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The extracts of mesenchymal stem cells induce the proliferation of periodontal ligament cells

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Transplantation of mesenchymal stem cells (MSCs) has been demonstrated to induce regeneration of periodontal tissues. Stem cell derivatives including paracrine factors, cell extract, and exosomes from MSCs are attracting attention as a novel cell-free treatment. We investigated the effect of cell extract from MSCs (MSC-extract) on the proliferation of periodontal ligament cells (PDLCs). We obtained MSC-extract from immortalized bone marrow-derived MSCs using freeze-thaw cycles and centrifugation. Water-soluble tetrazolium (WST)-8 assay and immunostaining of Ki67 were used to examine cell proliferation and detect cells in a proliferation state, respectively. Cycloheximide (CHX) was used to inhibit protein synthesis of MSCs. In the WST-8 assay, we found that MSC-extract increased the proliferation of PDLCs in a concentration dependent manner. MSC-extract significantly increased the number of Ki67-positive cells after 72 hours. Additionally, heat treatment of MSC-extract diminished the proliferation of PDLCs, and the MSC-extract obtained from CHX-treated MSC showed significantly lower proliferative effect on PDLCs. These results suggest that MSC-extract enhances the proliferation of PDLCs through its protein components. Our new findings may provide important information for the development of novel periodontal regenerative therapy. (J Osaka Dent Univ 2023; 57: 119-124)

Key words: Periodontal ligament cells; Mesenchymal stem cells; Periodontal regeneration

INTRODUCTION

Periodontitis is a chronic inflammatory disease caused primarily by gram-negative bacterial infection. The high incidence of this disease worldwide makes it a major oral health concern.[1, 2] The progression of periodontal disease leads to progressive loss of the attachment apparatus between the tooth and alveolar bone that supports the periodontal tissues, eventually leading to tooth extraction.[3, 4] Many therapies have been developed to regenerate periodontal tissues lost due to the disease. However, the extent of periodontal regeneration that can be achieved by these conventional regenerative therapies was limited, making the development of new periodontal regenerative therapies highly desirable. The periodontal ligament is thin soft tissue that connects the teeth to alveolar bone and is responsible for physically buffering occlusal forces applied to the teeth. In addition to its physical buffering function, the periodontal ligament has been shown to play an important role in wound healing and homeostasis of periodontal tissues by supplying progenitor/stem cells.[5] It has also been demonstrated that periodontal tissue healing is dependent on the cell types that initially occupy the healing site, and that periodontal regeneration occurs only when periodontal ligament-derived cells proliferate and migrate to the wound area.[5, 6] The guided tissue regeneration (GTR) method has proved that periodontal regeneration occurs by inducing proliferation and migration of periodontal ligament cells to the root surface using a physical barrier membrane.[7, 8] This demonstrated that proliferation and

migration of periodontal ligament cells are important phenomena for periodontal regeneration.

Mesenchymal stem cells (MSCs) are a stem cell population with multi-differentiation potential and are used for regenerative therapies for various diseases.[9, 10] In periodontal disease, transplantation of MSCs has been shown to regenerate periodontal tissues in many animal studies.[11-14] However, the regenerative mechanism by MSCs remains largely unknown. Recently, it has been revealed that some of the therapeutic mechanisms of MSCs depend on humoral factors released by MSCs, and paracrine factors derived from MSCs have attracted attention as a novel cell-free regenerative material.[15, 16] We previously reported that periodontal tissues are regenerated by the transplantation of culture supernatants containing paracrine factors from MSCs.[17] MSC paracrine factors are stored intracellularly before being released from the cells and may be recovered as cell extracts from cultured MSCs. However, the function of MSC cell extracts is still largely unknown.

The purpose of this study was to investigate the effect of cell extracts from MSCs (MSC-extract) on the proliferation of periodontal ligament cells (PDLCs), which play an important role in periodontal tissue regeneration.

MATERIALS AND METHODS

Cell culture

The bone marrow-derived MSC line UE7T-13 and human PDLCs were purchased from Riken Bioresource Research Center, Tokyo, Japan, and Lonza, Basel, Switzerland, respectively. The UE7T-13 line was originally established as an immortalized bone marrow-derived MSC line by transduction of papillomavirus type 16 protein E7 and human telomerase reverse transcriptase cloning.[18] The cells were cultured in α-minimum essential medium (αMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 15% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), GlutaMax (Thermo Fisher Scientific) and antibiotic-antimycotic solution (Thermo Fisher Scientific). To inhibit the synthesis of intracellular proteins, in some experiments, UE7T-13 cells were cultured in the presence of the protein synthesis inhibitor, cycloheximide (CHX; Nacalai Tesque, Kyoto, Japan).

Harvest of cell extracts from MSC

UE7T-13 cells were harvested using 0.05 % trypsin -EDTA (Thermo Fisher Scientific) and washed with αMEM without FBS. The cells were then suspended in phosphate buffered saline (PBS) at a density of 10^6 cells/100 μL of PBS. The cell suspensions were placed alternately in a water bath at 37°C and liquid nitrogen. This freeze-thaw cycle was repeated three times. The suspensions were then centrifuged at 15,000 rpm for 30 min at 4°C to deposit cell membranes and other debris at the bottom of the tubes. Cell extracts were obtained as the supernatants after filtration through a 0.22 μm pore size filter (Kurabo, Osaka, Japan). The protein concentration of the MSC-extract was measured using a BCA protein assay kit (Toyobo, Osaka, Japan).

Water-soluble tetrazolium (WST)-8 proliferation assay

The WST-8 proliferation assay was used to examine the cell proliferation of the PDLCs. The cells were seeded in 96-well plates (3000 cells/well) in αMEM containing different concentrations of MSC-extract. After 3 days, proliferation of the PDLCs was examined using the WST-8 detection reagent (Dojindo, Kumamoto, Japan) according to the protocol provided. The cell proliferation rate was expressed as the absorbance at 450 nm.

Ki67 immunocytochemistry

In order to investigate the cells in the proliferation state, immunocytochemical staining of Ki67 was utilized. PDLCs were fixed with 4% paraformaldehyde (Nacalai Tesque) and permeabilized in 0.25% Triton-X (Nacalai Tesque) in PBS for 15 min. After blocking of the cells in 10% goat serum (Vector Laboratories, Burlingame, CA, USA) -PBS for 1 hour, the cells were incubated with mouse anti human Ki67 antibody (DAKO, Tokyo, Japan) and goat anti mouse IgG Alexa 488 secondary antibody (Thermo Fisher Scientific). Following nuclear stain-

ing with Hoechst 33342 (Dojindo), images of the stained cells were taken by a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The percentage of Ki67-positive PDLCs was calculated using ImageJ software from the total nuclei count and the number of Ki67-positive cells in 10 images of the microscope field taken randomly.

Statistical analysis

Student's t-test and Tukey's test were used for statistical analysis of comparisons between the two groups and among more than three groups, respectively. All statistical data were examined using Graph Pad Prism 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA). In this study, a difference of $p < 0.05$ was considered significant.

RESULTS

Effect of MSC-extract on PDLC proliferation

For the collection of cell extract, MSC were subjected to three freeze-thaw cycles of the cells to disrupt the cell membrane. Most cells were disrupted and the cell extracts were collected as a clear supernatant after centrifugation. First, the effect of MSC-extract on the proliferation of PDLCs was examined. As demonstrated in Figure 1, three days after the MSC-extract treatment, we found that the MSC-extract promoted the proliferation of PDLCs in a concentration dependent manner.

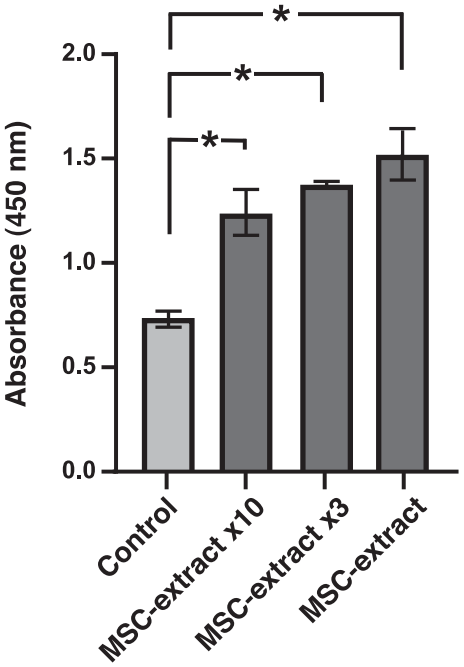

Fig. 1 Effect of MSC-extract on PDLC proliferation. This shows the results of WST-8 assay after 3 days treatment of PDLCs with different concentrations of MSC-extract. MSC-extract increased the proliferation of PDLCs in a concentration-dependent manner (*$p < 0.05$ by Tukey's test).

Effect of MSC-extract on Ki67 expression in PDLCs

Since Ki67 is a protein specifically expressed by cells in the proliferative state, we examined the effect of MSC-extract on the number of PDLCs in the proliferative phase.[19] As shown in Figure 2, the percentage of Ki67-positive cells was significantly increased in PDLCs treated with MSC-extract compared to the control group.

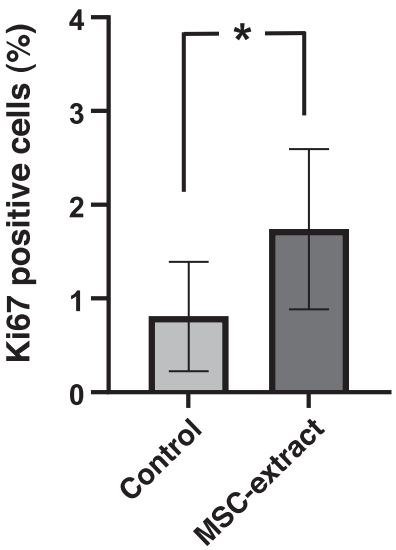

Fig. 2 Effect of MSC-extract on Ki67 expression in PDLCs. This shows the results of Ki67 immunostaining 3 days after PDLC exposure to MSC-extract. Ki67 expression was presented as the percentage of Ki67 positive cells to total cells. MSC-extract increased the number of Ki67 positive cells in the PDLCs (*$p < 0.05$ by Tukey's test).

Effect of heat treatment of MSC-extract on PDLCs proliferation

Various cell-derived components are contained in the cell extract. In order to study the factors that can promote the proliferation of PDLCs in MSC-

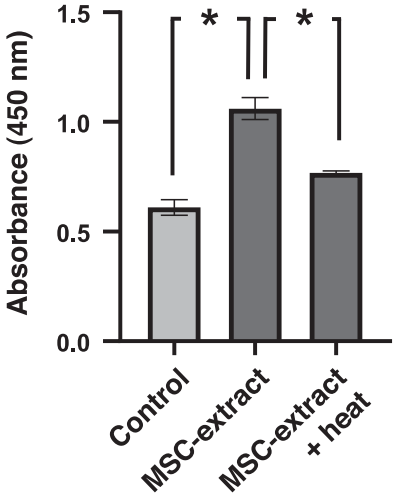

Fig. 3 Effect of heat treatment of MSC-extract on PDLC proliferation. This shows the results of the WST-8 assay 3 days after exposure of the PDLCs to MSC-extract and to MSC-extract with heat treatment. Heat treatment diminished MSC-extract-elicited proliferation of the PDLCs ($*p < 0.05$ by Tukey's test).

extracts, we heat-treated MSC-extracts at 65°C for 30 minutes and examined the changes in the proliferative activity of the cell extract. As shown in Figure 3, heating MSC-extract significantly reduced its effect on PDLC proliferation. Since 65 degrees is a well-known protein denaturation temperature, this result suggested that the protein components of MSC-extract may play a role in this effect.

Effect of CHX on MSC-extract-elicited PDLC proliferation

To examine the effect of the protein component in MSC-extract, we examined the cell proliferative activity of MSC-extract recovered from UE7T-13 cells treated with CHX, a protein synthesis inhibitor. As shown in the Table 1, CHX treatment significantly reduced the protein concentration in MSC-extract. As shown in Figure 4, the cell proliferative activity of PDLCs was significantly reduced in MSC-extract treated with CHX compared to MSC-extract collected without CHX. These results indicate that MSC-extract promotes PDLC proliferation through its protein component.

Table 1 Effect of CHX on protein production in PDLCs

Total protein in MSC-extract	
Control	CHX treatment
1217.62 μg	669.41 μg

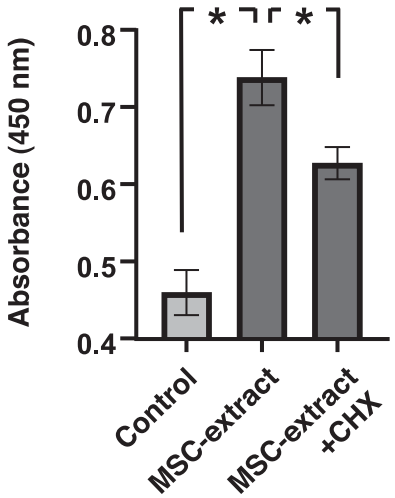

Fig. 4 Effect of CHX treatment of MSC-extract on PDLC proliferation. This shows the results of the WST-8 assay after 3 days treatment of the PDLCs with MSC -extract collected in the presence or absence of CHX. CHX treatment reduced MSC-extract-elicited proliferation of PDLCs ($*p < 0.05$ by Tukey's test).

DISCUSSION

In this study we examined the effect of MSC-extract on PDLC proliferation. The results showed that MSC-extract significantly enhanced the proliferation of PDLCs. Heat treatment of MSC-extract at 65°C and treatment of MSC with protein synthesis inhibitor significantly reduced the cell proliferative activity of MSC-extract. Since almost all cells undergo necrosis due to membrane disruption by the freeze-thaw cycle, MSC-extract is thought to contain various intracellular components. These include such substances as DNA, RNA, intracellular organelles, lipids, and proteins. Therefore, the effect of MSC-extract is considered to be the sum of the effects of these various cell-derived factors. The results of this study showed that the protein components of MSC-extract are mainly responsible for the proliferative effect of MSC-extract on PDLCs. However, in this study, we could not clarify which of the pro-

tein components played the most important role in MSC-extract-elicited PDLC proliferation. Because MSC-extract contains a wide variety of proteins, it would be difficult to identify which one plays the central role. Since MSCs are known to produce various cell growth factors, the growth factors contained in MSC-extract may play an essential role, such as fibroblast growth factor, platelet-derived growth factor and vascular endothelial growth factor.[20] This is an area for further study.

In this study, we found that MSC-extract promoted PDLC proliferation. PDLC proliferation is known to be an important phenomenon underlying periodontal regeneration. EMDOGAIN®, an enamel matrix protein derivative, has been used in the clinical treatment of periodontal disease to regenerate periodontal tissues, and has been reported to enhance proliferation of PDLCs.[21, 22] In addition, basic fibroblast growth factor has been clinically applied under the name REGROTH® and has been shown to induce periodontal tissue regeneration and PDLC proliferation.[23-25] Taken together, the results and findings of the present study suggest that MSC-extract may also have potential for use in periodontal regeneration therapy. This point will be investigated using animal experiments in the near future. We used UE7T-13 cells, which are immortalized MSCs, instead of primary cultured MSCs in this study. Primary MSCs are known to undergo cellular senescence and lose their stem cell properties during cell culture. When considering the application of MSC-extract in regenerative medicine, immortalized MSCs are considered to be a useful cell source to recover MSC-extract in terms of cell extract quality, since they grow stably and cellular changes are minimized during the cell culture. In addition, because the cost of cell culture is lower than that of primary MSCs, the application of MSC-extract from UE7T-13 for periodontal regenerative therapy has some advantages over primary MSC.

The present study showed that MSC-extract promoted the proliferation of PDLCs, which is an important phenomena associated with periodontal tissue regeneration, through its protein component. The results of this study provide new insights into the effects of stem cells on PDLCs and may also serve as a basis for the development of novel periodontal regenerative therapy.

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Conflicts of interest
The authors declare no conflicts of interest associated with this study.

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