CellR4 2015; 3 (3): e1581

Human Adipose Tissue Derived Mesenchymal Stromal Cells Modulate Intervertebral Disc Degeneration-Induced Inflammatory Response and Differentiate to Nucleus Pulposus-Like Cells: Perspective for Use in the Treatment of Intervertebral Disc Degeneration

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Abbreviations:

Ad-MSC: adipose derived mesenchymal stromal cells
IVD: intervertebral disc
NP: nucleus pulposus
LBP: low back pain
DDD: degenerative disc disease
ECM: extracellular matrix
FC: flow cytometry
MSC: mesenchymal stromal cells
PBS: phosphate-buffered saline
RT-PCR: reverse transcriptase polymerase chain reaction

Abstract

Introduction: **Recent studies have shown encouraging progress toward the use of syngenic and allogenic mesenchymal stromal cell (MSCs) to arrest, or even reconstruct the normal structure and function of degenerated intervertebral discs (IVDs) in various animal models. Here we demonstrate that human adipose-derived mesenchymal stromal cells (Ad-MSCs) modulate the inflammatory response of degenerated IVD and differentiate into a chondrogenic fate.**

Patients and Methods: **IVD fragments obtained from patients undergoing discectomy were placed on the bottom layer of a 12-wells co-colture system, while Ad-MSCs were placed on the upper layer. At different time-point co-colture medium was harvested and human cytokines were detected and analysed using a multiplex bead-based assays. To verify the Ad-MSCs differentiation into a chondrogenic fate, RNA isolated from Ad-MSCs co-coltured in presence of IVD fragments was analysed by RT-PCR analysis.**

Results: **Herein we tested the capability of human adipose-derived mesenchymal stromal cells (Ad-MSCs) to modulate the inflammatory status of degenerating IVD fragments obtained from patients undergoing discectomy; in addition we co-cultured in vitro Ad-MSCs and nucleus pulposus (NP) cells obtained from degenerated IVD tissue to understand whether Ad-MSCs are able to differentiate into an NP-like cell phenotype and/or to have a stimulatory effect on native NP cells.**

Conclusions: **Our results demonstrate that Ad-MSCs elicit immunomodulation on IVD fragments, and that Ad-MSCs co-cultured with IVD fragments show a commitment towards a NP-like phenotype. These data suggest a potential role of human Ad-MSC in regenerative therapy of IVD.**

Keywords: Islet transplantation, Diabetes, Multidisciplinary approaches, Bioengineering, Biologic.

Introduction

Degenerative disc disease is one of the major risk factors of chronic low back pain (LBP)[1]. Although the causes of LBP are thought to be multifactorial[2], in almost all cases there is evidence of intervertebral disc (IVD) degeneration which makes curing disc degeneration (DDD) one of the most important socioeconomic imperatives facing modern health care today.

The IVD acts as a shock absorber during compressive loading of the spine. As the disc degenerates, the function of the spinal column motion segment is altered with less load-bearing potential[3]. On the cellular level, the degeneration process is characterized by cellular dysfunction accompanied by reduced synthesis of extracellular matrix (ECM)[4]. Despite the recent developments in therapies to treat IVD, including laser beam therapy or needle aspiration, in most time treatment involves either removal of tissue or fusion of the affected disc level. However, these therapies relieve the symptoms rather than repair the tissue procedures[5,6]. Furthermore such treatment modalities are often short-term solutions that frequently lead to complications due to alterations in the biomechanics of the spine that neither arrest the progression of degeneration nor restore the native functional state of the intervertebral disc.

With the advent of tissue engineering and regenerative medicine, novel cell-based therapies are being investigated with the ultimate aim of replacing or regenerating the nucleus pulposus cells (NP) producing a matrix with similar or improved biological and biomechanical properties compared with the original ones[7,8].

Stem cell transplantation can reconstruct the normal structure and function of IVDs by supplementing the loss of NP cells and promoting the formation of extracellular matrix[9,10]. Recent studies have shown encouraging progress toward the use of autogenic and allogenic mesenchymal stem cells (MSCs) to arrest and lead to partial rescue of IVD in various animal models[11,12]. However, this approach is still in its infancy and further developments are required to clarify many issues before clinical translation.

The present study aimed to evaluate if human adult adipose-derived mesenchymal stem cell (Ad-MSCs), characterized by their relatively easy availability, are a good candidate as source for the IVD treatment.

Ad-MSCs are undifferentiated, multipotent cells that have the ability to differentiate into a number of cell types (cartilage, bone, adipose tissue) including the chondrocyte-like cells found within the NP of the intervertebral disc[13]. Adipose tissue is an easy accessible source to isolate MSCs that can be expanded *in vitro* to large quantities for therapeutic purposes[14].

Because factors associated with disc degeneration include up-regulated inflammatory cytokines and loss of resident NP cell populations, we focused our study on the capability of human Ad-MSCs *in vitro* to affect the inflammatory reaction present in IVD fragments, obtained from patients undergoing discectomy. The modulation of the microenvironment could offer a mean to affect cell function in order to promote tissue regeneration and attenuate the catabolic processes that contribute to the degenerative state.

In addition, we investigated whether Ad-MSCs are able to differentiate to an NP-like cell using an *in vitro* co-culture model system combining Ad-MSCs and IVD specimens. We hypothesized that the molecules/signals produced from both ECM and cells within the IVD fragment may provide a niche-specifying environment to enhance the chondrogenic differentiation of Ad-MSCs.

Patients and Methods

Patients

From November 2013 to April 2014, 14 patients (6 male and 8 female; mean age 48±27SD) affected by DDD associated with disc erniation were included in this study. All 14 patients did not respond to conservative treatment (physical and medical) lasting at least 6 months. In all patients the spinal MRI (T1 and T2-weighted axial and sagittal images) showed a disc erniation that was at L4-L5 level in 7 patients and L5-S1 in the last 7 patients.

In the same period in 5 patients (3 male and 2 female; mean age 56±37 SD) of which 3 affected by Normal Pressure Hydrocephalus undergoing to ventriculo-peritoneal shunt placement, and 2 patients in which abdominal fat graft was used in preventing iatrogenic cerebrospinal fluid rhinorrhea after transsphenoidal surgery, a sample of periombelical adipose tissue was harvested (1 g) for the following mesenchymal stromal cell (Ad-MSC) isolation.

Surgical operation was performed according to all modern surgical (including microscopy) and anesthesiological standards. No perioperative adverse events occurred.

Written informed consent was obtained from all patients.

Adipose-derived mesenchymal stromal cell (Ad-MSC) isolation

Harvested human periombelical fat tissue was washed in saline and digested in 0.1% collagenase at 37°C for 1h. The harvested fat tissue was treated with an equal volume of low-glucose Dulbecco's modified Eagle's medium (DMEM: Gibco, Invitrogen) containing 10% fetal bovine serum (Gibco, Invitogen) and 1% penicillin/streptomycin (Gibco, Invitrogen). Cells were collected by centrifugation for 10 min at 300xg and the supernatant was discarded. The pellet was suspended in a 75-cm^2 flask (Nunc, Invitrogen) and incubated at 37°C under 5% CO_2. Non-adherent cells were removed by replacing the medium. Cells were fed every 3 days with 10ml of complete DMEM media. When this primary culture of Ad-MSCs reached 80% confluence, cells were harvested using 0.25% Trypsin-EDTA and washed in 10 ml of phosphate-buffered saline (PBS). Cell viability was assessed by Trypan blue staining.

Identification of Ad-MSCs

Fluorescence-activated cell sorting (FACS) was used to analyze the surface markers of human Ad-MSCs. After 3 passages, the cells were trypsinized and resuspended in DMEM containing 10% fetal bovine serum (FBS). Samples were counted, centrifuged and resuspended in PBS. The cells were placed into tubes at $1x10^6$ cells per 1.5 mL, washed twice with PBS and incubated for 1hour at 4°C with the following fluorochrome conjugated antibodies CD34, CD45, CD31, CD73, HLA-DR (BD Pharmigen, San Jose, CA, USA), CD90 (Millipore, Temecula, CA, USA), CD105 (AbDSerotec, Raleigh, NC, USA). In control groups, the cells were incubated in PBS without antibodies. The samples were then washed twice with PBS and analyzed by FACS.

Adipogenic, osteogenic, chondrogenic differentiation of Ad-MSCs

Human adipose mesenchymal stem cells differentiation was induced as indicating using the media supplements induced in the Human Mesenchymal Stem Cell Functional Identification Kit (Catalog# SC006).

Intervertebral disc fragment harvest

Following informed consent, 14 human intervertebral disc (IVD) samples were obtained from 14 donors who underwent disc surgery. Harvested IVD fragments were washed twice in PBS, cut manually to sections with the same size and co-cultured with Ad-MSCs. Separate parts from the same disc fragment were distributed to both control and treated sample groups.

Co-cultures of Ad-MSCs and IVD fragments

A 12-well co-culture system (Nunc, USA) was used. IVD fragments obtained from patients undergoing discectomy were placed on the bottom layer, while Ad-MSCs were placed at 10,000 cells/well densities on the upper layer. The cells were incubated for 15 days in DMEM media plus 10% FBS.

Basal release of cytokines in Ad-MSC/IVD coculture

After 6 hours, 7 days and 15 days of co-culture, 100 μL of co-culture medium was harvested. Human cytokines were detected using multiplex bead-based assays (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Plex Human Group II Cytokines 23-Plex Panel; Biorad Laboratories). Appropriate controls were included to confirm the results.

RT-PCR analysis

For RNA isolation, media was aspirated and the membrane of the 12-well co-culture system (Nunc, USA) removed and placed in a tube. Total RNA was extracted according to RNeasy Mini Kit protocol (Qiagen). 1 μg of RNA were reverse transcribed according to the supplier's instructions of iScript cDNA Synthesis Kit (Biorad). To compensate forslightly variable RNA and cDNA yields, the amount of cDNA synthesized was calibrated by using the relative expression level of the housekeeping gene GAPDH as a standard. Amounts of cDNA yielding equivalent amounts of GAPDH amplification product (between 1/20 and 1/80 of the RT reaction) were used in subsequent PCR reactions.

cDNAs were amplified for 30 or 35 cycles using the following primers:

- GAPDH: 5'-ACGGGAAGCTCACTGGCA-3'; 5'- CCTGCTTCACCACCTTCTTGAT-3'
- SOX9: 5'-GACCTTCGATGTCAACGAGTTTG-3'; 5'- TGCTGCTTGGACATCCACAC-3'
- Col1α1: 5' AGTCACCCACCGACCAAGAA-3'; 5'- CACGAGGACCAGAGGGACC-3'
- Col1α2: 5'- GGTGAAATTGGAGCTGTTGGTAA-3'; 5'- CACCAACAGGGCCAGGAA T-3'
- Col2α1: 5'- GGTCCTCCTGGAAATCCTGG-3'; 5'- TCAGGTCTCTGCAGGTGCG-3'

PCRs were performed in 20 ml in standard conditions (15 mM MgCl2, dNTP 2 mM each, 1 U Taq polymerase). The semi-quantitative evaluation of cDNA bands is performed using the densitometry analysis (ImageJ software, NIH)

Statistics

Analysis of data was performed using SPSS statistical package for Windows (SPSS Inc., Chicago, IL, USA). A 2-tailed *p* value less than 0.05 was considered significant.

Study approval

All patients gave informed consent for the investigations. The ethical committee of the Istituto Neurologico Carlo Besta approved the protocols and investigations. Investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Results

Human Ad-MSCs characterization and differentiation

Adipose mesenchymal stem cells used in this study were obtained from the fat deposit in the abdominal area (periumbilical) from healthy donors (n=5) and validated following the minimal criteria for defining multipotent mesenchymal stromal cells[15].

The adipose tissue extracted Ad-MSCs were initially characterized by the ability to create colony forming units (CFU) and their elongated fibroblastic cellular morphology (Figure 1 A).

Flow cytometry analysis of multiple surface epitopes showed that Ad-MSCs did not express (≤2%) CD34 and CD45 hematopoietic cell markers, CD31 endothelial marker and HLA-DR immunological markers. In contrast, Ad-MSCs highly expressed (≥90%) MSC markers, such as CD73, CD90 and CD105 (Figure 1,C).

Cells showed a classical growth pattern with an early lag-phase in the first 7 days and a subsequent exponential growth. After 30 days culture cells reached a 12x expansion of the initial seeded number (data not shown).

The same cells were induced to differentiate into adipocytes, osteocytes and chondrocytes and representative results are shown in Figure 1 B.

Human Ad-MSCs modulate degenerating IVD fragments inflammatory response in an in vitro co-cultured system

Human IVD fragments (n=14) obtained from patient undergoing discectomy were co-cultured in a cell culture system without contacts as shown in a schematic diagram (Figure 2A) with Ad-MSCs for 2 weeks.

We further analyzed the level of expression of cytokines and chemokines involved in sustaining the inflammatory state using a multiplex bead-based assays.

For this aim we compared the culture medium of IVD fragments after co-culture with Ad-MSC to the one obtained from the culture of IVD alone (Figure 2B).

The presence of Ad-MSCs decreases the concentration of the pro-inflammatory proteins IL-3, IL-9, IL-16 and IL12p40 produced by IVD in a IVD and Ad-MSCs co-cultures already after 6 hours and this attenuation effect is maintained during all the 15 days of coculture.

The secretion of cytokines involved in anti-inflammatory processes was also measured in the Ad-MSCs and IVD fragments co-culture medium. The expression of IL-4, IL-15, IL-13 and IL-1ra starts to be up-regulated by the presence of Ad-MSCs already after 6 hours and reached a 6 fold increase at day 7. Interestingly the amount of anti-inflammatory cytokines is higher in the medium collected from co-cultures (Ad-MSC/IVD) compared to the one detected in the Ad-MSC culture medium. This may suggest an Ad-MSC anti-inflammatory response stimulated by the presence of a degenerated IVD microenvironment.

"NP like" phenotype differentiation of Human Ad-MSCs after co-culture with IVD fragments

We also determined the "NP like" cell phenotype commitment of Ad-MSC by RT-PCR.

Ad-MSC co-cultured with IVD fragment showed a significant increase in the gene expression of collagens types Col1α1, Col1α2 and Col2α2 compared to the one showed in Ad-MSC cultured alone (Figure 3).

The increase is already visible after 6 hours and was monitored for 21 days proving to grow over time. Gene expression of the transcription factor Sox-9, which is crucial for differentiation of MSCs to chondrocyte-like NP cells was also measured. Notably, Sox9 showed an increased expression after 6 hours and returned to basal levels at 7 days. These findings suggest that Sox9 up-regulation is required to drive the first stage of chondrocyte differentiation in order to activate the expression of Col1α1, Col1α2 and Col2α2.

Ad-MSC stimulate NP cells growth and chondrogenic differentiation

In order to verify that Ad-MSCs have a positive effect on the native NP cells we set up a co-culture experiment in which NP cells isolated from IVD fragments were seeded on the bottom of wells. In the upper insert IVD fragments were placed to recreate at the best the degenerated IVD microenvironment and Ad-MSCs to evaluate their stimulatory effects (Figure 4).

In the presence of Ad-MSCs was visible as the NP cells enhance their capability to increase the levels of expression of Col1α1, Col1α2, Col2α2. The expression levels of these genes increase significantly already after 7 days showing how the presence of Ad-MSC can induce also a differentiation of native NP cells towards a chondrogenic fate.

Discussion

Adipose tissue is an abundant, accessible and easily available source of adult stromal cells. Ad-MSCs have potential applications for the repair and regeneration of acute and chronically damaged tissue in several disease models14,16.

Previous studies reported that MSCs directly penetrate into damaged tissue and secrete or indirectly induce the release of various cytokines that are known to facilitate tissue regeneration[17]. Other studies revealed also that MSCs are able to induce replacing properties of endogenous stem cells in different tissues[14].

We have previously demonstrated the survival and differentiation capability of human Ad-MSCs transplanted into the IVD of a rodent model[18]. In this study for the first time we showed that human adult Ad-MSCs have the intrinsic ability to control the human degenerated IVD inflammatory microenvironment and to differentiate into a NP-like phenotype by an *in vitro* co-culture system.

In an attempt to understand the underlying inflammation regulation mechanisms and dynamics, we investigated the expression level of different pro-inflammatory cytokines. Particularly, IL-3, IL-9, IL-12 and IL-16 in co-cultured Ad-MSCs reduced their expression in comparison to the ones found in the IVD culture alone.

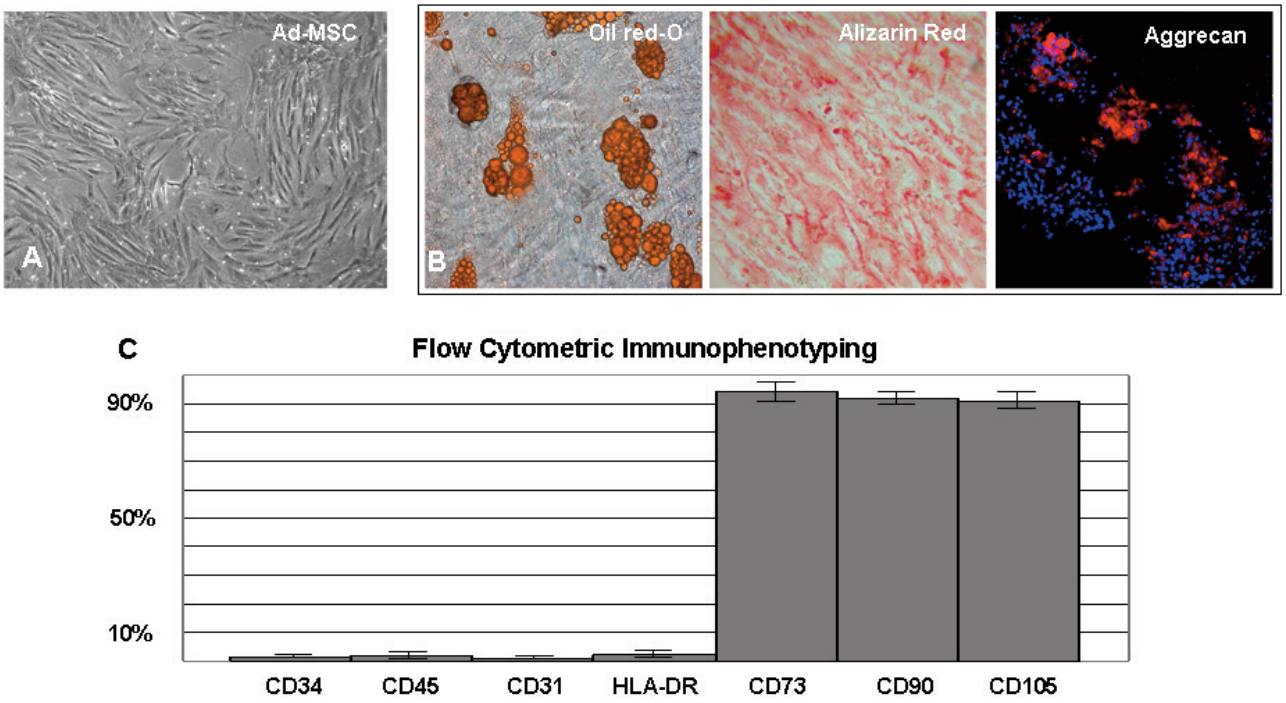

Figure 1. Phenotopic characterization of Ad-MSCs. *A,* Images of cultured human Ad-MSCs at passage 3 were taken on subconfluent culture of 5 different preparation of Ad-MSCs. Note the typical fibroblastic-like morphology of cells. *B,* After induction, Ad-MSCs exhibited adipogenic, osteogenic and chondrogenic potentials which are demonstrated by staining of lipid droplets (Oil red-O), calcium modules stained (Alizarin Red) and aggrecan deposition. *C,* Flow cytometry analysis of multiple surface epitopes showed that Ad-MSCs minimally expressed (≤2%) hematopoietic cell markers CD34, CD45, endothelial marker CD31 and immunological marker HLA-DR and highly expressed (≥90%) MSC markers such as CD73, CD 90 and CD105.

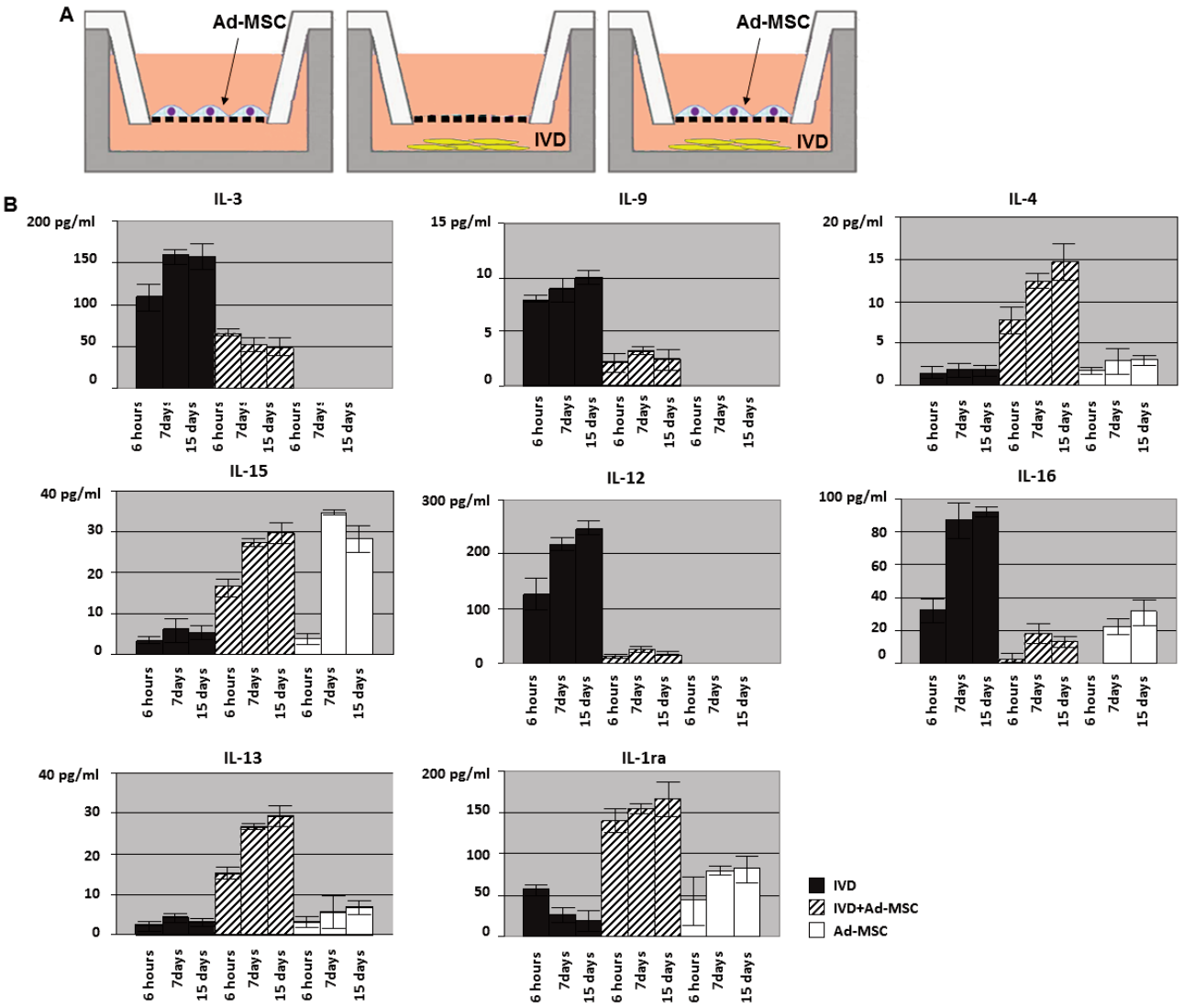

Figure 2. *A,* **Schematic representation of IVD+Ad-MSCs co-colture system.** IVD fragments (n=14) were placed on the bottom layer of a12-well co-culture system while Ad-MSCs (10,000 cells/well) were placed on the upper layer. IVD fragment and Ad-MSCs alone represent internal experimental controls. *B,* **Analysis of the level of expression of cytokines and chemokines involved in sustaining the inflammatory state.** The level of expression of cytokines and chemokines involved in sustaining the inflammatory state were analyzed after 6 hours, 7 and 15 days. The presence of Ad-MSCs decreases the concentration of the pro-inflammatory proteins (IL-3, IL-9, IL-16 and IL12p40) while increases the production of cytokines involved in anti-inflammatory processes (IL-4, IL-15, IL-13 and IL1ra).

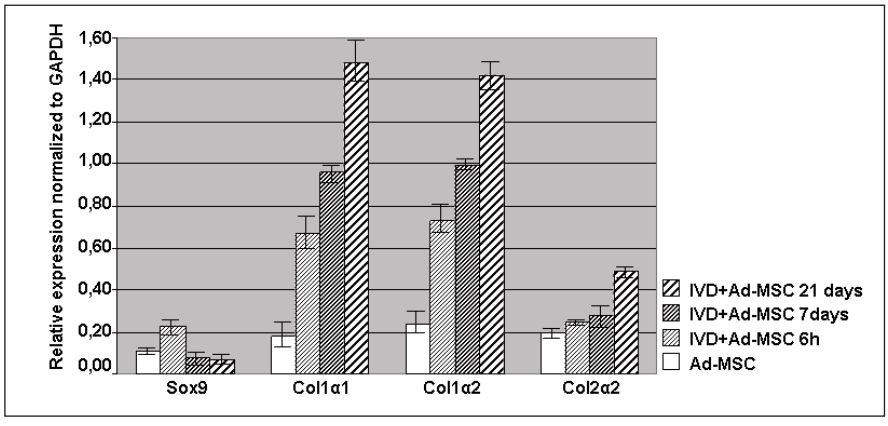

Figure 3. Semiquantitative expression of chondrogenic markers in a IVD+Ad-MSCs co-colture system. Equally amount of cDNA isolated from Ad-MSC co-cultured with IVD fragment (n=8) was amplified for SOX9, Col1α1, Col1α2 and Col2α2. The chondrogenic differentiation program genes (Col1α1, Col1α2 and Col2α2) increase their expression along the time (21 days) of IVD+Ad-MSC. Peculiarly Sox9 increases its expression after 6 hours and returns to control levels (Ad-MSC alone) after 7 days of co-colture.

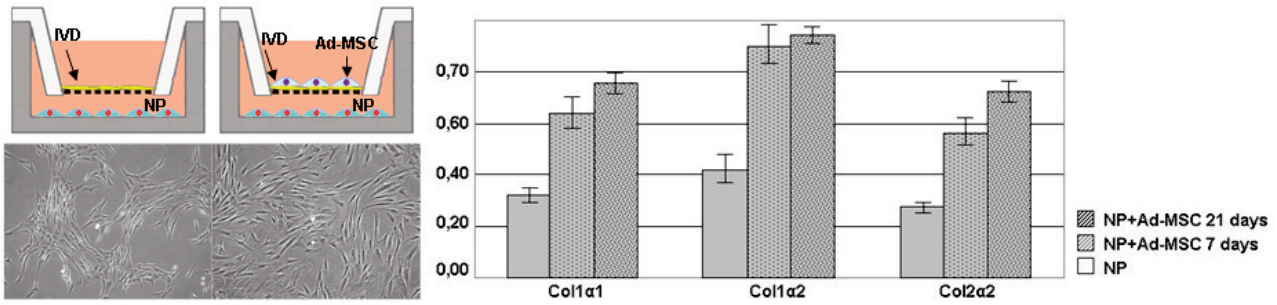

Figure 4. *A,* **Schematic representation of NP+Ad-MSCs co-colture system.** NP cells isolated from IVD fragments were placed on the bottom layer of a12-well co-culture system, while IVD fragments with or without Ad-MSCs (10,000 cells/well) were placed on the upper layer. *B,* **Semiquantitative expression of chondrogenic markers in a NP+Ad-MSCs co-colture system**. Equally amount of cDNA isolated from NP cells (n=8) was amplified for Col1α1, Col1α2 and Col2α2 genes. The chondrogenic markers increase their expression progressively along 21 days of NP+Ad-MSC co-colture.

We also investigated whether co-cultured Ad-MSCs modulate the inflammatory responses, by expressing themselves anti-inflammatory molecules. As expected co-cultured Ad-MSCs increased the expression of IL-4, IL-15, IL-13, IL-1ra. Thus, a contemporary regulation of both pro-inflammatory and anti-inflammatory molecules may be invoked as the potential mechanism by which Ad-MSCs are able to reduce the inflammatory response[19,20].

This finding confirms the already demonstrated immunomodulating properties of Ad-MSCs in other tissues[21]. The reduction of inflammation could be a key element for inhibition of the activity of the enzymes involved in the process of tissue degradation, and possibly in pain control.

The second goal of this study was to elucidate the effects that co-culture had on the differentiation capability of both human Ad-MSCs and NP cells. In general, chondrogenic differentiation of MSCs has been performed by the application of growth factors from the transforming growth factor-β (TGF-β) superfamily[22,23], in the presence of ascorbate and dexamethasone. For the first time, our cell co-cultures showed a large increase in Ad-MSCs gene expression of Sox-9 and a number of matrix molecules in particular type I and type II collagen without growth factors stimuli. The steady increase in the time of gene expression Col1α1, Col1α2, Col2α2 demonstrates how Ad-MSCs assume a chondrogenic fate[24,25] only in presence of NP cells. Interestingly, Sox9 expression showed a 0.2 fold increase after few hours of co-culturing supporting the hypothesis that Sox9 up-regulation is the result of NP cells co-cultures and represent the first stage of the chondrogenic differentiation program. Sox9 expression returned to basal levels after 7 days suggesting the potential of Ad-MSCs in carrying out the chondrocyte hypertrophy process.

Further studies are necessary to understand in details the role of Sox9 in the early stages of Ad-MSCs chondrogenic differentiation to better clarify the molecular basis of this process.

We finally verified the effect of Ad-MSC on NP cells in our *in vitro* system trying to recreate a degenerated IVD microenvironment using IVD fragment.

We observed a significant up-regulation in Col1α1, Col1α2, Col2α2 expression in NP cells harvested from IVD showing how these cells are probably committed to produce matrix molecules in the co-culture system. Similar results were obtained in the direct cell-to-cell contact co-culture system between NP cells and bone marrow-derived stromal cells derived from rabbits[26]. Further analysis are needed in order to understand the actual ability of Ad-MSC in stimulating the NP cells present in the degenerated IVD and to elucidate the real effectiveness of an hypothetical human therapy.

Conclusions

Human Ad-MSC may be an attractive source for regeneration therapy in degenerative IVD. The detection of anti-inflammatory and differentiating capabilities of these cells may provide insights into the mechanism of MSC action for the repair of IVD degeneration in vivo.

Competing interests

The authors declare that they have no financial competing interests.

Authors' contributions

ALMF: manuscript writing, collection and assembly of data, final approval of manuscript. M PS: collection of IVD fragments and adipose tissue, manuscript writing. VT: carried out RT-PCR tests and molecular data. VC: carried out the mesenchymal differentiation tests. VS: collection of histochemestry data. SV: collection of IVD fragments and adipose tissue. PF: collection of IVD fragments and adipose tissue. AB: conception and design, manuscript writing. EP: administrative support, final approval of manuscript. All authors read and approved the final manuscript

Acknowledgements:

We thanks Dr. Giulio Alessandri for the critical reading of the manuscript. This work was supported by IRCCS Foundation Neurological Institute "C. Besta".

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