-FLS) can replace TD-FLS as a cell source for experiments addressing this lineage. There are few studies comparing functionality of FD-FLS and TD-FLS of patients with OA, but almost no studies that have utilised cells from fluid and tissue from the same patient.

The aim of this study was to compare, baseline and after stimulation with IL-1β and TNF-α, the production of MMP-1, MMP-3 and IL-6 by TD-FLS and FD-FLS from patients with primary osteoarthritis.

Materials and methods

Sample collection and cell culture

Liquid and synovial tissue samples from the same patients with primary knee osteoarthritis classified according to the 1986 American College of Rheumatology criteria (6) (n=4) were collected during their total knee arthroplasty. The mean age of patients was 65 years (range 55–79 years). Samples of synovial fluid were centrifuged at 1200 rpm for 15 minutes, the supernatant discarded and the pellet seeded into cell culture flasks with Dulbecco's

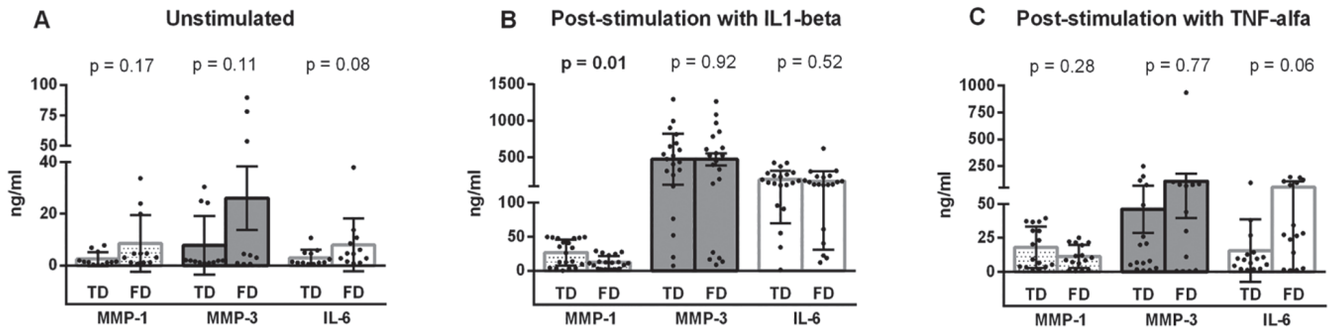

Fig. 1. Production of metalloproteinases (MMP)-1, -3 (and interleukin (IL)-6 without stimulation (**A**) and after stimulation with IL-1β (**B**) and TNF-α (**C**) by fibroblast-like synoviocytes from tissue derived (TD) and synovial fluid derived (FD) of patients with osteoarthritis (n=4).

Modified Eagle Medium (DMEM, Gibco, Life Technologies, USA), plus 10% fetal bovine serum (Gibco, Life Technologies, USA), 1% streptomycin/penicillin/amphotericin B solution (Gibco, Life Technologies, USA) and 1% non-essential amino acids (Gibco, Life Technologies, USA). Tissue samples were separated from adjacent cartilaginous and adipose structures, cut into 2 mm thick pieces and incubated with 0.1% collagenase type IV (Gibco, Life Technologies, USA) in overnight DMEM medium with shaking of 100 rpm at 37°C. At the end of the collagenase digestion, the material was centrifuged and the pellet transferred to culture bottle with the same culture medium described above for liquid samples.

Cultures were maintained in an oven at 37°C and 5% CO_2 with exchange of the culture medium every three days until freezing of appropriate passages. For all experiments, cultures were used in the 9th passage (P9).

All patients enrolled in the study signed a free and informed consent form, approved by the local Research Ethics Committee (ETIC 0637.0.203.00-10), prior to any study procedure.

Stimuli and dosages

The cells were transferred to 24-well plates (150,000 cells / well) and maintained for 24 hours immersed in DMEM medium enriched with 1% fetal bovine serum and 1% streptomycin/penicillin/amphotericin B solution and kept in the 37°C incubator and 5% CO 2 to be stimulated (and non-stimulated) with TNF-α at concentrations 1; 5; 10 and 50 ng/ml and IL-1β at concentrations 0.1; 0.2; 0.3; 0.5; 1 ng/ml. After 22 hours of stimulation with these agonists, the supernatant was harvested for the detection of IL-6, MMP 1 and 3 by ELISA (human MMP-3 DuoSet® - R&D systems, USA, human total MMP-1 DuoSet®- R&D systems, USA and human IL-6 DuoSet® - R&D systems, USA) following the manufacturer's protocols.

Immunophenotyping

For human FLS, monoclonal antibodies, including anti-human CD14 PerCP-Cyanine 5.5, anti-human CD45 V450, anti-human CD90 PE, isotype control antibodies labelled with PE, V450 and PerCP (all human monoclonal antibodies are from BD Biosciences), and a non-labelled cell control, were included in all experiments. Cells were stained with these antibodies for 20 min at 4°C in dark, washed, resuspended in PBS and analysed by a flow cytometer in FACS Canto II (BD Biosciences). At least 100,000 events were used for each sample, and the acquisition was processed using the software Diva (BD Biosciences). Data were analysed with Flowjo software (Tree Star).

Statistical analysis

It was used the paired *t*-test, the Spearman correlation and the Bland Altman concordance analysis, for the comparisons between the concentrations of MMP-1, MMP-3 and IL-6 measured in the culture supernatant of FLS derived from synovial fluid and synovial tissue. GraphPad Prism v. 6.01 was used for data analysis and graph production. It was considered evidence of significance, effects with *p*-values <0.05.

Results

The cell immunophenotyping showed that OAFLS cells from synovial fluid and from synovial membrane were anti-CD90 positive. The anti-CD45, expressed on leukocytes, and anti-CD14, expressed on monocytes and macrophages, did not mark the FLS. The positivity for the CD90 marker confirmed the FLS origin and cell culture pureness (data not shown).

Figure 1 shows production of MMP-1, MMP-3 and IL-6 by TD- and FD-FLS at baseline and after stimulation with IL-1β and TNF-α. At baseline, TD- and FD-FLS constitutively produced metalloproteinases and IL-6 with no statistical difference between samples (Fig. 1A). Stimulation of TD- and FD-FLS with IL-1β (Fig. 1B) and TNF-α (Fig. 1C) resulted an increase release of MMP-1, -3 and IL-6. When production of metalloproteinases and IL-6 by TD- and FD-FLS were compared, both at baseline and after pro-inflammatory stimulation, there was no statistical difference between cells, except for MMP-1 after stimulation with IL-1β (p=0.01). Additionally, for all three products (MMP-1, MMP-3 and IL-6) we found a positive correlation between FD-FLS and TD-FLS values (r=0.40 and p<0.0008; r=0.66 and p<0.0001; r=0.76 and p<0.0001, respectively) (Fig. 2). Using the Bland-Altman plotting, we found a homogeneous distribution of the values and low mean disagreement rates between all the observations of TD- and FD-FLS (23.1%, 56.8% and 48.1%, respectively) (Fig. 2).

Discussion

Recent advances in management of

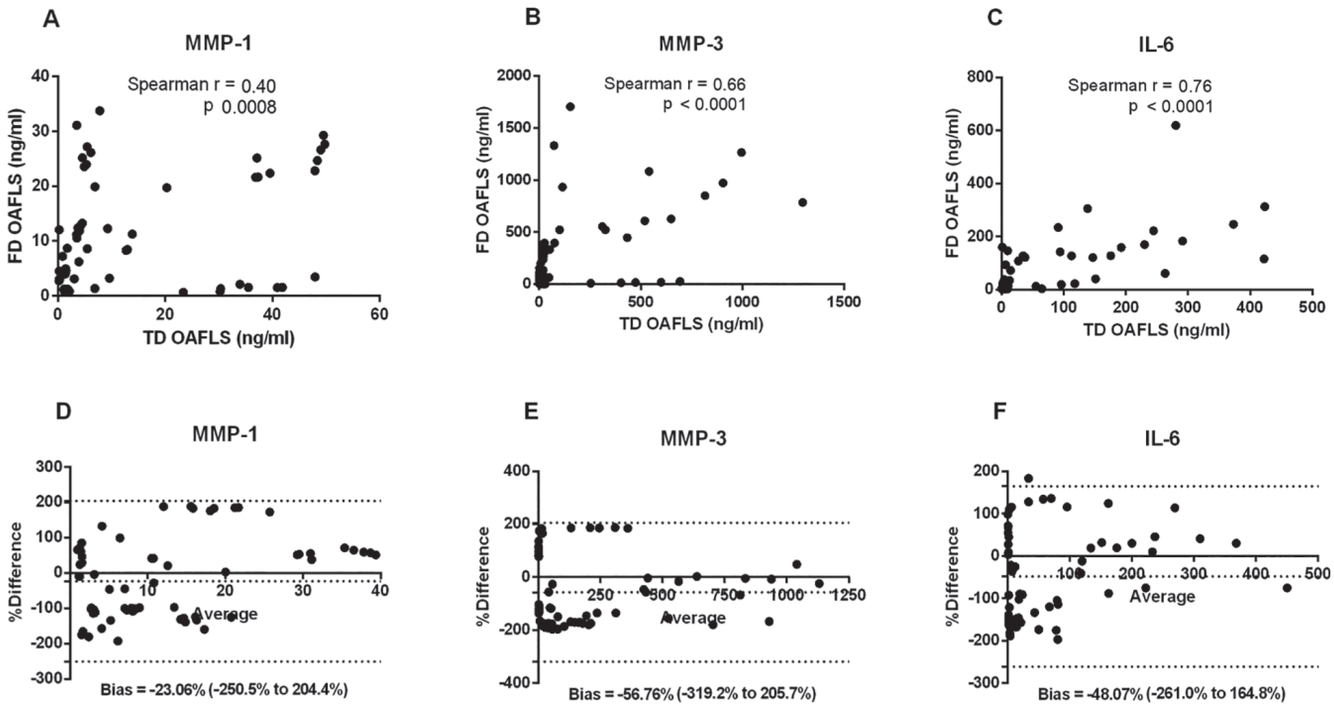

Fig. 2. Correlations between concentrations of MMP-1 (**A**), MMP-3 (**B**), and IL-6 (**C**) in supernatant of fibroblast-like synoviocytes cultures from synovial fluid-derived (FD) and tissue derived (TD) of patients with osteoarthritis (OAFLS) (stimulated and non-stimulated). Bland-Altman plot for percentage of discordance (Bias) between supernatant concentrations of MMP-1 (**D**), MMP-3 (**E**) and IL-6 (**F**) on fibroblast-like synoviocytes cultures from synovial tissue and fluid of osteoarthritis patients.
MMP-1: metalloproteinase-1; MMP-3: metalloproteinase-3; IL-6: interleukin-6.

patients with OA have resulted in reduced access by investigators to synovial tissue. As a result, there is a substantial difficulty in obtaining TD-FLS from patients with OA. Thus, a more accessible source of synovial cells than the tissue from replaced joint may increase the availability of FLS to perform *in vitro* studies. Additionally, it is more convenient to obtain cells at different times (before and after treatment) from synovial fluid from the same patient with rheumatic disease through synovial joint effusion approach.

In the present study, we have shown similar responses of FLS from tissue and fluid at baseline and after stimulation with IL-1β and TNF-α. Also, our results show that IL-6, MMP-1 and MMP-3 are produced constitutively by FD-FLS and TD-FLS and their synthesis is up-regulated by IL-1β and TNF-α. IL-1β and TNF-α are considered important players in OA because they stimulate the synthesis and secretion of many proteases and MMPs (7). IL-1β is related to the destruction of cartilage, and TNF-α is associated to induction of downstream inflammatory cascade. These two cytokines induce the production of a number of inflammatory diseases and catabolic factors (8).

MMP-1 and MMP-3 are expressed by TD-FLS and production of these enzymes increases markedly when exposed to these pro-inflammatory cytokines (9). Previous studies demonstrated that both tissue and fluid synovial fibroblasts secrete metalloproteinases, which it is most likely to be important in the process of cartilage degradation (7, 10). They found consistent secretion of MMP-1 and MMP-3 in all synovial fibroblast cultures with greater increased levels after cytokine stimulation (8). MMP-3 levels are found increased in OA synovial fluids (7) and the significant concentrations of MMP-3 released at baseline and after stimulation in our study and theirs indicate a synovia inflammation as a source of this protease (7). In contrast to our study, some differences were found in production of MMP-1 and -3 after stimulation with IL1-β and TNF-α, but with no difference at MMP-3 baseline levels (7). Although we observed a difference of MMP-1 production after stimulation with IL-1β, we still believe these cells are similar, due the Spearman correlation and Bland Altman analysis have shown similarity. Other studies with fluid and tissue-derived FLS from rheumatoid arthritis patients showed similar production of MMP-1 and -3 after IL-1β stimulation (10), however they did not utilised cells from the same patient. For comparison, we believe to be important methodologically to use cells from tissue and fluid-derived FLS from the same patient as in our study.

Local levels of pro-inflammatory cytokines such as IL-6 produced by FLS are detectable in OA (11, 12). The production of IL-6 in the tissues of the inflamed joint is usually in response to IL-1β and TNF-α and is mainly released by FLS and macrophages (13, 14). In accordance to our data, one study reported that levels of IL-6 expression are identical for TD-FLS and FD-FLS from different patients with rheumatoid arthritis when stimulated by TNF-α (15).

Conclusion

Considering our findings, FD-FLS and TD-FLS produce MMPs and IL-6

at baseline and after stimulation, that MMPs have the potential to degrade cartilage matrix components which provides strong evidence for a major impact of synovial MMPs in the pathophysiology of OA. The expression pattern of MMP-1, -3 and IL-6 in FD-FLS seems to be equivalent to that of TD-FLS and may replace tissue-derived synovial cells as a source to perform *in vitro* studies and test potential new pharmacological molecules for primary osteoarthritis.

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