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The Comparison of the Differentiation Potential of Periodontal Ligament and Dental Pulp Mesenchymal Stem Cells in the Inflammatory Synovium Microenvironment

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Abstract

BACKGROUND/AIMS: Dental mesenchymal stem cells are easily accessible sources for mesenchymal stem cells (MSCs) and can rapidly proliferate in culture conditions. Rheumatoid arthritis (RA) is a chronic and multi-systemic autoimmune inflammatory disease that results in cartilage damage. The present study aimed to investigate the regenerative potential of dental MSCs in the synovial fluid microenvironment of patients with RA.

MATERIALS AND METHODS: Synovial fluid samples (8-10 mL/patient) were collected from patients with RA (age; 48–67 years). Dental pulp (DP) and periodontal ligament (PL) tissues were collected from healthy individuals, and the tissues were enzymatically digested in 3 mg/mL collagenase type I. MSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM). The cells were cultured in the presence and absence of the synovial fluid samples of the patients with RA and subjected to flow cytometry analysis for cell surface expressions of positive markers for chondrogenesis (CD49e) and an osteogenic marker (alkaline phosphatase; ALP). The differentiation capacity of MSCs was evaluated with osteogenic or chondrogenic stimulation media and analyzed by staining the cells with Alizarin Red or Alcian Blue, respectively.

RESULTS: The cytokines interleukin (IL)-1β (61.1±9.8) and IL-6 (2386.7±397.4) were significantly higher in the end-stage RA-SF samples, compared to the early-stage RA-SF samples (IL-1β:35.2±4.8, IL-6:561.3±197.6) ($p<0.05$, $p<0.001$, respectively). DP-MSCs were significantly differentiated to osteocytes and formed calcium deposits cultured with end-stage RA-SF samples, whereas PL-MSCs were differentiated to osteocytes in limited levels, and low concentrations of calcium deposits were observed. Chondrocytes were observed in DP and PL-MSCs, and cartilage formation was observed only in DP-MSCs when cultured with end-stage RA-SF samples. The neutralization of IL-1β or IL-6 tended to decrease osteogenic marker expressions of DP-MSCs cultured in the presence of end-stage RA-SF samples.

CONCLUSION: The present study showed the differentiation potential of DP-MSCs into osteogenic and chondrogenic lineage in the inflammatory microenvironment of SF. DP-MSCs may be candidates for tissue regeneration, especially in patients with RA with bone or cartilage erosions.

Keywords: Mesenchymal stem cells, synovial fluid, dental pulp

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INTRODUCTION

Distinct types of cells have been described in the oral cavity, and most of them share different characteristics from commonly used bone marrow or adipose tissue mesenchymal stem cells (MSCs). Thus, each source of MSCs may contribute to variable differentiation capacity in a different inflammatory environment[1]. The therapeutic potentials of MSCs have been explored to date in various degenerative and inflammatory disorders, such as Crohn's disease, rheumatoid arthritis (RA), diabetic nephropathy and allergic diseases[2]. The effects of MSCs in regulating inflammation and tissue regeneration are multifaceted, but it is generally thought that these cells have a strong anti-inflammatory and regenerative potential in the inflammatory microenvironment[3]. Recent studies have demonstrated that MSCs, when exposed to an inflammatory environment, have a local and systemic immunoregulatory effect through the release of various mediators, including many metabolites, exosomes, growth factors, and chemokines. Studies have shown that MSCs isolated from different sources could represent variable immunoregulatory and regenerative responses according to the inflammatory niche[4].

RA is a chronic inflammatory disease which has destructive effects on joint cartilage and subchondral bone. RA is divided into four specific stages: i) early stage: join pain and stiffness, (ii) moderate-stage: inflammation in the synovium causing damage to the joint cartilage, (iii) severe: damage in the cartilage and bones and (iv) end-stage: no longer any inflammation in the joints, bone erosions and mobility loss[5]. Differentiation of naive T cells into Th1 or Th17 cells contributes to synovitis. The pro-inflammatory cytokines interleukin (IL)1β and tumor necrosis factor α (TNF-α) are the two main mediators in the initial phase of RA. In addition, IL-6, IL-8, IL-15, IL-17 and interferon-γ (IFN-γ) produced in the joints have progressive effects and cause joint destruction in RA[6]. These cytokines in the synovial fluid (SF) may vary in the different stages of RA. While TNF-α, IFN-γ and IL-6 are predominantly secreted in the early stage of RA, they tend to decrease in the later stages[7]. Nowadays, non-steroidal anti-inflammatory agents, corticosteroids and disease-modifying anti-rheumatic drugs are used in RA[8]; however, most of these medications have side effects, such as loss in bone density or susceptibility to infections[9]. Therefore, new therapeutic options are required which target inflammatory responses and tissue regeneration without side effects.

In previous studies, MSCs have shown promising trends for clinical application in RA. The MSCs used are mostly isolated from bone marrow, adipose tissue or the umbilical cord. In the present study, we evaluated the differentiation potential of dental pulp and periodontal ligament MSCs in the synovial fluids of different stages of patients with RA to investigate whether they can be new cellular candidates for tissue damage.

MATERIALS AND METHODS

Study Population

This study was approved by the Muğla Sıtkı Koçman University Clinical Ethics Committee with reference number 02/IV, 30.01.2020. Six patients with RA (aged between 48 and 67 years old) with synovial fluid aspiration participated in the present study. The patients had bone or cartilage erosions (end-stage RA). The patients were included in this study according to their ACR and EULAR diagnostic criteria for RA, and the additional inclusion criteria for the present study were as follows: 1) patients with RA without comorbidity, 2) positive for rheumatoid factor and 3) Elevated C-Reactive Protein (CRP) values. The minimum number of patients for statistical analyses was determined using G-power analysis. The total number of patients was calculated to be n=6 with the parameters as follows: α-err probability was 0.05 and power (1-β err probability) was 0.80. The demographic data of the patients and healthy individuals for dental tissue collection are given in Tables 1 and 2, respectively.

Dental Pulp and Periodontal Ligament MSCs Isolation

Tissues were obtained from four healthy individuals who applied to the Muğla Sıtkı Koçman University Faculty of Dentistry. The isolation of MSCs from the dental tissues was performed as described before[10]. In brief, dental pulp and periodontal ligament tissues were mechanically fragmented into 1x1 mm^2 pieces and enzymatically digested in collagenase type I solution (3 mg/mL) in phosphate buffer solution (PBS, pH=7.4) (Thermofisher, US) for 45 minutes at 37°C. The tissue fragments were passed through a 70-micron filter and washed twice with DMEM-Low Glucose 1.0 g/L (Pan Biotech, Germany) supplemented with 10% fetal bovine serum (FBS, Pan Biotech, Germany) and 1% Penicillin/Streptomycin (100 U/mL, 100 µg/mL) (Thermofisher, US) and thereafter referred to as "complete DMEM," and centrifuged at 1200 rpm for five

Table 1. Demographic data of RA patients and healthy individuals

	Rheumatoid arthritis	Healthy subjects
Male/Female	1/5	3/5
Age (years)	48–67	26–42
Duration of illness over 3 years (number of patients)	3	None
Bone or cartilage erosions	3	NA
Treatment		
Infliximab	1	None
Adalumimab	1	None
Methotrexate	2	None
Corticosteroid	3	None
Duration of illness under 3 years (number of patients)	3	None
Bone or cartilage erosions	0	NA
Treatment		
Hydroxychloroquine sulfate	1	None
Methotrexate	2	None
Corticosteroid	2	None
ANA (positive)	100%	NA
RF (IU/mL)	34.55±23.52	NA

ANA: anti-nuclear antibody, RF: rheumatoid factor, NA: not applicable.

Table 2. Data of healthy individuals for dental pulp and periodontal ligament tissue collection

Male/Female	**3/5**
Age (years)	**26–42**
Dental pulp tissues (number of healthy subjects)	4
Periodontal ligament tissues (number of healthy subjects)	4
Inflammatory or genetic diseases (number of healthy subjects)	0
Abscess or microbial contamination (number of healthy subjects)	0

minutes. Cell pellet (3000 cells/cm^2) was transferred to a T25 flask and incubated in 5 mL of complete DMEM until it reached 70%–80% confluence approximately for 10 days. MSCs were cultured until the third passage by transferring cells to T75 flasks containing 3,000 cells/cm^2 in 10 mL of complete DMEM. The third passage cells were analyzed for positive cell surface markers of CD29, CD90 and CD105, and negative markers of HLA-DR, CD3 and CD28, by staining the cells with anti-CD29 (APC), anti-CD90 (PerCp), anti-CD105 (FITC), anti-HLA-DR (APC, anti-CD3 (PerCp), and anti-CD28 (PE), respectively. All antibodies were purchased from BD Biosciences, US. Analysis was performed via flow cytometry (BD Accuri C6 Plus), and the data were recorded as mean fluorescence index (MFI) %.

Cytokine Analysis of SF Samples

The cytokine profiles were analyzed from 50µL of each SF sample using the CBA Th1/Th2/Th17 kit (BD Biosciences, US) via flow cytometry. The analysis was performed as described in the kit protocol and as described previously[11]. In brief, standard beads were prepared with serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256) in 12x75 mL in standard tubes. The standard curve was obtained via flow cytometry. SF samples were diluted in 1:5 with assay diluent in the CBA kit. The dilution factor was calculated for each of the samples. The pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ, IL-17) were analyzed with BD Accuri C6 plus software. Data were recorded as pg/mL.

Culture Conditions

The culture conditions were determined as described previously[12] with some modifications. DP-MSCs or PL-MSCs (5x10^4 cells/cm^2) were cultured in six well-plates (Corning®, US) for two separate analyses, one of the culture plates was used for osteogenic or chondrogenic differentiation analysis, and one of the culture plates was used for cell surface marker analysis (5x10^4 cells/cm^2). DP-MSCs or PL-MSCs in the third passage were cultured with each of the fresh synovial fluid samples with osteogenic or chondrogenic stimulation medium (1:1 v/v) at 37°C and 5% CO_2 incubation for three weeks for the osteogenesis or chondrogenesis, using osteogenic or chondrogenic differentiation kits (StemPro™, Thermofisher, US). After the culture period, adherent cells were washed with PBS, and fixation was done with 10% formaldehyde solution. Osteogenic differentiation potential was evaluated by staining the cells with Alizarin Red (Sigma-Aldrich, Germany), and chondrogenic differentiation was evaluated by staining the cells with Alcian Blue (Sigma-Aldrich, Germany). Complete DMEM was used as a negative control medium, and osteogenic differentiation or chondrogenic differentiation media were used as positive control media[10].

The results of the samples of the patients with RA were evaluated by comparing the calcium deposits or cartilage formation in the negative and positive control media.

Cell Surface Marker Analysis for Osteogenic or Chondrogenic Differentiation

At the end of the culture period, the adherent cells were trypsinized with 0.25% Trypsin EDTA solution and subjected to cell surface markers for osteogenic or chondrogenic differentiation by staining cells with anti-ALP (Alexa Fluor 488) or anti-CD49e (PE), respectively, and analyzed via flow cytometry as described before[13]. The cell population was gated for CD90+ cells and these cells were analyzed for ALP or CD49e expressions as MFI%.

Additionally, a neutralization assay was performed to determine whether the high expressed cytokines affect the differentiation potential of DP-MSCs in the osteogenic or chondrogenic lineage. 5 mM anti-IL-6 neutralizing antibody (eBioscience, US) or 5 mM anti-IL-1β antibody (Invivogen, France) were added in the end-stage patients with RA-SF and the samples were cultured with DP-MSCs, and cultured for 21 days with the osteogenic or chondrogenic stimulation medium (1:1 v/v) for 21 days at 37°C in 5% CO_2 incubation. The cells were trypsinized and subjected to flow cytometry analysis for cell surface expressions of CD49e or ALP in the CD90+ cell population.

Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) with the GraphPad Prism 9.0 software. Data were presented as mean ± standard deviation. The Mann−Whitney U test was performed to investigate the difference between two sample data. $P<0.05$ was considered significant.

RESULTS

Characterization of DP-and PL-MSCs

The MSCs isolated from the dental pulp and periodontal ligament tissues formed fibroblast-like colonies in passage 0 (P0), P1, P2 and P3. The third passage cells expressed the positive cell surface markers of CD29, CD90 and CD105 over 95% and lacked the expression of the negative markers of HLA-DR, CD3 or CD28 (Figure 1).

Cytokine Levels in the SF Samples

The levels of secreted cytokines were analyzed for the evaluation of inflammatory microenvironment in the different stages of the patients with RA. The end-stage RA-SF samples showed significantly high levels of IL-1β (61.1±9.8) and IL-6 (2386.7±397.4), compared to the early-stage RA-SF samples (IL-1β: 35.2±4.8, IL-6: 561.3±197.6) ($p<0.05$, $p<0.001$, respectively). TNF-α, IL-17 and IFN-γ levels were slightly higher (TNF-α: 981.6±182.3, IL-17: 17.9±2.6, IFN-γ: 92.5±12.8) in the early-stage patients' RA-SF samples compared to the end-stage patients with RA (TNF-α: 648.3±212.9, IL-17: 12.1±3.2, IFN-γ: 96.7±27.3), but no significance was observed between the two groups ($p>0.05$) as shown in Figure 2.

The Osteogenic and Chondrogenic Differentiation Potential of DP-MSCs Increased in the SF Samples of End-Stage Patients With RA

The cultured DP-MSCs with end-stage RA-SF samples showed a high expression of ALP in the CD90+ cell population (59.1±5.3), while PL-MSCs expressed a low expression of the osteogenic marker (19.5±7.2). The low expression of ALP was significant in DP-MSCs cultured with the early-stage RA-SF samples (18.7±4.2) in comparison to the end-stage RA-SF samples and DP-MSCs cultured with osteogenic medium (51.1±3.8) ($p<0.005$). The chondrogenic marker expression was significantly higher in DP-MSCs cultured with the end-stage RA-SF samples (39.2±2.5) when compared to those in the early-stage patients with RA (19.7±3.2) ($p<0.01$). Additionally, DP-MSCs cultured with the SF samples of the end-stage patients with RA formed osteogenic colonies and calcium deposits to a greater extent compared to the PL-MSCs. Cartilage formation was observed in DP-MSCs cultured with the SF samples of the end-stage patients with RA, while sparse colonies of chondrocytes were observed in DP-MSCs cultured with the early-stage patients' RA-SF samples. Also, chondrocytes were observed in DP-MSCs or PL-MSCs cultured with

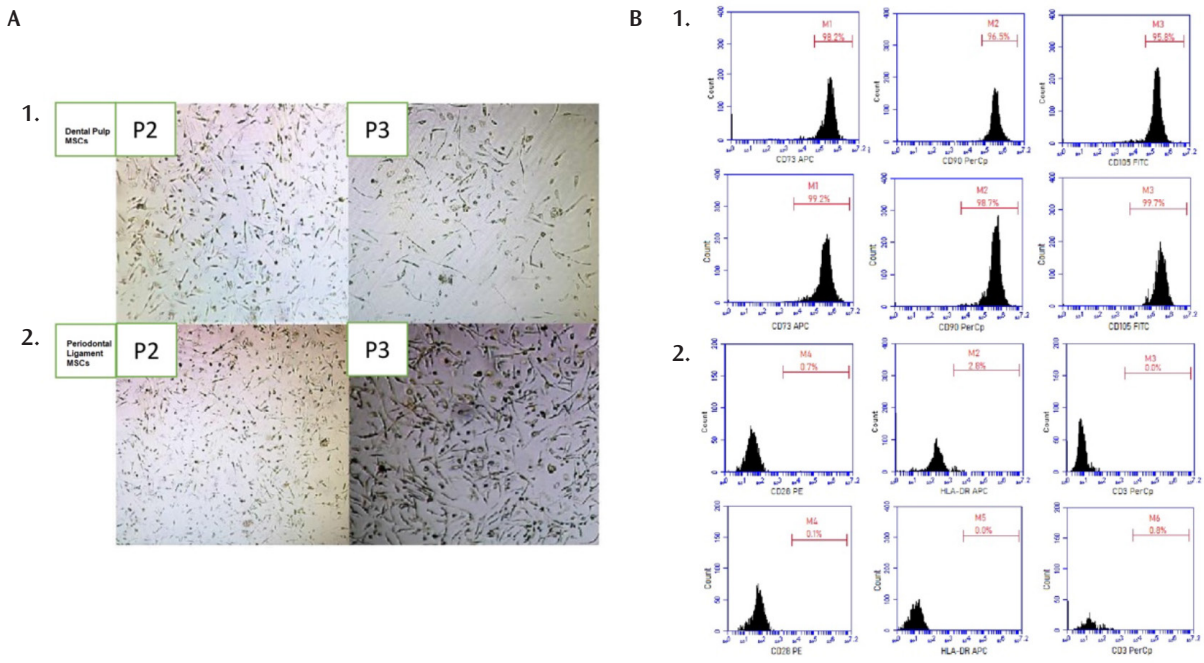

Figure 1. Characterization of MSCs. **A)** The fibroblast-like colony formation of A1) DP-MSCs or A2) PL-MSCs in the second and third passages. **B)** The cell surface markers for MSCs B1) DP-MSCs or B2) PL-MSCs expressed positive markers (CD73, CD90, CD105) over 95%, and lack the expression of hematopoietic markers.

PL-MSCs: mesenchymal stem cells derived from human placenta, DP-MSCs: mesenchymal stem cells from the exfoliated decidu-ous teeth dental pulp

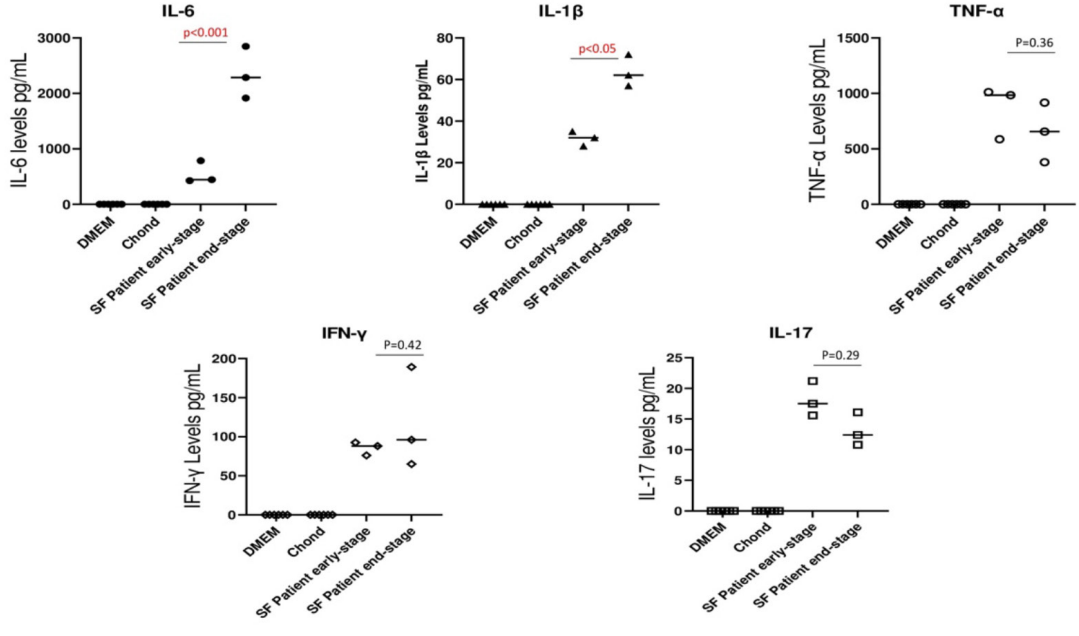

Figure 2. Cytokine levels of SF samples of RA patients. IL-1β and IL-6 levels were significantly high in the end-stage RA-SF samples compared to the early-stage patients ($p<0.05$ and $p<0.001$, respectively). TNF-α, IFN-γ and IL-17 levels were closer in the end-stage and the early-stage RA-SF samples, and no significant difference was observed ($p>0.05$).

RA: rheumatoid arthritis, RA-SF: rheumatoid arthritis synovial fibroblasts, TNF-α: tumor necrosis factor-alpha, IFN-γ: cytokine interferon-gamma, IL: interleukin

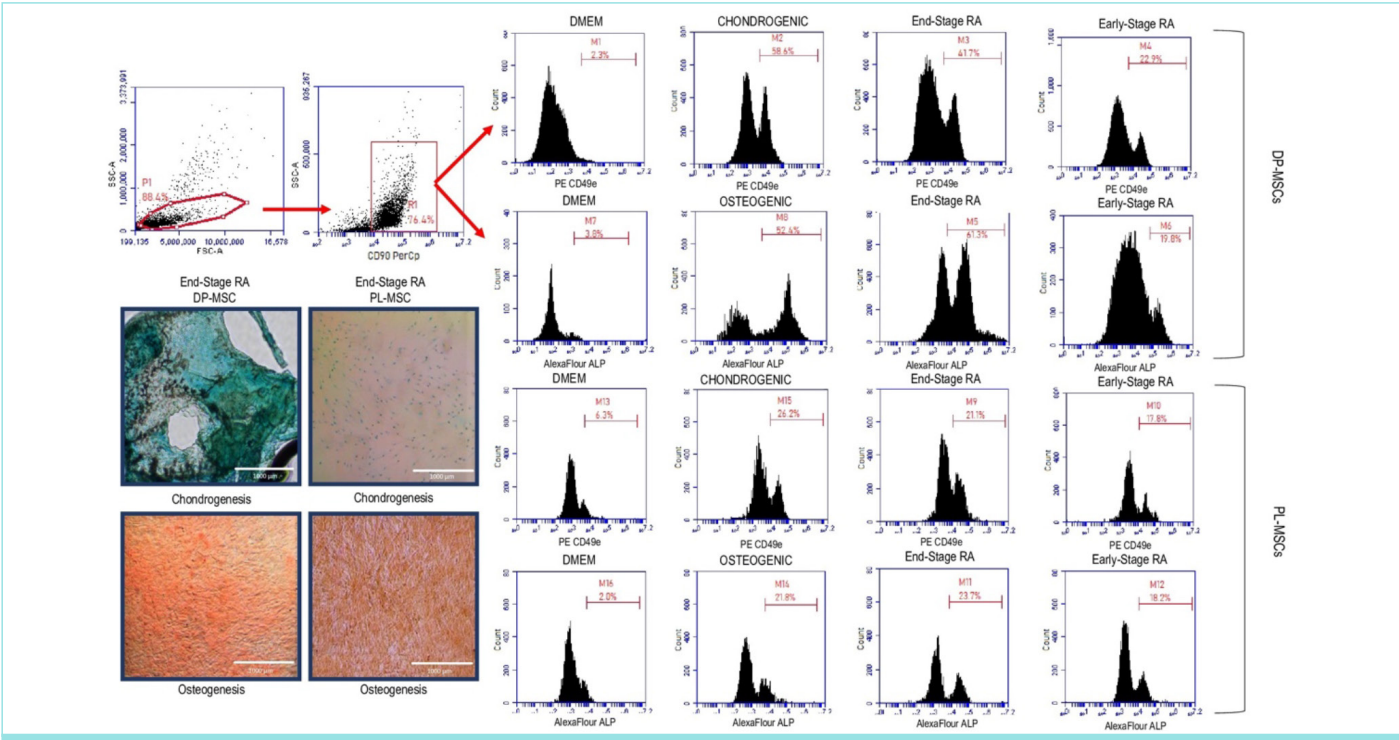

Figure 3. The analysis of the differentiation potential of DP- and PL-MSCs. Gating strategy for positive cell surface markers of DP- or PL-MSCs. CD90+ cells were gated from the total cell population to analyze the CD49e or ALP ratio in DP- or PL-MSCs from 21-day of cultured cells. Positive cell surface markers of chondrogenic (CD49e) or osteogenic (ALP) differentiation of MSCs. The differentiation potential of DP- or PL-MSCs cultured with end-stage RA-SF samples. DP-MSCs showed cartilage formation and tight clusters with high intensity of calcium deposits, while PL-MSCs showed sparse colonies of chondrocytes and looser clusters of osteogenic colonies.

PL-MSCs: mesenchymal stem cells derived from human placenta, DP-MSCs: mesenchymal stem cells from the exfoliated decidu-ous teeth dental pulp, RA-SF: rheumatoid arthritis synovial fibroblasts

the SF samples of the early-stage RA patients, but the chondrogenic differentiation marker of CD49e was significantly higher in DP-MSCs (39.2±2.5) compared to those in the PL-MSCs (25.7±3.8) ($p<0.05$). As a result, DP-MSCs fully participated in the osteogenic and chondrogenic differentiation process with high expression of osteogenic and chondrogenic markers and formed osteogenic colonies and cartilage, while PL-MSCs weakly differentiated into osteogenic or chondrogenic lineage in the end-stage and early-stage RA-SF microenvironments (Figures 3 and 4).

The Neutralization of IL-1b Tended to Downregulate the Osteogenic Differentiation of DP-MSCs

To evaluate the effects of cytokine profiles on the osteogenic or chondrogenic differentiation potential of DP-MSCs in which ALP or CD49e expressions were observed in cells cultured with end-stage RA-SF, we performed neutralization assays for the highly secreted cytokines IL-1β and IL-6.

The osteogenic marker expression tended to decrease with the neutralization of IL-1β in DP-MSCs (48.6±3.9) when compared with un-neutralized cultures (57.7±2.7) ($p>0.05$), and the neutralization of IL-6 tended to decrease the ALP expression of DP-MSCs (49.3±2.8), but no significant change was observed compared to un-neutralized cultures ($p>0.05$).

The chondrogenic marker expression of DP-MSCs slightly reduced with the neutralization of IL-1β (54.4±1.7) or IL-6 (46.7±3.1), but no significant difference was observed when compared with un-neutralized cultures ($p>0.05$) as shown in Figure 5.

DISCUSSION

MSCs are adult stromal cells which can be isolated from bone, adipose tissue, umbilical cord, placenta or dental tissues. These cells have the capacity to differentiate into a variety of tissue types, including cartilage, tendon, fat and muscle[14]. In addition to their multiple differentiation capacity, MSCs are candidates for the cellular treatment of many inflammatory diseases, with the anti-inflammatory responses they generate in inflammatory conditions[15,16]. In addition to suppressing inflammation, their use in damaged tissue regeneration has been shown in previous studies[17-19]. In the present study, we investigated the differentiation capacity of periodontal ligament and dental pulp MSCs in the synovial fluid microenvironment aspirated from two distinct stages of patients with RA to investigate the cellular therapeutic benefits of these cells in cartilage or bone damage in RA.

MSCs can develop variable anti-inflammatory or regenerative responses depending on the source from which they are isolated and the inflammatory conditions in which they are found. In previous studies, it has been reported that umbilical cord MSCs (UC-MSCs) support M2 macrophage differentiation to a greater extent compared to bone marrow MSCs, thus exhibiting a high regulatory effect on immune responses[20]. In a study conducted on bone regeneration, it was shown that, unlike BM-MSCs, UC-MSCs were persistent in implants for up to

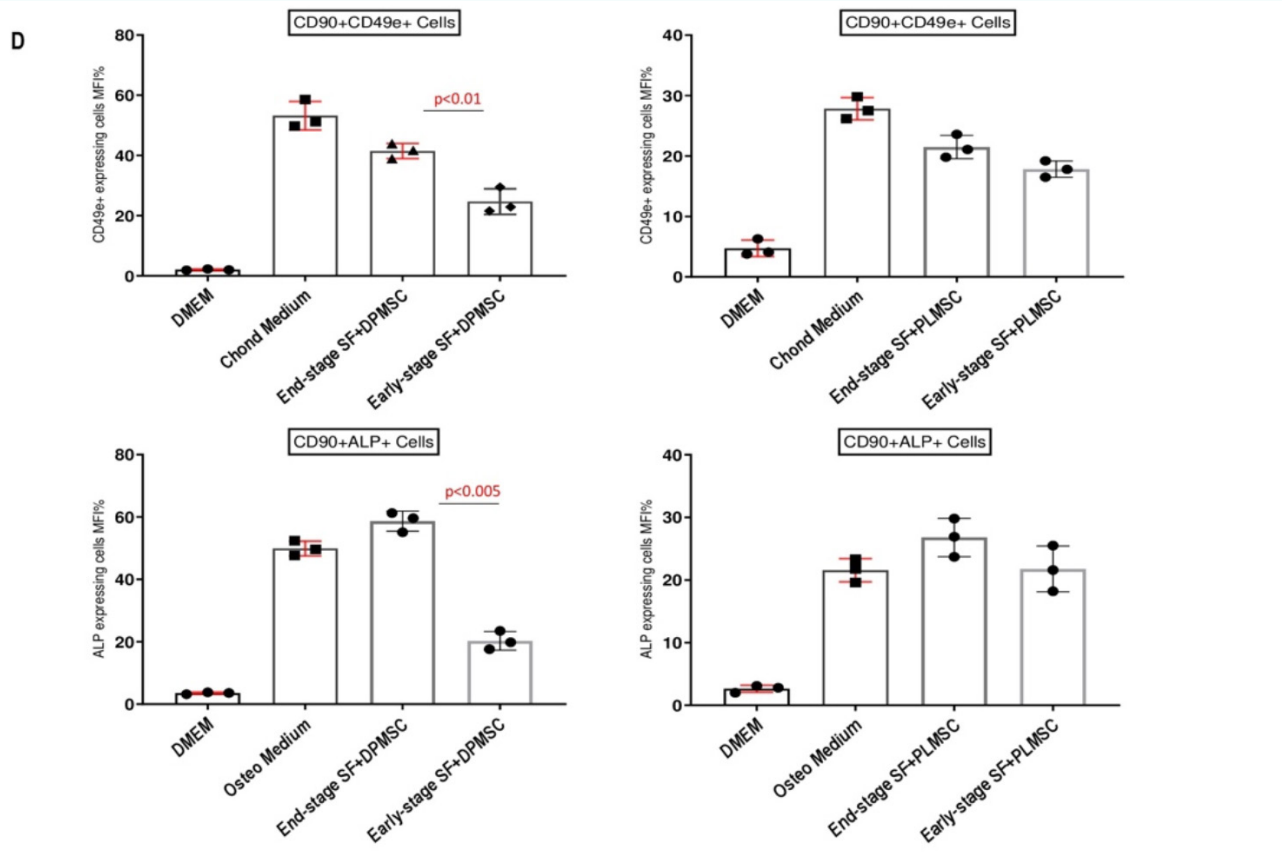

Figure 4. The statistical analysis of cell surface markers for osteogenic or chondrogenic differentiation of DP- and PL-MSCs. The ratio of chondrogenic and osteogenic differentiation markers (CD49e and ALP) in the flow cytometry analysis were significantly high in DP-MSCs cultured with the end-stage RA-SF samples, compared to the early-stage RA-SF samples (CD49e: $p<0.01$, ALP: $p<0.005$).

PL-MSCs: mesenchymal stem cells derived from human placenta, DP-MSCs: mesenchymal stem cells from the exfoliated decidu-ous teeth dental pulp, RA-SF: rheumatoid arthritis synovial fibroblasts

three weeks and achieved bone formation in the local injured site. However, unlike BM-MSCs, UC-MSCs activated the host cells for bone regeneration, highlighting the effects of the microenvironment in determining cell differentiation and response[20,21]. Studies conducted to date have demonstrated the protective effects of intraperitoneally injected MSCs against joint destruction in collagen-induced arthritis (CIA) in RA and similar inflammatory arthritis models. In one of these studies, it was reported that after intravenous injection of bone marrow or UC-MSCs, recovery occurred in 3–6 months and this effect was achieved by increasing the Treg cell population[22]. In addition, it has been shown that locally applied MSCs are more effective than systemic application in tissue regeneration in RA cartilage damage.

In the current study, we found that the cytokine profiles of patients with RA change the differentiation potential of PL-MSCs and DP-MSCs towards chondrogenesis or osteogenesis. DP-MSCs differentiate to osteogenic colonies and expressed a high ratio of ALP in the SF samples of the end-stage patients with RA with high levels of IL-1β and IL-6. PL-MSCs showed slight osteogenic differentiation potential in the same inflammatory niche. These data indicate the variable differentiation potentials of PL-MSCs and DP-MSCs in the inflammatory microenvironment of RA-SF. Also, these results demonstrate that DP-MSCs are a more suitable source for MSCs for cartilage and bone repair in the RA-SF microenvironment since RA is an inflammatory disease characterized by cartilage and bone damage. Additionally, the differentiation potential of MSCs may change with the origin of the source and due to the presence of progenitor cells of different types[23]. Although the mechanisms that determine the osteogenic behavior of adipose tissue MSCs and bone marrow MSCs are complex, their differentiation potential is entirely dependent on the inflammatory cytokines secreted in the damaged tissue. In one study, BM-MSCs were shown to downregulate osteogenic differentiation in an environment with inflammatory cytokines, such as IL-6 and TNF-α[24]. In contrast, IL-6 was shown to support the differentiation of BM-MSCs towards osteogenesis and was mentioned as a central mediator of bone homeostasis, and it was shown to positively affect skeletogenesis and bone formation[25]. In another study, it was concluded that IL-1β activates osteogenic differentiation of BM-MSCs with the activation of the BMP/Smad pathway[26]. However, contrasting results have been reported for the effects of IL-1β on the osteogenic differentiation capacity of murine BM-MSCs. In a previous study, IL-1β or TNF-α stimulation of bone marrow MSCs inhibited the osteogenic differentiation potential *in vitro*[27]. These results in the previous studies indicate that the differentiation potential of MSCs may be influenced by distinct inflammatory conditions or the source of the MSCs. We, therefore, conducted neutralization assays for the highly secreted IL-1β and IL-6 cytokines, which were at the same time prominent cytokines in RA. Although statistically significant change was not observed, the slight downregulation of osteogenic and

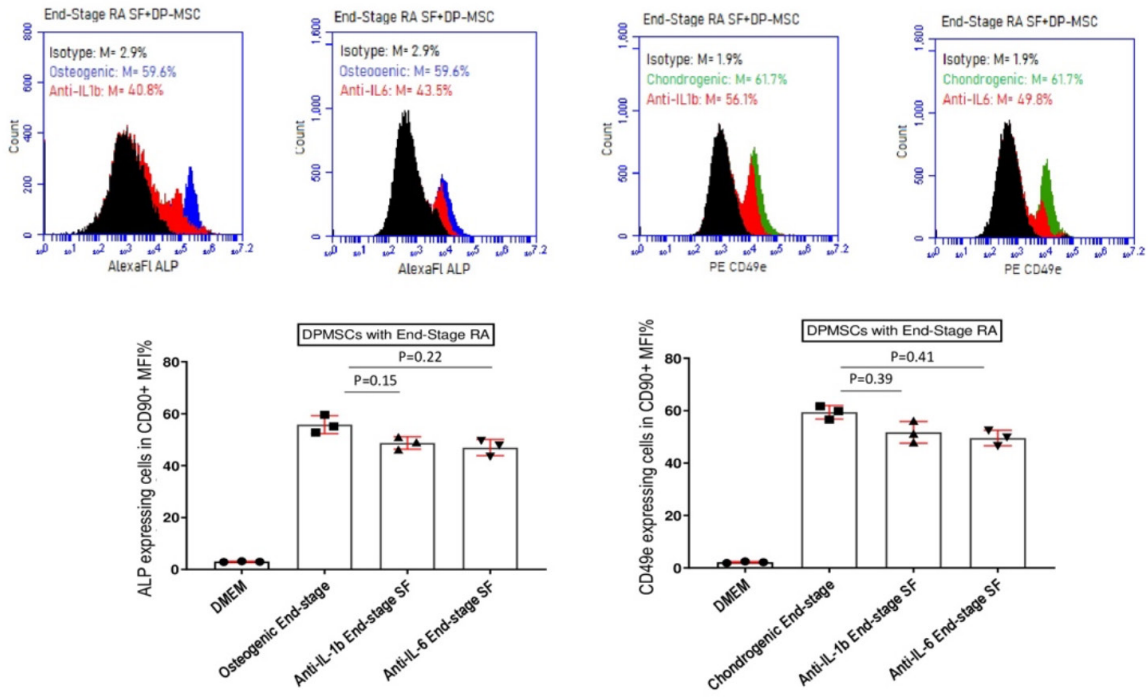

Figure 5. Neutralization of IL-1β and IL-6 in end-stage RA-SF samples cultured with DP-MSCs. The neutralization of IL-1β and IL-6 tended to decrease the CD49e or ALP expression ratio of CD90+ cell population in the 21 days of the culture period.

IL: interleukin, RA-SF: rheumatoid arthritis synovial fibroblasts

chondrogenic markers in DP-MSCs due to neutralization of IL-1β or IL-6 suggests that these cytokines may also be important in DP-MSCs differentiation potential.

CONCLUSION

The present study shows that the DP-MSCs can differentiate towards osteogenic cells in the end-stage RA-SF microenvironment, but the osteogenic or chondrogenic differentiation potential of PL-MSCs is limited in the RA-SF niche. There may be other inflammatory factors, such as exosomes, inflammatory molecules or proteins, which play a role in the differentiation process. Thus, further analysis can be performed to highlight the differentiation mechanisms in DP-MSCs or PL-MSCs. In addition, DP-MSCs may be used for damaged tissue regeneration in patients with RA with bone or cartilage erosions, and for this purpose, further *in vivo* studies can be performed.

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MAIN POINTS

- Dental pulp mesenchymal stem cells can differentiate both into osteocytes or cartilage in the inflammatory synovial fluid microenvironment.
- Periodontal ligament mesenchymal stem cells can differentiate into chondrocytes in the inflammatory microenvironment of synovial fluid.
- Dental pulp mesenchymal stem cells can be a candidate for bone or cartilage repair in rheumatoid arthritis.

ETHICS

Ethics Committee Approval: This study was approved by Muğla Sıtkı Koçman University Clinical Research Ethics Committee with the number of 02/IV, 30.01.2020.

Informed Consent: All participants gave informed consent to participate.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.G., Design: D.G., Data Collection and/or Processing: S.S., A.A., E.F.T., Analysis and/or Interpretation: D.G., B.G., Literature Search: D.G., B.G., S.S., A.A., E.F.T., Writing: D.G.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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