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Factors secreted from dental pulp stem cells show multifaceted benefits for treating experimental rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial hyperplasia and chronic inflammation, which lead to the progressive destruction of cartilage and bone in the joints. Numerous studies have reported that administrations of various types of MSCs improve arthritis symptoms in animal models, by paracrine mechanisms. However, the therapeutic effects of the secreted factors alone, without the cell graft, have been uncertain. Here, we show that a single intravenous administration of serum-free conditioned medium (CM) from human deciduous dental pulp stem cells (SHED-CM) into anti-collagen type II antibody-induced arthritis (CAIA), a mouse model of rheumatoid arthritis (RA), markedly improved the arthritis symptoms and joint destruction. The therapeutic efficacy of SHED-CM was associated with an induction of anti-inflammatory M2 macrophages in the CAIA joints and the abrogation of RANKL expression. SHED-CM specifically depleted of an M2 macrophage inducer, the secreted ectodomain of sialic acid-binding Ig-like lectin-9 (ED-Siglec-9), exhibited a reduced ability to induce M2-related gene expression and attenuate CAIA. SHED-CM also inhibited the RANKL-induced osteoclastogenesis *in vitro*. Collectively, our findings suggest that SHED-CM provides multifaceted therapeutic effects for treating CAIA, including the ED-Siglec-9-dependent induction of M2 macrophage polarization and inhibition of osteoclastogenesis. Thus, SHED-CM may represent a novel anti-inflammatory and reparative therapy for RA.

1. Introduction

The precise etiology of rheumatoid arthritis (RA) remains unclear, however monocyte lineage cells, including macrophages and osteoclasts, respectively play central roles in the inflammation and bone resorption of RA [1,2]. Recent studies indicate that differentially activated macrophages are involved in the pathophysiology of various types of intractable diseases [3]. The pro-inflammatory M1 cells and anti-inflammatory M2 cells are thought to represent the extreme activation states on each end of a continuum [3–5]. Classically activated M1 cells initiate inflammation, promote osteoclast differentiation, and accelerate tissue damage by releasing high levels of pro-inflammatory cytokines, reactive oxygen species, and nitric oxide [6]. In contrast, M2 cells counteract pro-inflammatory M1 conditions by secreting anti-inflammatory cytokines and scavenging cellular debris. In general wound repair, M1- and M2-like cells are involved in initiating and resolving inflammation, respectively [7]. Thus, strategies designed to modulate macrophage polarity may provide significant therapeutic benefits for RA.

Stem-cell transplantation represents a new therapeutic strategy for treating RA [8,9]. The transplantation of mesenchymal stem cells (MSCs) derived from bone marrow [10], adipose tissue [11], umbilical cord [12], or gingival tissue [13] improves arthritis symptoms in mouse models of RA *via* paracrine, trophic, and/or immunomodulatory mechanisms. However, the therapeutic effects of soluble factors secreted from various types of MSCs have not been reported.

Human adult dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs) are self-renewing MSCs residing within the perivascular niche of the dental pulp [14–16]. These cells are thought to originate from the cranial neural crest, and express both MSC and neural stem cell markers [17]. Studies of engrafted SHEDs and DPSCs in various animal disease models, including myocardial infarction [18], systemic lupus erythematosus [19], ischemic brain injury [20,21], and spinal cord injury [17–22,23], demonstrated that these cells can promote significant recovery through paracrine mechanisms, which enhance endogenous tissue-repairing activities [24]. We recently reported the therapeutic effects of intrathecally administered serum-free conditioned medium derived from SHED (SHED-CM) for severe spinal cord injury in rat. The efficacy of SHED-CM was associated with an immunoregulatory activity that induced anti-inflammatory M2-like macrophages through the synergistic action of monocyte chemoattractant

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Table 1
Mouse primers for real time q-PCR.

Primer	Sequence (forward 5′–3′)	Sequence (reverse 5′–3′)
GAPDH	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
TRAP	TCCTGGCTCAAAAAGCAGT	ACATAGCCCACACCGTTCTC
Cathepsin K	CAGCAGAGGTGTGTACTATG	GCGTTGTTCTTATTCCGAGC
NFATc1	CGGGAAGAAGATGGTGCTGT	TTGGACGGGGCTGGTTAT
RANK	CGAGGAAGATTCCCACAGAG	CAGTGAAGTCACAGCCCTCA
RANKL	ATGAAAGGAGGGAGCACGAA	GGAAGGGTTGGACACCTGAA
TNF-α	CCCTTTACTCTGACCCCTTTATTGT	TGTCCCAGCATCTTGTGTTTCT
IL-1β	AGTTGACGGACCCCAAAAGA	ACAGCTTCTCCACAGCCACA
IL-6	CCAAGAACGATAGTCAATTCCAGA	CATCAGTCCCAAGAAGGCAAC
iNOS	AGCCAAGCCCTCACCTACTTC	GCCTCCAATCTCTGCCTATCC
MMP3	GGCCTGGAACAGTCTTGGC	TGTCCATCGTTCATCATCGTCA
MMP9	GGACCCGAAGCGGACATTG	CGTCGTCGAAATGGGCATCT
F4/80	CCAGAAGGCTCCCAAGGAT	TCTGCTCACTTTGGAGTATCAAGTC
CD206	TCTCCCGGAACCGACTCTTC	AACTGGTCCCCTAGTGTACGA
Arginase1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Fizz1	CCAATCCAGCTAACTATCCCTCC	CCAGTCAACGAGTAAGCACAG

protein-1 (MCP-1) and the secreted ectodomain of sialic acid-binding Ig-like lectin-9 (ED-Siglec-9) in SHED-CM [25]. However, the therapeutic potential of SHED-CM for RA has not been examined.

Here, we examined the therapeutic benefits of SHED-CM for arthritis induced by an anti-collagen type II antibody (CAIA). CAIA is a rapid arthritis model, which represents the effector phase of arthritis. Our data showed that SHED-CM improved arthritis symptoms and inhibited tissue damage by inducing M2 macrophage polarization in CAIA mice. Our findings suggested that SHED-CM has multiple therapeutic anti-inflammatory effects and inhibits osteoclastogenesis in mice. Thus, SHED-CM may represent a novel and powerful approach for treating RA and other inflammatory and autoimmune diseases.

2. Materials and methods

2.1. Cells

Human SHEDs were isolated as described previously [16,17]. Briefly, exfoliated deciduous teeth (from 6- to 12-year-old individuals) were collected at Nagoya University Hospital, under approved guidelines set by Nagoya University (H-73, 2003). Ethical approval was obtained from the Ethics Committee of Nagoya University (permission number 8-2). All study participants provided written informed consent. After separating the crown and root, the dental pulp was isolated and then digested in a solution containing 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 h at 37 °C. Single-cell suspensions ($1–2 \times 10^4$ cells/ml) were plated on culture dishes in DMEM supplemented with 10% fetal bovine serum, then incubated at 37 °C in a humidified atmosphere of 5% CO_2.

BMSCs (from 20- to 22-year-old individuals) at passage 5 were obtained from Lonza.

Table 2
Therapeutic factors in SHED-CM for RA.

	Ratio (vs DMEM)	Ratio (vs BMSC-CM)	References
Immunosuppression			
HGF	23.28	3.21	29
IL-22	10.20	3.31	30
Furin	3.88	3.18	33
Anti-inflammation			
IL-1Ra	0.93	0.94	31
RAGE	1.67	1.96	32
Anti-osteoclastogenesis			
OPG	412.05	3.70	28
Macrophage differentiation			
MCP-1	120.25	1.54	25
ED-siglec-9	2.08	1.79	25

2.2. Preparation of conditioned medium (CM)

At passage 5–9, SHEDs or BMSCs at 70–80% confluence were washed with PBS and serum-free DMEM for two times, then the culture medium was replaced with DMEM. After 48 h incubation at 37 °C in a humidified atmosphere of 5% CO_2, the medium was collected and centrifuged for 3 min at 440 *g* at 4 °C. The supernatants were collected and centrifuged for 3 min at 1750 *g* at 4 °C. The supernatants were used as CM for CAIA treatment or assays.

2.3. Animals

Male 8-week-old DBA/1 J mice were obtained from Japan SLC. The animals were housed in plastic cages (4 mice per cage), with wood tip bedding, and maintained under specific pathogen-free conditions in a temperature- and humidity-controlled room (23 ± 2 °C and 55 ± 10%, respectively) under a fixed 12 h light/dark cycle (9:00 a.m. to 9:00 p.m.). The animals were given free access to standard laboratory food and water. The animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Nagoya University School of Medicine.

2.4. Induction of arthritis and experimental design

The arthritogenic mouse anti-collagen type II monoclonal antibody (mAb) cocktail (Chondrex), contains equal amounts of 5 mAbs (A2-10, F10-21, D8-6, D1-2G, and D2-112). On day 0, the mice were intraperitoneally injected with 1.5 mg of the cocktail. On day 3, the mice were intraperitoneally injected with 50 μg of lipopolysaccharide (LPS). On day 5, 500 μl of SHED-CM, BMSC-CM, or serum-free DMEM was injected into the tail vein of CAIA mice. On day 7 or 14, at least five mice per treatment group were sacrificed under deep anesthesia. From days 0 to 14, the mice were blindly inspected for disease progression (Fig. 1A).

2.5. Arthritis evaluation

From days 0 to 14, the mice were blindly inspected for disease progression. The severity of arthritis in each paw was graded on a scale of 0–4, as follows: 0, normal; 1, mild swelling; 2, moderate swelling; 3, severe swelling; 4, pronounced edema of the entire paw. The cumulative score from 4 paws (maximum score of 16 per mouse) was used as the overall disease score.

2.6. Histology

On day 14, the mice were sacrificed and the hind paws were fixed in 4% paraformaldehyde, decalcified in 10% EDTA for 3 weeks, and embedded in paraffin. Serial 5-μm sections were stained with hematoxylin and eosin (H–E) and toluidine blue, and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit according to the manufacturer's instructions (Sigma-Aldrich).

2.7. Histological scoring

The samples were evaluated for synovial inflammation, bone erosion, and cartilage damage in a blinded manner, as described previously, with minor modifications [26].

Synovial inflammation was scored as follows: 0, no inflammation; 1, slight thickening of the lining layer with some infiltrating cells in the sublining layer; 2, moderate thickening of the lining layer with a moderate number of infiltrating cells in the sublining layer; 3, extensive thickening of the lining layer with a moderate number of infiltrating cells in the sublining layer and the presence of inflammatory cells in the synovial space; 4, substantial influx of inflammatory cells into the synovium.