

Activation of synovial fibroblasts with osteoarthritic synovial membrane and Hoffa's fat pad in comparison with synthetic cytokines

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Abstract Osteoarthritis (OA) belongs to the most common degenerative joint diseases in adults. Nowadays, there are mostly symptomatic treatments and therefore, is necessary to create an appropriate in vitro OA model to analyse new therapeutic options. In this study, we used synovial fibroblasts (SFs) isolated from synovial membrane (SM) and for their stimulation we used cytokines IFN γ , TNF α + IL-1 β or OA tissues (SM, Hoffa's fat pad (HFP), cartilage). We analysed influence of inflammatory agents on SFs proliferation, morphology and secretion. Significant results in proliferation rate were observed in groups stimulated with IFN γ and HFP. In groups stimulated with HFP, SM and TNF α + IL-1 β were detected higher levels of inflammatory cytokines IL-6, IL-8, MCP1, GRO. These findings suggest that stimulation of SFs with OA tissues is comparable with cytokines and could be used for future OA research.

Keywords osteoarthritis, synovial fibroblasts, in vitro model

1. INTRODUCTION

Osteoarthritis (OA) belongs to the most common degenerative joint diseases in adult population, nowadays considered as a whole joint disease. In the present, there are mostly symptomatic treatments, therefore research community is trying to find a new approaches to treat this disease against conventional treatment. Mesenchymal stem

cells or their extracellular vesicles as a „cell free“ therapy have a good properties and potential to be used for OA treatment. To analyse this therapeutical effect it is necessary to create an appropriate in vitro model of OA.

OA as a degenerative joint disease, affect articular cartilage, subchondral bone, ligaments, capsule and synovial membrane (Martel-Pelletier, et al. 2016). Synovial membrane is one of the main structure which influence progression of OA significantly. With the content of metabolically highly active cells play an important role in knee physiology via synovial fluid (Sellam & Berenbaum, 2010). Inflammation of synovial membrane (synovitis) lead to cartilage degradation by releasing proinflammatory cytokines and pro-matrix metalloproteinases. Inflammatory cytokines are critical mediators involved in catabolism of joint tissues present in OA patients (Chevalier, et al. 2013). Tumor necrosis factor α (TNF α) and interleukin 1 β (IL-1 β) are key cytokines involved in inflammation present in OA. These cytokines induce the production of inflammatory and catabolic factors. It seems that IL-1 β promote cartilage destruction and TNF α drive the inflammatory cascade (Kapoor, et al. 2010).

1.1 Synovial membrane in OA

The synovial membrane is a layer of cells that lines the joint cavity and regulate transfer of molecules between joint and surrounding tissues. Synovial membrane is responsible for the maintenance of

synovial fluid composition by releasing nutrients and lubricant factors (mainly lubricin, hyaluronic acid) and for the maintenance of fluid volume. Synovial membrane is also involved in chondrocyte nutrition via synovial fluid (Scanzello & Goldring, 2012) (Martel-Pelletier, et al. 2016).

Common feature of OA is synovial membrane inflammation characterized by hyperplasia and infiltration of membrane with T and B lymphocytes and macrophages (Scanzello & Goldring, 2012) (Nakamura, et al. 1999). In OA synovium the formation of new vessels is present which may together with inflammation affect disease progression and pain (Walsh, et al. 2007). OA synovium is involved in progression of disease by releasing proteases like MMP-3, disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs), and cytokines IL-1 β and TNF α , that causing the cartilage matrix damage (Sellam & Berenbaum, 2010).

1.2 Proinflammatory cytokines in OA

Inflammatory cytokines are compounds participating in the OA pathogenesis by influencing cells in joint tissues through cellular pathways. The most described are IL-1 β , TNF α , IL-6, IL-15, IL-17, and IL-18 (Wojdasiewicz, et al. 2014). IL-1 β and TNF α induce the production of inflammatory and catabolic factors that confirm their position as a key molecules of OA pathophysiology (Kapoor, et al. 2010). They have synergistic effect and combination of them have a stronger impact on cartilage destruction, demonstrated in vivo (Henderson & Pettipher, 1989). They are secreted by the same joint cells and their levels are elevated in the same joint elements.

IL-1 β activate cells by binding membrane receptor and through molecular pathways activate transcription factors results in the expression of genes coding cytokines, chemokines, adhesion molecules, inflammatory mediators, and enzymes (Wojdasiewicz, et al. 2014) (Roman-Blas & Jimenez, 2006). IL-1 β also influence the chondrocyte synthesis of extracellular matrix components and group of metalloproteinases (MMPs), which damage cartilage (Vincenti & Brinckerhoff, 2002). In the joint cells IL-1 β can induce self secretion or stimulate the synthesis of other cytokines (e.g. TNF α , IL-6, IL-8) (Aigner, et al. 2005) (Lotz, et al. 1992). TNF α activate the same group of intracellular signalling pathways like IL-1 β results in inflammation and catabolism increase in joint tissues (Roman-Blas & Jimenez, 2006).

2. METHODS

The work flow of our experiments is indicated on Figure 1.

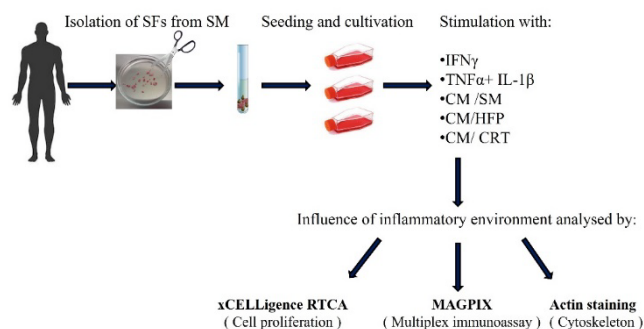


Figure 1 Scheme of the study design

2.1 Samples collection

For simulating OA environment, we used conditioned media (CM) obtained from tissues of OA patients undergoing total knee arthroplasty or inflammatory cytokines. Synovial fibroblasts (SFs) were isolated from synovial membrane and cultured in vitro. All tissues were collected with the approval of the local ethical committee with obtained informed consent from patients.

2.2 Synovial fibroblasts isolation

Synovial fibroblasts (SFs) were isolated from synovial membrane by enzymatic digestion with Collagenase type II (Gibco™) overnight. Synovial fibroblasts were then seeded and cultivated in vitro with cultivation media (1% Antibiotic Antimycotic Solution (Sigma-Aldrich®), 10% Fetal bovine serum (Sigma-Aldrich®), MEM α , nucleosides, GlutaMAX™ Supplement (Gibco™) and DMEM, high glucose (Gibco™) half-and-half) in condition of 5% CO₂ and 37°C.

2.3 Conditioned media collection from OA tissues

Tissues obtained from 5 OA patients undergoing total knee arthroplasty were washed using Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich®) and cut into small pieces. Then the weight of tissue pieces were measured and calculated amount of media according to sample (1g tissue /~7 ml media). Dulbecco's Modified Eagle's Medium - high glucose without phenol red (Sigma-Aldrich®) was then added to samples and incubated for 24 hours in condition of 5% CO₂ and 37°C.

2.4 Cell proliferation measurement (xCELLigence RTCA system)

Synovial fibroblasts were seeded on 96-well xCELLigence RTCA E-plate (7000 cells/ well) and cultivated in vitro with cultivation media (described above). When cells adhere on well surface, inflammatory environment was simulated by adding cytokines (IFN γ , TNF α + IL-1 β) in concentration 10ng/ml or CM obtained from OA tissues (synovial membrane, Hoffa's fat pad (HFP), cartilage) in 50% concentration to cells. Influence of inflammatory environment on cells proliferation was measured in real time by xCELLigence system. xCELLigence RTCA software and GraphPad were used for data analysis.

2.5 Actin staining of activated SFs

SFs were seeded on 6-well plate (10 000 cells/ well) and cultivated in vitro. When the cells reached 80% confluency, they were stimulated with cytokines (IFN γ , TNF α + IL-1 β) or with CM obtained from OA tissues (synovial membrane, Hoffa's fat pad (HFP), cartilage) within 24 hours. Changes in morphology of SFs after stimulation were determined by light microscopy (Leica DMI 3000B) and immunofluorescence detection of cytoskeleton. After inflammatory activation, cells were washed twice with PBS (Sigma Aldrich, Germany). Then the cells were fixed with 4% paraformaldehyde in PBS, washed with wash buffer (0.05% (v/v) Tween-20, Sigma Aldrich, Germany), permeabilized with 0.1% (v/v) Triton X-100 (Sigma Aldrich, Germany) and washed with wash buffer. Blocking solution 1% (v/v) BSA, Sigma Aldrich,

Germany) was added for 30 min. and then the cells were incubated with TRITC conjugated Phalloidin (Millipore, Burlington, MA, USA) for 30 min. and washed with PBS. Nuclei staining was performed by DAPI. Fluorescence images were visualized with Cytell Cell Imaging System (GE Healthcare, Life Sciences).

2.6 Protein release measured by multiplex immunoassay

Protein release in CM from stimulated SFs was evaluated by multiplex immunoassay. Concentrations of 11 biomarkers (EGF, Eotaxin, FGF-2, GRO/CXCL, IL-10, IL-1Ra, IL-8, IP-10, MCP-1, PDGF-AB/BB, RANTES) were measured using MILLIPLEX® Assays (Merck KGaA, Germany) according to the manufacturer's protocol and the MAGPIX Luminex platform. xPONENT software and Bio-Plex Manager 6.1 were used for data analysis. Once standard curves were generated, concentrations were interpolated for each sample using a 5-parameter curve fit equation and expressed as pg/ml.

3. RESULTS AND DISCUSSION

Proliferation rate of activated synovial fibroblasts (SFs) was statistically significant in group with IFN γ against control after 24 hour (Fig.2A) incubation.

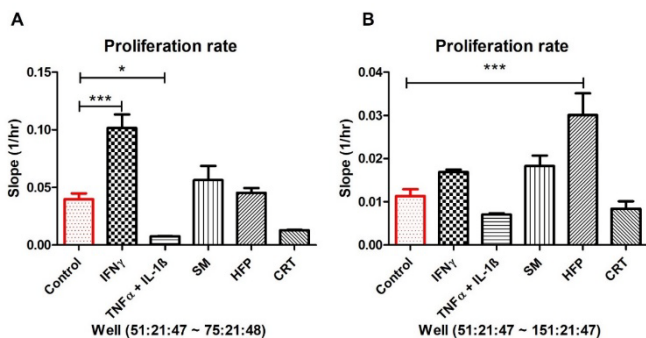


Figure 2 Proliferation rate of activated Synovial fibroblasts (A – 24hours, B – 100hours from adding cytokines / CM)

In the other hand, group stimulated with conditioned media from Hoffa's fat pad showed statistically significant changes in proliferation rate against control in longer cultivation (Fig.2B, Fig.3). These findings suggest, that HFP could promote synovial fibroblasts proliferation and synovial membrane hypertrophy in knee OA, but molecular mechanism involved in OA initiation and progression is still unclear. Hoffa's fat pad or infrapatellar fat pad is adipose tissue located extrasynovially and intracapsularly in the knee (Clockaerts, et al. 2010). TNF α and IL-1 β are proinflammatory cytokines involved in pathophysiology of OA, (Kapoor, et al. 2010) but they had just low influence on synovial fibroblasts proliferation. In this group, statistically significant changes in proliferation rate were observed 24 hours after adding and they had opposite effect against IFN γ (Fig.2A).

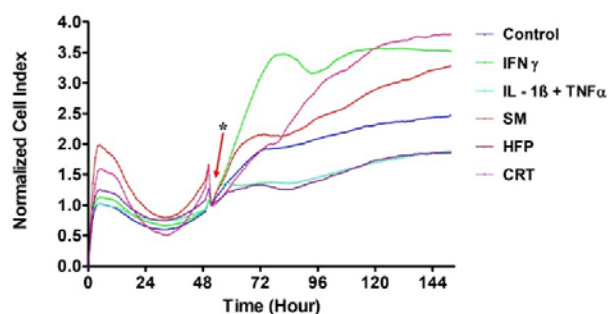


Figure 3 Proliferation curves of activated synovial fibroblasts (* Adding Cytokines / CM)

From 11 biomarkers, we measured the main changes in 4 of them against control, namely IL-6, IL-8, MCP-1 (Monocyte Chemoattractant Protein-1), GRO (Growth-Related Oncogene) (Fig. 4). We detected the highest levels of these cytokines in group stimulated with conditioned media from synovial membrane. Lisignoli et al. shows that MCP-1 is released by OA osteoblasts in response to stimulation with inflammatory cytokines such as IL-1 β , TNF- α and IFN γ (Lisignoli, et al. 2002), but in our results very low or no levels of inflammatory markers were measured in group stimulated with IFN γ . MCP-1 is chemokine attracting monocytes, that lead to accumulation of them and secretion of cell products to facilitate immune response in OA. At the end this lead to clinical symptoms (Hampel, et al. 2013). Cytokine IFN γ did not have influence on inflammatory proteins secretion, therefore using this cytokine is not appropriate for SFs activation in our conditions. In contrast, combination of TNF α and IL-1 β promote proteins release and we observed similarity between protein levels measured in this group and group stimulated with conditioned media from Hoffa's fat pad, but there was difference in proliferation rate (Fig. 2). Here, we did not detected correlation between proliferation rate and secretion of proteins by using these methods. In the other hand, synovial fibroblasts influenced by conditioned media from OA cartilage did not release inflammatory cytokines, seem to cartilage, did not promote inflammation of synovial membrane in OA knee but defend itself.

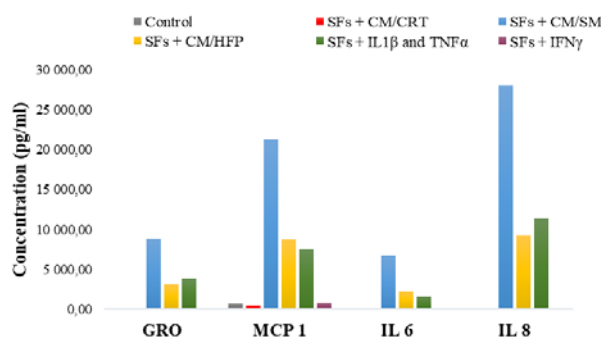


Figure 4 Levels of inflammatory cytokines IL-6, IL-8, MCP1, GRO in CM obtained from influenced synovial fibroblasts

We did not detect the significant differences in cytoskeleton using Phalloidin staining, between all mentioned groups (Fig.5). The main changes in cytoskeleton were seen in cells activated with IFN γ or conditioned media from Hoffa's fat pad, the cells were bigger with spread microfilaments compared to control group. (Fig.6). It seems that, IFN γ influences the morphology of cells and promote proliferation of cells (Fig. 2A), but not inflammatory proteins release (Fig. 4).

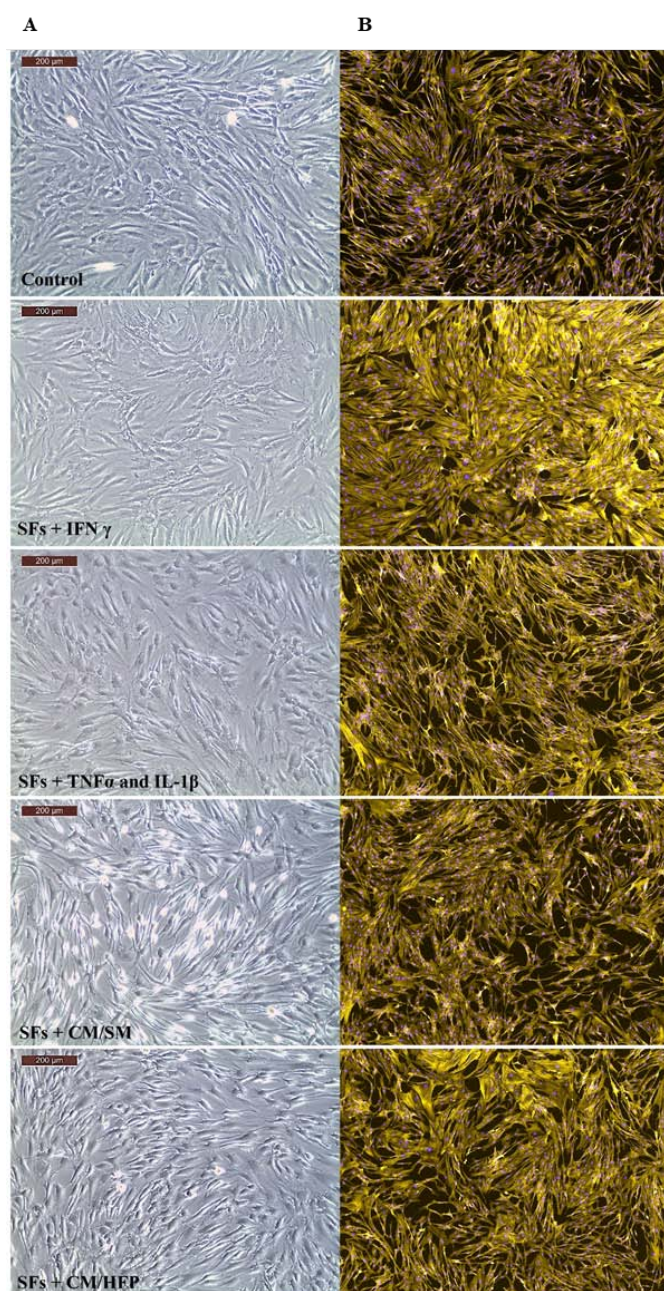


Figure 5 Influence on SFs morphology after stimulation of inflammatory environment. (A - Images from light microscopy (100x), B - Cytoskeleton stained with Phalloidin – yellow (100x)).

4. CONCLUSIONS

The aim of this study was to create an appropriate *in vitro* model of osteoarthritis and compare the impact of conditioned media from osteoarthritic tissues against to synthetic cytokines. In groups influenced with conditioned media from synovial membrane and Hoffa's fat pad and in group stimulated with cytokines TNF and IL-1 β higher levels of inflammatory proteins against control were observed. These stimulators of inflammatory environment could be used for next research of OA. Instead of IFN γ , which affected proliferation rate of synovial fibroblasts, but did not promote inflammatory protein release. We did not observed correlation between the influence on proliferation rate and protein release. We have found out that conditioned media from OA Hoffa's fat pad and

synovial membrane activated synovial fibroblasts and simulated inflammatory environment comparably with synthetic cytokines.

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