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ORIGINAL ARTICLE

Isolation and characterization of human gingiva-derived mesenchymal stem cells using limiting dilution method

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Abstract *Background/purpose:* Gingiva-derived mesenchymal stem cells (GMSCs) are attractive alternative MSC sources because of their relative abundance of sources and ease of accessibility. However, the isolation method for harboring GMSCs remains under discussion. The aim of the study was to isolate and explore *in vitro* characterization of human GMSCs, and compare stem cell properties with bulk-cultured gingival fibroblasts (GFs).
Materials and methods: GMSCs were isolated with limiting dilution method. Tissue-matched bulk-cultured GFs and GMSCs were evaluated in terms of their colony-forming abilities, population doubling capacities, cell surface epitopes, and multilineage differentiation potentials.
Results: GMSCs showed a significantly higher number of colony-forming units-fibroblast ($P < 0.001$) than bulk-cultured GFs, while the population doubling capacity of GMSCs reduced. Both types of cells were uniformly positive for MSC-associated makers CD44, CD73, CD90, CD105, and CD166, and were negative for hematopoietic markers CD14, CD34, and CD45. The only distinct marker was STRO-1, which was more highly expressed in GMSCs (13.4%) than in bulk-cultured GFs (0.02%). Upon induction, GMSCs displayed the capacity to undergo osteogenic, adipogenic, and chondrogenic differentiation. Real-time polymerase chain reaction showed related gene levels were significantly upregulated ($P < 0.001$). By contrast, bulk-cultured GFs lacked the capacity to undergo multilineage differentiation, and related gene levels showed no significant difference when compared with control groups.

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Conclusion: The data validate the effectiveness of limiting dilution method for GMSCs isolation. GMSCs, in contrast to bulk-cultured GFs, harbor stem cell characteristics and can act as alternative cell sources for tissue engineering.
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Introduction

Stem cell biology has become an important field in regenerative medicine and tissue engineering therapy since the discovery and characterization of mesenchymal stem cells (MSCs). MSCs represent a population of multipotent stem cells that can be isolated from many tissues, including bone marrow, adipose tissue, placenta, and umbilical cord blood.[1–4] All of these MSCs display fibroblast-like cell morphology, have self-renewal capacities, and multilineage differentiation potentials, such as giving rise to osteogenic, adipogenic, and chondrogenic lineages.[5,6]

MSC-like populations have also been isolated from human dental tissues, including dental pulp stem cells, stem cells from human exfoliated deciduous teeth, stem cells from apical papilla, dental follicle progenitor cells, and periodontal ligament stem cells.[7–11] Those dental tissue-derived stem cells possess potent capacities to differentiate into odontogenic cells and generate reassembly dental tissue structures. Given the innate capacity of dental-derived MSC-like cells to ectopically generate structures resembling the tissues from which they are derived *in vivo*, these progenitor cell populations represent promising candidates for oral tissue regeneration.[6–8] However, there are some drawbacks in using these stem cells for cell therapy, such as their limited tissue sources and the requirement for tooth extraction. Comparatively, a population of stem cells within gingival tissue, termed gingiva-derived mesenchymal stem cells (GMSCs), constitutes more appealing alternatives to other dental-derived MSCs for the accessibility and availability of human gingival tissues. GMSCs can be obtained from gingival tissue that are easily accessible from the oral cavity with minimal discomfort.[12,13] Gingival tissues also exhibit scarless wound healing properties and a regenerative capability with rapid constitution of the tissue architecture. Interestingly, GMSCs display stable phenotype and telomerase activity in long-term cultures, and are not tumorigenic.[14] Notably, GMSCs have demonstrated the capacity for self-renewal and the formation of connective tissue-like structures *in vivo*.[15] A few recent studies also demonstrated that GMSCs possessed osteogenic potential *in vivo* after incubation under osteoinductive medium *in vitro*.[16,17] These properties indicate that the clinical use of GMSCs is an attractive therapeutic option for tissue regeneration and repair. However, it is still problematic as to which isolation method is to be favored when obtaining GMSCs. Various processes have been reported to isolate MSCs, while cell sorting technologies with certain surface markers by flow cytometry or magnetic activated cell sorting (MACS) are the most common methods.[18,19] However, to date, no specific cell surface markers have been available for isolating GMSCs. Recent reports indicated that there is a decrease in the level of cell surface marker expression after MACS.[20–22] Previous studies have indicated that human bone marrow-derived MSCs generate single cell-derived colonies if plated at extremely low densities.[23–25] Digirolamo et al[23] showed that the replicative potential of the cells in culture was best predicted by a simple colony-forming assay when cells were plated at low densities, and the samples with the highest colony-forming efficiency also exhibited the greatest replicative potential. In addition, single cell-derived colonies obtained with low-density plating were able to differentiate into osteocytes, adipocytes, and chondrocytes under defined culture conditions, and had the capacity to generate reassembly tissue structures *in vivo*.[25–29]

Therefore, in this study, we selected single cell colonies of GMSCs using a limiting dilution method and established tissue-matched bulk-cultured GFs. Our study aimed to compare GMSCs with bulk-cultured GFs with regard to the colony-forming ability, population doubling capacity, cell surface epitopes, multilineage differentiation potentials, and related gene expression levels.

Materials and methods

Cell isolation

Human gingival tissues were obtained from three patients undergoing crown lengthening surgery with no history of periodontal disease at the School of Stomatology, Shandong University, Jinan, China. This study was approved by the Medical Ethics Committee of the Medical School, Shandong University (approval number: 2010015) and written informed consent was obtained from each individual patient. The gingival tissue samples were minced and digested in collagenase type I (Invitrogen, Carlsbad, CA, USA) and dispase II (Roche Diagnostics, Indianapolis, IN, USA) for 2 hours at 37°C. After that, the single cell suspension was filtered through a 70-μm cell strainer. Half of the single cell suspensions were plated at a concentration of 60 cells/cm^2 in 10-cm tissue culture dishes for the selection of single cell-derived colonies in α-minimal essential medium (α-MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 2mM L-glutamine (Sigma-Aldrich), 100μM L-ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd, Osaka, Japan), 1mM sodium pyruvate (Sigma-Aldrich), 50-μg/mL streptomycin with 50-U/mL penicillin G (JRH Biosciences Inc., Lenexa, KS, USA), and 2.5-μg/mL amphotericin B (Life Technologies, Grand Island, NY, USA) in a humidified atmosphere (37°C, 5% CO_2). The nonadherent cells were removed 3 days later and the basic medium was changed three times per week. Individual plastic-adherent, MSC-like colonies grown for 10–14 days in 10-cm tissue culture dishes

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