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Original Article

Protective effects of mesenchymal stem cell cond tional medium against inflammatory injury on human gingival fibroblast

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Abstract: Mesenchymal stem cells (MSCs) alleviate oxidative stress in kidney, lung and heart by secreting bioactive factors. Human gingival fibroblasts (HGFs) are important for repairing periodontal tissues. This study observed protective effects of MSCs conditional medium (MSCCM) on HGFs against inflammatory injury, to investigate anti-inflammatory function and mechanism of MSCCM on HGFs. Primary cultured HGFs were identified for sources by immunohistochemistry (IHC)-SP assay. *In vitro* cultured MSCs were extracted for MSCCM, which was used to pre-treat HGFs with inflammatory induction by 10 μg/L IL-6 or TNF-α for 4 h. ELISA quantified transforming growth factor (TGF)-β level in the supernatant, and superoxide dismutase (SOD) plus malondialdehyde (MDA) levels were measured by colorimetry. RT-PCR measured keratocyte growth factor (KGF) mRNA expression, and flow cytometry or Western blot measured apoptosis or Caspase-3 expression in HGFs, respectively. Compared to control group, MSCCM treatment group showed no significant change of SOD, MDA, TGF-β level, cell apoptosis, KGF mRNA or Caspase-3 expression ($P>0.05$). Inflammation treatment elevated all those indexes but decreased SOD ($P<0.05$). Compared to model group, MSCCM treatment further decreased these factors but increased SOD level ($P<0.05$). No significant difference was found between IL-6 and TNF-α treated cells. MSCCM can partially inhibit IL-6 induced inflammatory injury of HGFs via suppressing Caspase-3 and KGF expression.

Keywords: Mesenchymal stem cell conditional medium, human gingival fibroblast, cytokine

Introduction

Mesenchymal stem cells (MSCs) have potency for tissue repair and secretion, and its paracrine function is the major component for exerting repair functions. MSCs transplantation has certain specific differentiation potency in pulmonary edema model, and their paracrine function can improve pulmonary edema and alleviate pulmonary alveolar wall cell apoptosis [1, 2]. MSCs can alleviate oxidative stress injury of kidney, lung and heart tissues via secreting bioactive factors, and can alleviate oxidative stress injury of neuron cell line PC12 via synthesis and secretion of bioactive substances [3, 4]. However, its related functional roles have not been completely illustrated. In periodontal mesenchymal tissues, human gingival fibroblasts (HGFs) play the dominant role. HGFs are critical for maintain normal structure of gingival structures and remodeling, and are closely correlated with long-term stability of oral implant. Previous studies showed the participation of cytokines such as interleukin (IL) in body inflammation and post-trauma repair [5, 6]. Serum or gingival crevicular fluid IL-5 and IL-1β levels showed statistical significant difference between normal people and periodontitis patients. Occurrence and progression of periodontitis is correlated with inflammatory factors, as multiple inflammation mediators such as IL-6 and TNF-α can be detected in periodontal lesions. IL-6 can affect proliferation of osteoblast and suppress osteoclast cell production during bone reabsorption process, thus aggravating the condition of periodontitis [7, 8].

Previous study showed significant role of MSCs in ischemic heart disease such as angina pec-

Table 1. Primer sequence

Primer	Size	Forward sequence	Reverse sequence
KGF mRNA	381 bp	5'-GCAAAGTAAAAGGGACCCAAGAGA-3'	5'-CCAGTTCXAGCAGGGAGATTTCT-3'
Gapdh	307 bp	5'-TGAACGGGAAGCTCACTGG-3'	5'-TCCACCACCCTGTTGCTGGA-3'

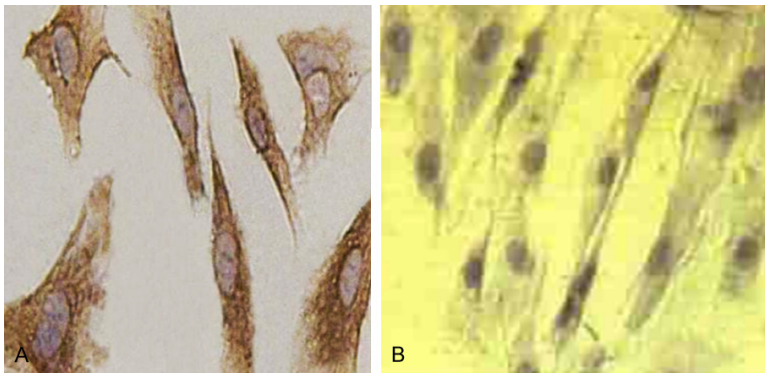

Figure 1. Identification of cell origin (×400). A. Positive staining for Vimentin; B. Negative staining for keratin.

toris or myocardial infarction. MSCs conditional medium (MSCCM) contains factors secreted by MSCs, which can alleviate oxidative stress injury. MSCCM can inhibit collagen synthesis of proliferative scar fibroblast cells or cell proliferation, but with minor effects on normal fibroblast, thus benefiting trauma healing and suppressing of scar formation. However, detailed mechanisms of MSCCM have not been completely illustrated [9, 10]. During the formation and regeneration process of tooth-implant interface, HGFs play critical roles, and their effective adhesion and proliferation are critical for repair of inflammation in peripheral tissues of tooth implant. Whether such paracrine role of MSCs can alleviate apoptosis of HGFs caused by inflammation damage is still unknown. This study thus observed the protective role of MSCCM on HGFs with inflammatory injury, to discuss potential role of MSCCM against inflammatory damage of HGFs and related mechanisms.

Materials and methods

Reagent and equipment

DMSO, high glucose DMEM, trypsin, sulfy-rhodamine B, fetal bovine serum (FBS), 2% type IV collagenase were purchased from Kemiou Chem (China), Gibco (US), Sigma (US), Boster Bio (China) and Gibco (US). Test kit and antibody including SOD, MDA kits, TGF-β ELISA kits, anti-keratin, anti-Vimentin, and rabbit anti-rat actin antibody were purchased from Jiancheng Bio (China) and Zhongshan Jinqiao Biotech (China). Cell apoptosis test kit (Roche, US), caspase-3 polyclonal antibody (Cell Signal, US). KGF primer (Invitrogen, US), reverse transcription kit, Bioneer amplificaoitn kit (Zhongshan Jinqiao Biotech, China). Major equipment including S570 scanning electron microscope, UV-2550 ultraviolet spectrometry was provided by Hitachi (Japan) and Shimadzu (Japan).

Primary culture of tissue cubes from HGFs using improved approach

Healthy gingival tissues collected from impacted tooth removal surgery in patients between 12 and 18 years old. Tissues were rinsed in Hank's solution containing dual antibiotics. Epidermal tissues were removed and cut into 1 mm^3 cubes, which were digested in trypsin and were centrifuged. Supernatants were added into FBS-containing medium for 37°C humidified incubation with 5% CO_2. Cell growth status was observed under an inverted microscope. Cells at 4^{th} to 8^{th} phase were used for further assays.

MSCCM preparation

With reference to previous method [11, 12], MSCs at third generation were collected for passage. After 24 h, culture medium was discarded and changed for serum-free medium. MSCs were placed in hypoxic and ischemic conditions for 48 h incubation. Culture medium was then collected, centrifuged and filtered.

Identification of HGFs origin

Cell motility was observed under an inverted microscope. IHC-SP approach was used to

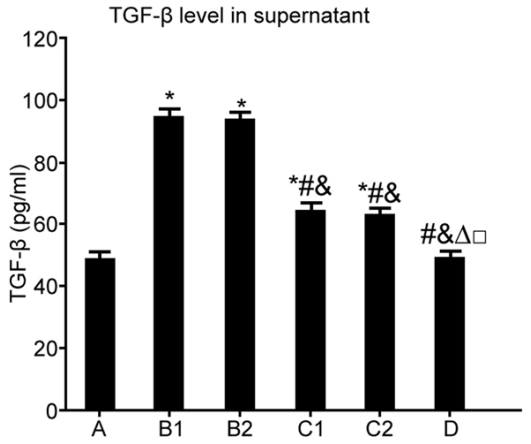

Figure 2. TGF-β levels in supernatant. A: Control group; B1: Model group 1; B2: Model group 2; C1: Model 1 + MSCCM group; C2: Model 2 + MSCCM group; D: MSCCM group. *, P<0.05 compared to group A; #, P<0.05 compared to group B1; &, P<0.05 compared to group B2; Δ, P<0.05 compared to group C1; □, P<0.05 compared to group C2.

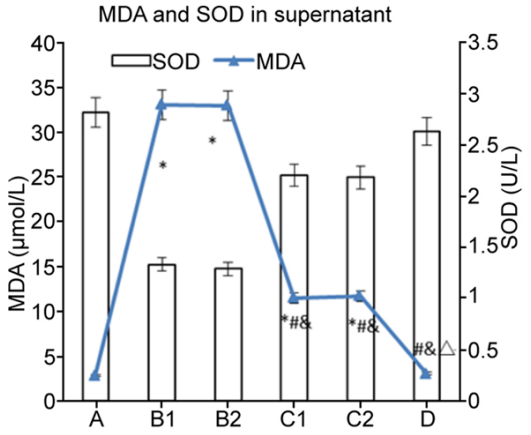

Figure 3. SOD and MDA levels in supernatant. A: Control group; B1: Model group 1; B2: Model group 2; C1: Model 1 + MSCCM group; C2: Model 2 + MSCCM group; D: MSCCM group. *, P<0.05 compared to group A; #, P<0.05 compared to group B1; &, P<0.05 compared to group B2; Δ, P<0.05 compared to group C1; □, P<0.05 compared to group C2.

detect expression of anti-keratin (1:100) and anti-Vimentin (1:100), followed by DAB development to determine cell origin.

Grouping

All cells were divided into six groups. In brief, HGFs were inoculated into sterile 6-well plate with sterile coverslips (5×10^5 cells per well). 2 ml complete medium was added into each well. Until reaching 50-70% confluence, cells were divided into group A (control group with serum-free medium only), group B1 (model group 1, with 10 μg/L IL-6 in serum-free medium), group B2 (model group 2, with 10 μg/L TNF-α in serum-free medium), group C1 (model group 1 + MSCCM, treated with 10 μg/L IL-6 in serum-free medium after MSCCM pre-treatment), group C2 (model group 2 + MSCCM, with 10 μg/L TNF-α in serum-free medium after MSCCM pre-treatment), group D (MSCCM group, with MSCCM in serum-free medium for pretreatment). Group C and D received MSCCM pre-treatment for 24 h. Group B1, C1 and B2, C2 received 10 μg/L IL-6 or TNF-α treatment for 4 h to mimic inflammatory injury model of fibroblast. Supernatant was collected from each group.

ELISA indexes in supernatant

ELISA was used to determine TGF-β level in supernatant following manual instruction. A microplate reader was used to test absorbance values at 540 nm wavelength. Standard curves were used to deduce sample concentration (pg/ml). Colorimetry quantified SOD and MDA level following manual instruction of test kit.

RT-PCR for KGF mRNA expression

Cultured supernatant was used to test total RNA by TransZol Up approach. Using cDNA by reverse transcription as the template, PCR amplification was performed under the following conditions: 95°C pre-denature for 10 min, followed by 40 cycles each containing 95°C denature for 10 s, 55°C annealing for 30 s, and 72°C elongation for 45 s. Using β as the internal reference, semi-quantitative analysis was performed by Rotor-Gene Q Series Software in triplicated experiment for each sample, using primers sequence as shown in **Table 1**.

Flow cytometry for HGFs apoptosis

Annexin V/PI double staining was used to test apoptotic cells following manual instruction. Five randomly selected fields were used to calculate apoptotic index (AI).

Western blot for Caspase-3 expression

Culture supernatant was mixed with RIPA lysis buffer to extract proteins, whose concentration was determined by Bradford method. Proteins were separated by SDS-PAGE, and were transferred to PVDF membrane. Following blocking

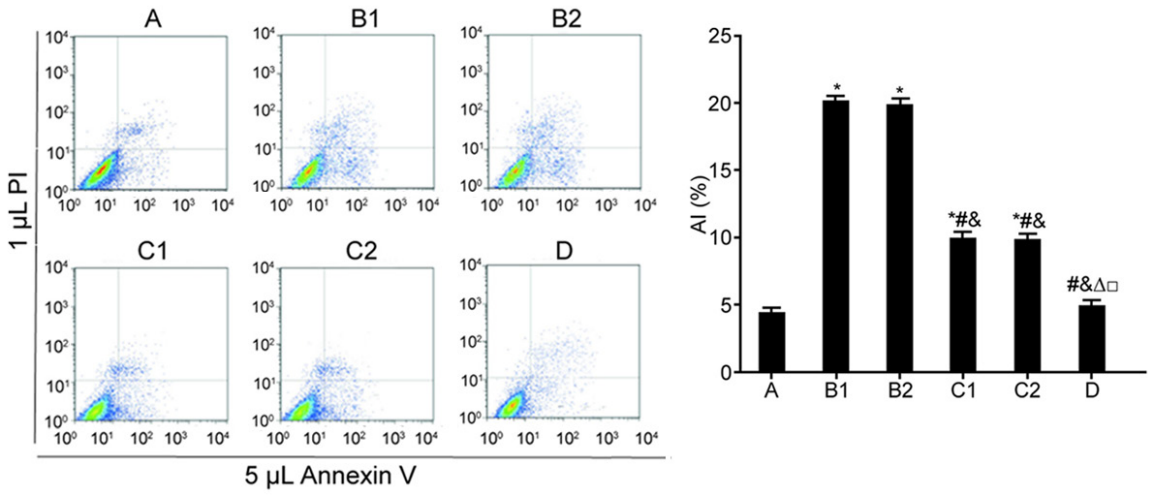

Figure 4. Apoptosis of HGFs. A: Control group; B1: Model group 1; B2: Model group 2; C1: Model 1 + MSCCM group; C2: Model 2 + MSCCM group; D: MSCCM group. *, P<0.05 compared to group A; #, P<0.05 compared to group B1; &, P<0.05 compared to group B2; Δ, P<0.05 compared to group C1; □, P<0.05 compared to group C2.

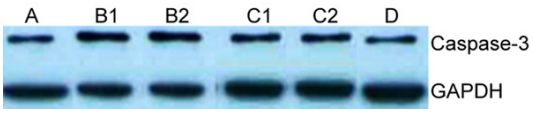

Figure 5. Western blot for Caspase-3 expression.

in defatted milk powder, Caspase-3 antibody (1:100) was added for overnight incubation, and was rinsed in TBST. Secondary antibody (1:200) was added for 1 h incubation, followed by development and exposure. Quantity One image analysis system was used to analyze color bands, which were shown as relative expression level as the gray value of target protein against that of β-actin.

Statistical methods

SPSS 19.0 software was used for analysis, Measurement data were tested for normality first, and those fitted normal distribution were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means among multiple groups. LSD test was used for within-group comparison. A statistical significance was defined when P<0.05.

Results

Identification of cell origin

IHC staining was performed on HGFs. Most cells showed positive staining for Vimentin, with dark-yellow cytoplasm but no staining in nucleus, and negative staining for keratin. All these results indicated cell originated from mesoderm, which fits the immune feature of gingival fibroblast (**Figure 1**).

SOD, MDA and TGF-β levels in supernatants

Compared to group A, group D had no significant difference in SOD, MDA or TGF-β levels (P>0.05). Group B1, B2, C1 and C2 had higher MDA and TGF-β levels but decreased SOD levels (P<0.05). No difference was observed between group B1 and B2, or between C1 and C2. Comparing to group B1 or B2, group C1 and C2 had lower MDA and TGF-β levels but higher SOD levels (P<0.05, **Figures 2** and **3**).

HGFs apoptosis

Compared to group A, group D had no significant difference of cell apoptotic rate (P>0.05), whilst group B1, B2, C1 and C2 had enhanced apoptosis (P<0.05). Compared to group B, group C had decreased apoptosis (P<0.05). No statistical significance was found between group B1 and B2, or between group C1 and C2 (P>0.05, **Figure 4**).

KGF mRNA and Caspae-3 expression

Compared to group A, group D showed no significant difference of KGF mRNA or Caspae-3 expression (P>0.05). Group B1, B2, C1 and C2 had elevated KGF mRNA and Caspase-3 ex-

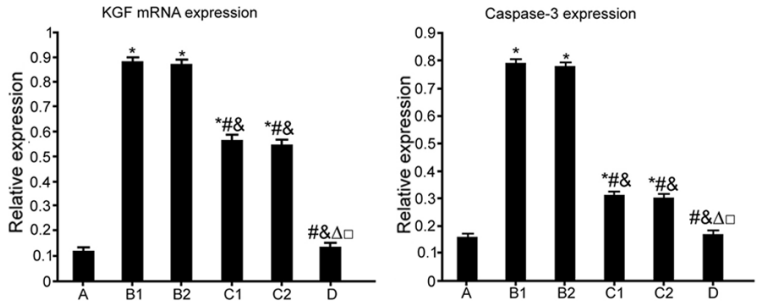

Figure 6. Relative expression of KGF mRNA and Caspase-3. A: Control group; B1: Model group 1; B2: Model group 2; C1: Model 1 + MSCCM group; C2: Model 2 + MSCCM group; D: MSCCM group. *, P<0.05 compared to group A; #, P<0.05 compared to group B1; &, P<0.05 compared to group B2; Δ, P<0.05 compared to group C1; □, P<0.05 compared to group C2.

pression (P<0.05). Compared to group B, group C had lower KGF mRNA and Caspase-3 expression (P<0.05). No statistically significant difference was observed between group B1 and B2, and between group C1 and C2 (P>0.05, **Figures 5** and **6**).

Discussion

HGFs play crucial roles in the formation and regeneration of gingival interface of tooth implants. At the early stage of gingival interface healing, HGFs secrete thread-like compounds to cover the surface of tooth implant to facilitate trauma repair via adhesion. After healing of tooth implants, HGFs maintain normal function of biological closed interface, and participate in repair and reconstruction of tooth implants to achieve closure of tooth [13, 14]. Therefore, effective adhesion of HGFs and proliferation play critical roles in repairmen of peripheral inflammation around tooth implant. Currently abundant studies have been performed regarding IL-6 and periodontitis. Periodontal tissue can produce IL-6, which further destructs periodontal tissues via facilitating T lymphocyte differentiation and inhibit fibroblast growth, thus inducing pathological bone reabsorption in periodontitis and playing important roles in inflammatory injury of periodontal tissues [15, 16]. MSCCM has different effects on fibroblast with different origins, as it has minor effects on normal fibroblast, but significantly suppress proliferation of scar-forming fibroblast via changing extracellular matrix constructs by TGF-β/Smad signal pathway, and fight against oxidative stress injury of H9C2 cells by activating MAPK, PI3K/Akt signal pathway against H_2O_2. Such different roles might be related with unique cytokine expression patterns of MSCCM in different cells. However, MSCs transplantation has anti-apoptotic roles in injury organs [17, 18]. This study applied MSCs extracts for assays and ruled out the role of MSCs directed differentiation, in addition to the observation of the effect of MSCs paracrine rules on HGFs inflammatory injury by IL-6 and TNF-α. This study showed elevated MDA level, enhanced Caspase-3 expression or cell apoptosis, plus lower SOD levels in model group. In MSCCM group, MDA level in supernatant was decreased, with depressed Caspase-3 expression or cell apoptosis, plus higher SOD level, indicating that MSCCM could alleviate oxidative stress in HGFs. Such roles might be correlated with initiation of apoptosis via suppressing Caspase-3 activation, indicating that MSCs could exert anti-apoptotic roles under hypoxic conditions via secreting cytokines.

KGF expression is limited in mesenchymal cells only. KGF receptor is expressed by epidermal cells. KGF exerts signal transduction function via specific function on epidermal cells to facilitate their proliferation, and synergistic effects for the interaction of mesenchymal-epithelial cells. Previous study showed that inflammatory mediator can stimulate KGF expression in fibroblast, and facilitate expression of inflammatory mediator or induce KGF expression in autocrine manner via direct function on KGF or TOLL like receptor [19, 20]. Therefore, KGF up-regulation might be related with stimulus of pro-inflammatory factors such as IL-6, and KGF might be involved in inflammatory factor-mediated pathology of periodontal tissues. This study showed elevated KGF mRNA expression in the supernatant of fibroblast inflammatory injury model, and lower KGF mRNA in MSCCM supernatant, indicating possible involvement of KGF in HGFs inflammatory injury induced by inflammation factors such as IL-6 and TNF-α. MSCCM can alleviate HGFs inflammatory injury possibly via inhibiting KGF expression. By down-regulation of KGF gene expression, inflammatory damage of HGFs can be alleviated. In this study,

model group 1 and group 2 had no significant difference among all indexes, indicating that IL-6 or TNF-α induced HGFs inflammatory injury has no specificity. TGF-β can suppress extracellular matrix degradation via facilitating its expression, thus playing important roles in cell proliferation and differentiation [21, 22]. Model group had elevated TGF-β level, and MSCCM can suppress TGF-β expression, which further affects gene transcription of inflammatory factors such as IL-6 and TNF-α, to facilitate their expression in human fibroblast, indicating that MSCCM could suppress TGF-β production via secreting active factors, thus alleviating HGFs inflammatory injury. This study, however, only investigated the protective effects of MSCCM on HGFs with inflammatory injury under interference by inflammatory factors such as IL-6 and TNF-α of HGFs, leaving detailed mechanisms to be further elucidated.

Conclusion

MSCCM can alleviate HGFs inflammatory injury induced by IL-6 or TNF-α via suppressing expression of Caspase-3, KGF and TGF-β.

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Disclosure of conflict of interest

None.

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