Effect of mesenchymal stem cells combined with chondroitin sulfate in osteoarthritis

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Abstract

Introduction: Osteoarthritis (OA) is a degenerative joint disease affecting the whole joint structure. Many authors have focused on the factors responsible for the development of inflammatory processes involved in OA. Adipose tissue-derived mesenchymal stem cells (ASCs) represent a promising alternative of cell-based therapy strategy in the treatment of OA which could be combined with any drugs. Chondroitin sulfate plays a protective role in the joint based on the decrease of pro-inflammatory cytokines, thus having an important role in the activation and inhibition of metabolic pathways in chondrocytes.

Aims: In this study, the effectiveness of chondroitin sulfate and ASCs in the treatment of knee OA have also been evaluated.

Materials: Cytokines and factors which are involved in OA as well as specific cartilage gene expression after adding ASCs and chondroitin sulfate have been discussed in detail.

Results: Our results show a decrease in the expression of all genes related to the pro-inflammatory cytokines analysed. Although there was no increase in the expression of the specific genes of the cartilage matrix, such as collagen type II and aggrecan.

Conclusions: This study shows the effectiveness of association of ASCs and chondroitin sulfate for the treatment of OA.

Introduction

Osteoarthritis (OA) is considered to be the fourth leading cause of disability by the year 2020 [1]. OA is a destructive joint disease causing degeneration of cartilage [2, 3]. Currently, inflammation is considered to be involved in the development and progression of OA in the early stages [4]. Emerging experimental evidence shows that secreted inflammatory factors such as pro-inflammatory cytokines are critical mediators of the disturbed metabolism and enhanced OA catabolism [5]. These cytokines mediate cartilage destruction through the upregulation of inflammatory or catabolic genes and the downregulation of anti-inflammatory or anabolic genes in articular chondrocytes [6]. In particular, IL-1 reduces the expression of type II collagen (Col2A1) [7] and increases the production of matrix metalloproteinases (MMPs) [8, 9], prostaglandin E₂ (PGE₂), cytokines, chemokines, reactive oxygen species, and nitric oxide (NO) [10, 11]. These substances enhance the catabolic activity of the chondrocytes and cause the destruction of the cartilage matrix.

Chondroitin sulfate (CS) is a major component in the extracellular matrix in many connective tissues [12, 13]. Commonly referred to “symptomatic slow-acting drug in OA” (SySADOA), CS is extensively used in the management of OA patients [14]. *In vitro*, CS has been shown to have anti-inflammatory and anti-catabolic properties on chondrocytes [15] and to be a structure/disease modifying anti-osteoarthritis drug (S/DMOAD) [16–18].

Cellular therapies for treating early to late stage OA have been thoroughly researched into for over two decades. Tissue engineering using stem cells emerged as an alternative method for treating OA over the last 10 years [19, 20]. In this way, mesenchymal stem or stromal cells (MSCs) have been extensively researched mainly for their regenerative potential [21–23]. Interestingly enough, stem cells are capable of secreting a wide range of trophic mediators which can exert paracrine effects on other cell types. Thus adipose tissue-derived MSCs (ASCs) are an interesting alternative MSCs source (instead of bone marrow stem cells, BMSCs) which can be easily collected using liposuction [24, 25].

The injected or infused MSCs based on two activities: immunomodulation and trophic activities. The immunomodulation of these cells has been shown to be mediated by both secreted bioactive molecules and by cell-cell contact and could involve suppression of T-cell proliferation in response to alloantigens or mitogens, inhibition of B cell proliferation, as well as dendritic cell maturation, and promoting the generation of T-regulatory cells [26]. Some of those cytokines and factors are transforming growth factor beta (TGF- β), indolamine 2,3-dioxygenase (IDO), interleukin 6 (IL-6) which are involved in MSCs immunomodulation [27] as well as others involved in the renewal of the extracellular matrix, such as type II collagen [28, 29].

The aim of this work is to study the effect of ASCs and CS on inflammatory mediators and proteolytic enzymes induced by TNF and related to cartilage catabolism.

Materials And Methods

Ethical Disclosure

The authors state that the experimental procedures developed in this work were approved by the Medical Committee of the University Hospital of León. Written consent was obtained from all patients following to the Helsinki Declaration of 1975, as revised in 2008.

Materials

In this study, human cells were obtained from three patients with symptoms of OA (n= 3 donors; a 74-years-old male and two females aged 67 and 55). Chondrocytes were obtained from femoral cartilage. ASCs were obtained from Hoffa adipose tissue.

Methods

Isolation and Culture of ASCs and Chondrocytes

ASCs were isolated from adipose tissue obtained from the knee fat deposit region, also known as the Hoffa fat pad [30]. Cells were collected and plated in 25cm² culture flasks (IWAKI®).

Cartilage was isolated from femoral biopsies samples. Pieces were cut into small fragments and incubated in 0.25% trypsin solution (Sigma-Aldrich®) for 30 min at 37°C and 5% CO₂. After centrifugation,

samples were incubated with 0.025% collagenase II (Sigma-Aldrich®) for 8h at 37°C. Cells were resuspended in culture medium comprising of DMEM (Sigma-Aldrich®) supplemented with 10% fetal bovine serum (FBS) (Gibco®) and 1% antibiotic-antimycotic solution (Gibco®) at 37°C in 5% CO₂, 90% humidity, and medium was renewed every 2-3 days.

ASCs–Chondrocytes co-cultures

ASCs and chondrocytes were co-cultured (ratio 1:1) in a 6-well plate. After 24h when they reached confluence, medium was removed and DMEM without phenol red, TNF (10 µg/ml) and/or CS (200 ng/mL) (Bioibérica®) was added as shown in Table 1. Cells and media were collected to analyze effect on inflammation.

Characterization of ASCs

Flow Cytometry Analysis

In order to confirm the identity of ASCs, we determined the expression of different surface markers: mouse anti-CD73, anti-CD90 and anti-CD105 (1:100) (Abcam®). Cells were stained with streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®). About 10⁴ events (minimum) were used for fluorescence capture.

Confocal Characterization

Cells were sub-cultured on 8-well Nunc Lab-Tek Chamber Slide System® (2×10³ cells/well). Cells were fixed with 2% paraformaldehyde for 15 minutes prior to incubation with primary mouse anti-CD73, anti-CD90 and anti-CD105 antibodies (1:100) (Abcam®) overnight at 4°C, and treated with secondary biotinylated antimouse antibodies (1:100) (Abcam®). They were then stained with streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®). Finally, chamber slides were mounted using Vectashield mounting medium containing DAPI.

ASCs differentiation

Isolated ASCs were cultured under conditions conducive to adipogenesis, osteogenesis and chondrogenesis to be assessed for multi-potentiality. After 15 days the cultured cells in adipogenic medium were stained with Oil Red O. ASCs were cultured under osteogenic culture conditions for 15 days. After induction, confirmation of osteogenesis was achieved by alizarin red staining. Finally, cell cultures were incubated under chondrogenic medium. Three weeks after induction confirmation of chondrogenesis was achieved through alcian blue staining and confocal microscopy using anti-Col2a1 (Abcam®) (1:100).

Fluorescence microscope proliferation assay

Density of 1×10^6 ASCs and chondrocytes were labeled with 5 μ M of CellTrace[®] Violet proliferation tracking dye and CellTrace[®] CFSE dye (Green) (Invitrogen[®]). Cell proliferation was analyzed at 24 and 36h. Two-dimensional images were digitally recorded for each surface at sample center as single topographical location with Nikon Eclipse TE2000-U inverted microscope (Nikon[®]).

Flow cytometry proliferation assay

1×10^5 cells were seeded in 24-well plate and were analyzed at 12, 24 and 36h. Cell fluorescence of cells stained with CellTrace CFSE dye (Green) and Violet Cell Proliferation Kit (Invitrogen[®]) was determined using a Beckman Coulter CyAn[®] ADP Flow Cytometer (Dako[®]) counting at least 1×10^4 events per sample. Excitation wavelength was 488 and 405 nm, and emission wavelength was 630 and 450 nm for green and violet stained cells, respectively.

Quantitative Real-Time PCR

Total RNA was extracted using the GeneMatrix Universal RNA Purification Kit (EurX[®]). Reverse transcription was accomplished on 1 μ g of total RNA using MultiScribe[®] RT (Applied Biosystems[®]) following the manufacturer's instructions of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems[®]). Gene expression of IL-6, TFG- β , IDO, MMP-13, COL2A1, iNOS and TNF were determined using qRT-PCR. Assays were carried out using Step One Plus RT-PCR (Applied Biosystems[®]) in a total volume of 25 μ l containing 0.7 μ l DNA template, 1X SYBR[®] Green (EURx[®]), 400nM ROX and 0.30U uracil-*N*-glycosylase (UNG) master mix, and 300nM of each primer.

Relative quantification was carried out normalizing to the housekeeping gene ACT- β . Primers were designed using OLIGO7[®] primer design tool (Table 2) which were provided by Integrated DNA Technologies (Coralville[®]).

ELISA

Chondrocytes and ASCs were stimulated with TNF (10 μ g/mL) for 12h. Concentration of prostaglandin E₂ (PGE₂) was measured by a specific ELISA with a goat anti-Mouse IgG microtiter plate, following the manufacturing instructions (Enzo Life Sciences Inc[®]). Measurements were carried out using a reader set Multiskan[®] GO Microplate Spectrophotometer (Thermo Fisher Scientific[®]) at 450 nm. Concentrations were calculated by comparing them to known standards.

Statistical Analysis

Each result from the study was expressed at the mean $\pm$ SD and was carried out with 3 experimental replicates. Statistical analysis was performed using IBM[®] SPSS[®] Statistics. Significant differences among groups were determined using ANOVA followed by post-hoc analysis for multiple group

comparisons or Student t-test for two group comparisons. Results with $p < 0.05$ were considered statistically significant.

Results

ASCs characterization

ASCs from infrapatellar Hoffa fat were characterized at passages 2 and 3. ASCs are adherent cells of fibroblastic morphology. With regard to cell pattern, it took 7–9 days to reach confluence in T75 culture flask, once they had been extracted.

Flow cytometric characterization showed a high expression of CD105 (endoglin), CD73 (ecto-5'-nucleotidase) and CD90 (Thy1). The fluorescence microscopy analysis reveals that cells displayed positive results for the surface markers CD90, CD73 and CD 105, as was expected. The percentages of positive cell markers and their histograms are shown in Fig. 1A-C.

The tri-lineage differentiation potential of the ASCs was confirmed

With regard to ASCs differentiation, after 15 days cells had differentiated towards osteocytes, chondrocytes and adipocytes. Spherical nodules were observed in chondrogenic cultures and deposits of acid mucopolysaccharides were confirmed with alcian blue staining. Similarly, under osteogenic induction conditions, dark ECM deposits have been detected after the induction period and mineralized calcium deposits stained orange-red with alizarin red S. ASCs, which underwent adipogenic differentiation, were characterized by an accumulation of cytoplasmic triglycerides and were represented as lipid droplets by Oil Red O staining (Fig. 1D-G).

Cell proliferation in ASCs and chondrocytes co-cultures

In order to study the behavior of ASCs and chondrocytes in co-cultures, a proliferation analysis was also performed using fluorescence microscopy and flow cytometry. Cell proliferation was analyzed at 12, 24 and 36 hours with or without TNF and CS. ASCs were stained in violet and chondrocytes in green. The results obtained using confocal microscopy and flow cytometry are shown in Fig. 2. In all experimental conditions chondrocytes proliferation rates were higher than ASCs. The proliferation rate was not the same between both cell lines due to the larger size of ASCs.

The addition of 10 $\mu\text{g/mL}$ of inflammatory agent (TNF) slightly reduces the viability of chondrocytes, which becomes stable when CS is added. The addition of CS does not significantly affect the proliferation of any cell types since the cell viability remains stable (Fig. 2C).

CS is capable to reducing the concentration of PGE_2 in previously inflamed cells

We observed the effect of CS on the release of PGE₂ in different experimental conditions using ELISA. In cells which did not receive an inflammatory stimulus, PGE₂ concentration remained at very low basal levels. However, in TNF-stimulated cells the level of PGE₂ production increased considerably (Fig. 3).

In a detailed analysis, our data showed that PGE₂ production only decreased when chondrocytes were co-cultured with ASCs and CS was added. The addition of CS into stimulated chondrocytes co-cultured with ASCs significantly reduces PGE₂ production when it is compared with the same co-culture without CS. Thus, the promising treatment with a combination of ASCs and the CS causes the greatest decrease in prostaglandin E₂ concentration, and an abatement in its release of 28.26% was observed.

IL-6, iNOS, TNF, MMP-13, IDO and TGF- β expression in ASCs-chondrocytes co-cultures

We analyzed the expression of IL-6, iNOS, TNF and MMP-13 in co-cultures of chondrocytes and ASCs with CS added. In the different experimental conditions inflammation was induced with TNF. Results are given in Fig. 4. The expression of IL-6 and iNOS significantly increased ($p \leq 0.005$) in chondrocytes cultures stimulated with TNF. However, when CS was added IL-6 expression significantly reduced. IL-6 expression increased in ASCs cultures when TNF was added but less than in chondrocyte cultures. Nonetheless, its expression reduced when chondrocytes were co-cultured with ASCs and treated with CS.

When iNOS expression was examined in chondrocytes after stimulation with TNF, it was found that chondrocytes showed extremely high iNOS levels. The treatment of chondrocytes with CS and ASCs dramatically reduced iNOS gene expression (Fig. 4). When CS was added to ASCs and chondrocytes co-cultures stimulated with TNF, iNOS expression was lower, though not significantly lower.

With regard to TNF and MMP-13 the same effect was observed in both ASCs and chondrocytes in response to inflammatory cytokine exposure, which reduced when treatment with CS occurs (Fig. 4), reaching the same levels as non-stimulated cells.

TGF- β levels increased slightly when activated with TNF (for ASCs and chondrocytes) but values were very close to those in the chondrocytes control group. In ASCs-chondrocytes co-cultures stimulated with TNF and when CS was added TGF- β levels were reduced significantly (Fig. 4).

IDO was over-expressed in stimulated chondrocytes and ASCs, but also when the treatment is initiated with CS its expression increases but not significantly with very different values as given in the error bar in Fig. 4. Regarding this gene, no reliable conclusions can be extracted with regard to this gene.

Specific Chondrogenic Gene expression evolution

The expression of specific chondrogenic genes such as SOX-9, aggrecan (ACAN) and collagen type II (Col2a1) was analyzed under the different experimental conditions stimulated and non-stimulated with TNF (Fig. 5). We observed that levels of Col2a1 and ACAN increased significantly when CS was added to chondrocytes cultures without TNF. However, the expression of the three specific genes of

chondrogenesis did not significantly change when TNF and CS were added. The same effect was detected in the co-cultures where an increase in the gene expression of Col2a1; ACAN and SOX-9 was obtained, though not significantly.

Discussion

MSCs are an attractive alternative candidate to conventional treatment for regenerative therapies [31]. They have been suggested as a new cell source for OA treatment due to their capability of differentiating in chondrocytes and the paracrine effects of secreted bioactive substances as well as their immunomodulatory effects [32]. CS is the major GAG component of native cartilage tissue which could provide cues to stimulate cells so as to proliferate, migrate, differentiate and produce the ECM compounds [33]. Moreover, CS has been shown to have anti-inflammatory effects reducing the concentration of pro-inflammatory cytokines such as TNF [34, 35] and IL-1 β [36]. We hypothesized that the combination of ASCs with CS should be capable of enhancing the cartilage regeneration and diminishing the inflammation caused in OA. We evaluated the immunomodulatory effect of CS combined with ASCs in co-culture with inflamed chondrocytes. The expression of specific cartilage genes was also analysed.

Most previous studies which examined chondrocyte depletion during OA progression discovered that a variety of factors (including TNF) have been reported to induce a progressive cartilage joint degeneration in OA [37, 38]. In a proliferation assay we observed that the addition of inflammatory cytokine (TNF) slightly reduces the viability of chondrocytes, which is stable when CS is added. The addition of CS does not significantly affect the proliferation of any cell types since the cell viability remains very stable.

It has been reported that MSCs could be induced to express enhanced levels of IDO and PGE₂. ASCs are known to constitutively produce PGE₂ and this production significantly increases in co-cultures [39–41]. However, in our work PGE₂ concentration remained at very low base levels in cells which did not receive an inflammatory stimulus. According to other references, CS had no effect on the basal PGE₂ release [42]. The effect of TNF and IL-1, both in chondrocytes [43] and in MSCs [39] causes the increase of PGE₂ expression. Although PGE₂ is considered a pro-inflammatory cytokine, there exist different theories on the beneficial or detrimental effect which it produces on OA [44]. Ronca *et al.*, 1998 [45], showed that the effects of CS in the treatment of OA are due to various mechanisms of action which decrease the concentration of prostaglandin PGE₂ in the joint c[46, 47]. However, we observed that in TNF-stimulated cells the level of PGE₂ production increased considerably under all conditions. The amount of PGE₂ produced was significantly reduced, although it remained high compared to the non-stimulated controls under conditions of co-culture with CS and TNF in the culture medium. Currently, PGE₂ seem to be involved in the up-regulation of the anti-inflammatory cytokine interleukin (IL)10 while reducing the secretion of TNF [48, 49]. We can therefore speculate that the inhibition of PGE₂ production by CS could decrease the degradative effect in the OA deep zone cartilage. Lastly, CS is shown to inhibit the expression of enzymes involved in PGE₂ synthesis, COX-2 and mPGES-1 [50].

As will be discussed later on, ASCs reduce levels of certain pro-inflammatory cytokines whose production is associated with PGE₂. Therefore, CS and ASCs jointly manage to reduce OA processes through several different routes. Our results agree with those obtained by other authors. Cytokine IL-6 is responsible for pain in OA [50], being one of the main inducers of inflammation. MSCs reduced the levels of pro-inflammatory cytokines, including IL-6 [42]. With regard to PGE₂ analysis, it is known that it accelerates expression of pain-associated molecules such as IL-6 e iNOS [50], but MSC-derived PGE₂ always acts independently of IL-6 [40]. In our work, we observed that the expression of IL-6 significantly decreased in co-culture of ASCs and chondrocytes previously inflamed and with the addition of CS, in comparison with the inflamed chondrocytes without CS. Previous studies showed these effects in which MSCs and CS reduced the expression of pro-inflammatory cytokines, among which were IL-6 [51]. The combination of ASCs and CS resulted in a marked reduction in the expression of IL-6, although it was not as important as that produced solely by CS. Although it has been shown that IL-6 is one of the main interleukins which induce inflammation, its role is currently being debated due to evidence that this interleukin could have an anti-inflammatory role [52].

Contrary to what happened in the previous case for IL-6, the expression of iNOS underwent a greater reduction in the cultures of ASCs combined with CS as a treatment. Both CS and ASCs significantly reduced their expression, this reduction was more than 40 times lower with the use of CS and up to 50 times less when CS and ASCs were combined. When iNOS expression was examined in chondrocytes after stimulation with TNF, it was found that chondrocytes expressed extremely high iNOS levels, which agree with Charles *et al.*, 1993 [53]. However, the expression level in ASCs was minimal, as predicted by Ren *et al.*, 2009 [54, 55]. As in the previous cases, the treatment of chondrocytes with CS and ASCs dramatically reduced iNOS gene expression.

With regard to metalloproteinase, activated chondrocytes also produced MMP-1 and MMP-13. Increased expression of IL-6 is related to the production of enzymes from the MMPs group [48]. Nevertheless, their levels were reduced in inflamed chondrocytes treated with CS and ASCs, particularly MMP-13 production. Deletion of the MMP-13 gene attenuated articular cartilage degradation (it targets: type II collagen), and it has been shown that it is critical downstream target gene of TGF- β signaling during OA development [56]. It has recently been shown that global MMP-13 knockout could prevent articular cartilage erosion [57].

Both ASCs and inflamed chondrocytes showed high TNF expression, which decreased when treatment with CS occurred. Its levels may not have been as high as expected since PGE₂ prevents proliferation of TNF [27]. This molecule together with IL-1 β is considered to be a key inflammatory cytokine involved in the pathophysiological processes occurring in OA, and it affects blocking the chondrocytes synthesis of proteoglycan components, and Col2a1. Moreover, it is responsible for the increased production of iNOS and IL-6 [48].

TGF- β levels slightly increased when ASCs and chondrocytes were activated with TNF, although values were very close to those from the chondrocytes control group. In the presence of CS, levels were minimally reduced. Observations by Shen *et al.*, 2014 [56]. have shown that TGF- β inhibition signaling in

chondrocytes leads to chondrocyte terminal differentiation and development of OA, as this cytokine is responsible for stimulating the production of proteoglycans, Col2a1 and chondrogenesis. Other publications also confirm that the amount of TGF- β is low or even undetectable in patients with OA [48]. Lee *et al.*, 2014 [41], have shown that the expression of IDO was induced in MSCs after tissue damage. Accordingly, in other tissues damaged or stimulated with TNF, the production of IDO increased [27]. This agrees with our results, which, after inducing cell inflammation, increased the expression of IDO, although it also increased with the addition of CS and ASCs. Our results showed that IDO is probably expressed as a natural protector against inflammation as shown by other authors [57].

During the more advanced stages of OA the amount of Col2a1 and ACAN decreases by denaturation [58, 59], which was influenced by the increase in the expression of MMPs. Levels of type II collagen and aggrecan increased significantly when CS was added to chondrocytes in inflamed co-cultures. This raises questions as to the connection of Col2a1 and ACAN to the increase of proteoglycans thanks to the CS, key components of ECM. CS is widely distributed in matrix where it forms an essential component of proteoglycans by covalent links with proteins [42], aiding the regenerative process. Our results confirm the boosting effect of CS on some gene expression of cartilage extracellular matrix (ACAN, Col2a1). Moreover, the expression of these genes was always higher when the ASCs were present compared to the inflamed chondrocytes without CS. There were no significant differences in SOX9 gene expression, a critical factor for chondrocyte differentiation that facilitates the expression Col2a1 [60].

Conclusions

To sum up, we can conclude that CS in combination with ASCs provides very encouraging results in OA treatment by means of inhibition the synthesis of pro-inflammatory and degradative mediators known to exert a deleterious effect on the cartilage. Further research is needed regarding the molecular pathways controlling the functional behaviour of cartilage under both physiological and pathological conditions to develop more effective strategies for the treatment of OA and other cartilage-related diseases.

Abbreviations

ACT- β : Actin Beta

AT-MSCs: Adipose Tissue derived Mesenchymal Stem Cells

BM-MSCs: Bone Marrow derived Mesenchymal Stem Cells

Col2A1: type II Collagen

COX-2: cyclooxygenase-2

CS: Chondroitin Sulfate

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extracellular Matrix

ELISA: Enzyme-Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

GAG: *Glycosaminoglycans*

IDO: *Indoleamine*-pyrrole 2,3-dioxygenase

IBMX: Isobutylmethylxanthine

IgG: Immunoglobulin G

IL-1 β : Interleukin-1 beta

IL-6: Interleukin-6

iNOS: *inducible Nitric Oxide Synthase*

ISCT: International Society for Cellular Therapy

MMPs: Matrix Metalloproteinase

MMP1: Matrix Metalloproteinase-1

MMP13: Matrix Metalloproteinase-13

mPGES-1: microsomal Prostaglandin E Synthase-1

MSCs: Mesenchymal Stem or Stromal Cells

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

OA: Osteoarthritis

PGE₂: Prostaglandin E2

RA: Rheumatoid Arthritis

RT-PCR: Real-Time Reverse Transcriptase-Polymerase Chain Reaction

SD: standard deviation

S/DMOAD: Structure/Disease Modifying Anti-Osteoarthritis Drug

SySADOA: Symptomatic Slow-Acting Drug on OA

TNF: Tumor Necrosis Factor

TGF- β : Transforming Growth Factor Beta

Declarations

Author Contributions

All the authors were involved in drafting the article or revising it critically for major intellectual content, and all the authors approved the final version to be published. Dr. Villar-Suárez has had full access to all the data in this study and is responsible for the integrity of the data and the accuracy of the data analysis. Conception and design of the study was made by Villar Suárez, Pérez-Castrillo, and González-Fernández. Data acquisition was carried out by Pérez-Castrillo, González-Fernández, Gutiérrez-Velasco, Sánchez-Lázaro, Esteban-Blanco and Álvarez-Suárez. Data analysis and interpretation were carried out by Villar Suárez, Pérez-Castrillo and González-Fernández.

Authorship

“All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.”

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Disclosure Statement

We confirm that none of the authors have any competing interests in the manuscript.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Tables

Table 1. Distribution of samples in 6-well plate. When cell confluence was reached, 5 µL of TNF (10 µg/mL) was added and incubated for 12 hours (Experiment 1). In experiment 2 when cell confluence was reached, TNF was added in a medium also containing CS (200 ng/mL).

Well	Experiment 1	Experiment 2
1	ASCs	ASCs + CS
2	Chondrocytes	Chondrocytes + CS
3	Chondrocytes + ASCs	Chondrocytes + ASCs + CS
4	ASCs + TNF	ASCs + TNF +CS
5	Chondrocytes + TNF	Chondrocytes + TNF +CS
6	Chondrocytes + ASCs + TNF	Chondrocytes + ASCs+ TNF +CS

Table 2. Gene primer sequences and conditions used for qRT-PCR.

Gene	NCBI RefSeq	Primer sequence (5'-3') (Forward/Reverse)	Melting temperature (°C)	Optimal concentration
ACT-β	KR710455	CCCTCCATCGTCCACCGCAAATGCT	79.1	50 nM
		CTGCTGTCACCTTCACCGTTCCAGT	73.2	50 nM
IL-6	HUMIFNB2A	ATAACCACCCCTGACCCAA	55.9	50 nM
		CCATGCTACATTTGCCGAA	53.4	50 nM
TGF-β	NM_000660	CTCCCGCAAAGACTTTTCCCCAGACCT	64.0	300 nM
		CCACGGAAATAACCTAGATGGGCGCGAT	63.7	300 nM
IDO	M58159	CATCCTGATTCTCTGCAAGCC	56.3	50 nM
		TCTGCTATGATAAAATGTGCTCT	52.0	50 nM
TNF	AB202113	CCTGAAAACAACCCTCAGACGCCACA	63.0	300 nM
		TCCTCGGCCAGCTCCACGTCCC	67.8	300 nM
MMP13	NM_002427	CCAGAACTTCCAACCGTATTGATGC	72.3	50 nM
		TGCCTGTATCCTCAAAGTGAACAGC	69.1	50 nM
COL2a1	X16711	CCCATCTGCCCAACTGACC	58.5	50 nM
		CACCTTTGTACACGATCCC	58.2	50 nM
iNOS	AF045478	AACGTTGCTCCCCATCAAGCCCTT	63.3	50 nM
		AGCAGCAAGTTCATCTTTCACCCACT	62.3	50 nM

Figures

Figure 1

Immunophenotyping analysis of ASCs by flow cytometry and immune-fluorescence. A) ASCs were positive to CD 90 (98.95 % expression), B) to CD73 (98.50 % expression) and C) to CD90 marker (98.95 % expression). In the fluorescence analysis (magnification 40x), all surface proteins are present in cells with the highest fluorescence intensity in the case of CD105. Nuclei were stained with DAPI. Tri-lineage differentiation potential of ASCs after 2 weeks of culture in differentiation medium (magnification 20x). D) Adipocytes stained with Oil Red O. Presence of intracytoplasmic lipid-rich droplets. E) Chondrocytes stained with Alcian Blue. F) Osteocytes visualized with Alizarin Red S. Matrix mineralization in induced cultures can be clearly seen. G) Positive cells stained with anti-Col2a1.

Figure 2

Monitoring of co-cultures proliferation by fluorescence microscopy (magnification 20x). A) ASCs (violet stained) and chondrocytes (green stained) co-cultures at 24 and 36 h in culture with and without TNF. B) ASCs (violet stained) and chondrocytes (green stained) co-cultures at 24 and 36 h in culture with and without TNF and CS added at 24 h. C) Quantification of cell proliferation in co-cultures at 12, 24 and 36 h after induction of inflammation and CS addition using flow cytometry. The viability percentages are maintained for the two cell types, with a slightly reduction in the case of inflamed chondrocytes without CS.

Figure 3

Effect of CS on TNF-stimulated cells PGE₂ production, both in monoculture and co-cultures of ASCs and chondrocytes. In an inflammatory microenvironment, CS and ASCs reduced the production of PGE₂. **P ≤ 0.01 with regard to the corresponding group without CS.

Figure 4

Relative expression of IL-6, iNOS, MMP-13, IDO, TNF and TGF- β in chondrocytes, ASCs and co-cultures of ASCs and chondrocytes. cell culture. CS was added to the cultures for 12 h and then inflammation was induced using TNF for 12 h. +++P $\leq$ 0.005) with regard to chondrocytes stimulated with TNF. # (P $\leq$ 0.05) ### (P $\leq$ 0.005) with regard to chondrocytes non-stimulated with TNF.

Figure 5

Relative gene expression of specific genes of chondrogenesis SOX-9, ACAN and Col2a1 in human chondrocytes, ASCs and co-culture of ASCs-Chondrocytes. CS was added to the cultures for 12 h and then inflammation was induced using TNF for 12 h. *** $P \leq 0.005$ with regard to chondrocytes non stimulated with TNF.