Evaluation of the secretion and release of vascular endothelial growth factor from two-dimensional culture and three-dimensional cell spheroids formed with stem cells and osteoprecursor cells

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Funding sources
This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, Information and Communication Technology & Future Planning (NRF-2017R1A1A05001307).

Conflict of interest
None declared

Received on October 26, 2016
Reviewed on January 19, 2017
Accepted on April 26, 2017
Published online on May 18, 2018

Abstract

Background. Co-culture has been applied in cell therapy, including stem cells, and has been reported to give enhanced functionality.

Objectives. In this study, stem-cell spheroids were formed in concave micromolds at different ratios of stem cells to osteoprecursor cells, and the amount of secretion of vascular endothelial growth factor (VEGF) was evaluated.

Material and methods. Gingiva-derived stem cells and osteoprecursor cells in the amount of $6 \times 10^5$ were seeded on a 24-well culture plate or concave micromolds. The ratios of stem cells to osteoprecursor cells included: 0:4 (group 1), 1:3 (group 2), 2:2 (group 3), 3:1 (group 4), and 4:0 (group 5).

Results. The morphology of cells in a 2-dimensional culture (groups 1–5) showed a fibroblast-like appearance. The secretion of VEGF increased with the increase in stem cells, and a statistically significant increase was noted in groups 3, 4 and 5 when compared with the media-only group ($p < 0.05$). Osteoprecursor cells formed spheroids in concave microwells, and no noticeable change in the morphology was noted with the increase in stem cells. Spheroids containing stem cells were positive for the stem-cell markers SSEA-4. The secretion of VEGF from cell spheroids increased with the increase in stem cells.

Conclusions. This study showed that cell spheroids formed with stem cells and osteoprecursor cells with different ratios, using microwells, had paracrine effects on the stem cells. The secretion of VEGF increased with the increase in stem cells. This stem-cell spheroid may be applied for tissue-engineering purposes.

Key words: vascular endothelial growth factor, osteoblast, co-culture techniques, cellular spheroids, stem cell research
Introduction

Co-culture has been applied in cell therapy, including stem cells, and has been reported to give enhanced functionality. Spheroids formed with primary hepatocytes and hepatic stellate cells showed a higher secretion of albumin than mono-culture hepatospheres. Additionally, the enzymatic activity of co-cultured heterospheres was higher than the activity of a mono-culture. Primary pancreatic islets and hepatocytes were applied as spheroids for the 3-dimensional co-culture model. It was shown that the 2 different types of cells supported each other’s functionality when compared with a mono-culture.

Our group isolated and characterized human mesenchymal stem cells from the gingiva. Gingiva-derived stem cells have been suggested as candidates in the tissue-engineering field. In this study, stem-cell spheroids were formed in concave micromolds at different ratios of stem cells to osteoprecursor cells, and the amount of secretion of vascular endothelial growth factor (VEGF) was evaluated. To the best of the authors’ knowledge, this report is the first to evaluate the secretion of VEGF from cell spheroids formed with human gingiva-derived stem cells and osteoprecursor cells.

Material and methods

Isolation and culturing of gingiva-derived stem cells

Gingiva-derived stem cells were obtained using a previously reported method. The Institutional Review Board of Seoul St. Mary’s Hospital, College of Medicine, Catholic University of Korea approved this study (KC11SISI0348). All participants signed informed consent.

Gingival tissues were de-epithelialized, minced into 1–2 mm² fragments and digested in an alpha-modified, minimal essential medium (α-MEM; Gibco, Grand Island, USA) containing dispase (1 mg/mL; Sigma-Aldrich, St. Louis, USA) and collagenase IV (2 mg/mL; Sigma-Aldrich). The cells were incubated in a humidified incubator at 37°C. The non-adherent cells were washed with phosphate-buffered saline (PBS) (Welgene, Daegu, South Korea) and replaced with a fresh medium every 2–3 days.

Co-culture of gingiva-derived stem cells and osteoprecursor cells

Gingiva-derived stem cells and murine calvarial osteoprecursor cells (MC3T3-E1) (ATCC, Manassas, USA) in the amount of $6 \times 10^5$ were seeded on a 24-well culture plate and grown with a growth medium α-MEM. The ratios of stem cells to osteoprecursor cells included: 0:4 (group 1), 1:3 (group 2), 2:2 (group 3), 3:1 (group 4), and 4:0 (group 5).
Evaluation of secretion of human vascular endothelial growth factor

The determination of human VEGF from 2- and 3-dimensional systems was performed using a commercially available kit (Quantikine® ELISA; R&D Systems). All reagents and samples were prepared according to the manufacturer’s recommendations. A media group served as the control.

Statistical analysis

A test of normality was performed with the Shapiro-Wilk test, and a one-way analysis of variance with Tukey’s post hoc test was performed to analyze the differences between the groups, using a commercially available program (SPSS 12 for Windows; SPSS, Chicago, USA), with the level of significance set at 0.05.

Results

Evaluation of 2-dimensional culture

The morphology of the osteoprecursor cells showed a fibroblast-like appearance on day 3 (Fig. 2A). No significant difference in the morphology was noted when the culture with gingiva-derived stem cells was at different ratios of stem cells to osteoprecursor cells: 1:3 (group 2), 2:2 (group 3), 3:1 (group 4), and 4:0 (group 5) (Fig. 2B, 2C, 2D, and 2E, respectively).

Secretion of human vascular endothelial growth factor from 2-dimensional culture

The secretion of VEGF was noted in groups 2, 3, 4, and 5 (Fig. 3), and it increased with the increase in stem cells. A statistically significant increase was noted in groups 3, 4 and 5 when compared with the media-only group (p < 0.05).

Evaluation of spheroid morphology

The morphology of the spheroids on day 3 is shown in Fig. 4. Osteoprecursor cells formed spheroids in concave microwells (Fig. 4A). Gingiva-derived stem cells and osteoprecursor cells also formed spheroids (Fig. 4B–E). No significant change in the morphology was noted with the increase in stem cells, at different ratios of stem cells to osteoprecursor cells: 1:3 (group 2), 2:2 (group 3), 3:1 (group 4), and 4:0 (group 5) (Fig. 4B, 4C, 4D, and 4E, respectively).

Determination of cell viability and maintenance of expression of stem cell markers

Most of the cells in the spheroids emitted green fluorescence; a small portion of red fluorescence was also noted (Fig. 5). Spheroids containing stem cells were positive for
Fig. 4. Morphology of 3-dimensional stem cells cultured with osteoprecursor cells on day 3 (osteoprecursor cells formed spheroids in concave microwells).

A – group 1; B – group 2; C – group 3; D – group 4; E – group 5; no significant change in the morphology was noted with the increase in stem cells, at different ratios of stem cells to osteoprecursor cells: 0:4, 1:3, 2:2, and 3:1.

Fig. 5. Determination of cell viability of cell spheroids

A–D – group 1; E–H – group 2; I–L – group 3; M–P – group 4; Q–T – group 5.
the stem-cell markers SSEA-4 and TRA-1-60(R) (Fig. 6). The green fluorescence showed a more intense assay with a higher number of stem cells.

**Secretion of human vascular endothelial growth factor from spheroids**

The secretion of VEGF from the spheroids was noted in groups 2, 3, 4, and 5 (Fig. 7), and it increased with the increase in the number of stem cells. A statistically significant increase in the secretion of VEGF was noted in groups 2, 3, 4 and 5 when compared with the media-only group (p < 0.05).

**Discussion**

In this report, stem cell spheroids were fabricated using microwells, and the paracrine effects of the stem cell spheroids were evaluated. This study showed that cell spheroids formed with stem cells and osteoprecursor cells with different ratios, using microwells, had different paracrine effects on the stem cells. It also proved that the secretion of VEGF increased with the increase in stem cells.

More recently, it has been shown that the beneficial effects of stem cells were reported to come from both cell restoration and paracrine effects. Mesenchymal stem cells were reported to secrete a vast array of proteins, including growth factors, cytokines, chemokines, and extracellular...
were applied for the treatment of erectile dysfunction due to the paracrine effect on surrounding tissues. The improvements of glucose intolerance were achieved from the factors secreted from dental pulp stem cells by increasing pancreatic β-cell function in streptozotocin-induced diabetic mice.

This study applied a 3-dimensional culture system. In a previous study, 3-dimensional stem cell spheroids were generated from gingiva-derived stem cells with concave microwells. Recently, a 3-dimensional human stem cell construct has been built by applying bioprinting. A 3-dimensional stem cell system may extend the knowledge about the structure–function relationship. It was suggested that the 3-dimensional system simulates reality more closely when compared to the 2-dimensional one. Additionally, it was suggested that 3-dimensional spheroids of mesenchymal stem cells showed enhanced survival effects.

Stem cells may be obtained from bone marrow, peripheral blood, adipose tissue, and umbilical cord blood. Stem cells from bone marrow are widely applied, but this may have limitations due to the low number of stem cells, pain of extraction and morbidity. Umbilical cord blood may be a good source, but the supply is very limited. Stem cells can be also obtained from the oral and maxillofacial area, including the maxilla and mandible. The gingiva can be considered a favorable source of stem cells, because the tissue can be obtained from daily dental procedures under local anesthesia.

This study showed that cell spheroids formed with stem cells and osteoprecursor cells with different ratios, using microwells, had paracrine effects on the stem cells. The secretion of VEGF increased with the increase in stem cells. This stem cell spheroid may be applied for tissue engineering purposes.

References


