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# Real-time quantitative polymerase chain reaction and flow cytometric analyses of cell adhesion molecules expressed in human cell–multilayered periosteal sheets *in vitro*

TOMOYUKI KAWASE[1], KOHYA UEMATSU[1,2], MANA KAMIYA[1,3], MASAKI NAGATA[2], KAZUHIRO OKUDA[3], DOUGLAS M. BURNS[4], KOH NAKATA[5] & HIROMASA YOSHIE[3]

*[1]Division of Oral Bioengineering, [2]Division of Oral and Maxillofacial Surgery and [3]Division of Periodontology, Institute of Medicine and Dentistry, Niigata University, Niigata, Japan, [4]Midwest Biomedical Research Foundation, Research Service, Department of Veterans Affairs Medical Center, Kansas City, Missouri, USA, and [5]Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata, Japan*

**Abstract**

*Background aims.* Cultured human periosteal sheets more effectively function as an osteogenic grafting material at implantation sites than do dispersed periosteal cells. Because adherent cell growth and differentiation are regulated by cell-cell and cell–extracellular matrix contacts, we hypothesized that this advantage is a result of the unique cell adhesion pattern formed by their multiple cell layers and abundant extracellular matrix. To test this hypothesis, we prepared three distinct forms of periosteal cell cultures: three-dimensional cell-multilayered periosteal sheets, two-dimensional dispersed cell cultures, and three-dimensional hybrid mock-ups of cells dispersed onto collagen sponges. *Methods.* Periosteal cells were obtained from human alveolar bone. Cell adhesion and extracellular matrix molecules were quantitatively determined at the messenger RNA and protein levels by means of real-time quantitative polymerase chain reaction and flow cytometry, respectively. *Results.* Real-time quantitative polymerase chain reaction analysis demonstrated that regardless of culture media α1 integrin, vascular cell adhesion molecule-1, fibronectin and collagen type 1 were substantially upregulated, whereas CD44 was strongly downregulated in periosteal sheets compared with dispersed cell monolayers. With increased thickness, stem cell medium upregulated several integrins (β1, α1 and α4), CD146, vascular cell adhesion molecule-1, fibronectin and collagen type 1 in the periosteal sheets. Flow cytometric analysis revealed that the active configuration of β1 integrin was substantially downregulated in the stem cell medium–expanded cell cultures. The cell adhesion pattern found in the mock-up cultures was almost identical to that of genuine periosteal sheets. *Conclusions.* Integrin α1β1 and CD44 function as the main cell adhesion molecule in highly cell-multilayered periosteal sheets and dispersed cells, respectively. This difference may account for the more potent osteogenic activity shown by the thicker periosteal sheets.

**Key Words:** *bone tissue engineering, cell adhesion, extracellular matrix, integrin, periosteal sheet*

## Introduction

Cell-based regenerative therapies have recently been developed for the treatment of periodontal disease with severe bone defect and have shown notable success (1,2). Among the various cell types used for clinical applications, we hypothesize that periosteal cells in the form of a cell-multilayered sheet are the most promising candidate for periodontal regenerative therapy (3–5). In previous studies (6,7), we demonstrated that periosteal cells within the periosteal sheet differentiate into osteogenic cells and produce various growth factors and cytokines that are involved in bone metabolism. Therefore, we have suggested that these periosteal sheets act as an effective osteogenic grafting material and also as an osteoinductive grafting material to influence the surrounding host tissues and cells.

At the present time, it is widely accepted that the implantation of mature osteogenic cells would yield the best results in skeletal regenerative treatments. However, it was recently reported that immature mesenchymal stromal or progenitor cells could be more potent at inducing osteogenesis at the implantation site than differentiated osteoblasts (8). Consistent with this finding, we previously demonstrated that through expanding cells positive for CD146, a

Correspondence: **Tomoyuki Kawase**, DDS, PhD, Division of Oral Bioengineering, Institute of Medicine and Dentistry, Niigata University, 2–5274 Gakkocho-dori, Chuo-ku, Niigata 951–8514, Japan. E-mail: kawase@dent.niigatau.ac.jp

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surface marker of immature osteoprogenitor cells, the thicker periosteal sheets prepared with the stem cell medium expressed significantly enhanced osteogenic activity at the site of subcutaneous implantation in mice (9). Determining why immature periosteal cells are expanded during the preparation of thicker periosteal sheets is important because such knowledge will improve the development of more potent periosteal sheets.

It is generally thought that cell proliferation and differentiation are intricately regulated by the combined influence of soluble factors, substratum stiffness, the topography of cell scaffolds and a multitude of cell-cell or cell-matrix contacts (10,11). Because the cell-multilayered periosteal sheets exhibit a thick three-dimensional (3D) structure containing numerous cells and a very abundant extracellular matrix (ECM), it is reasonable to assume that cell-cell and the cell-matrix contacts increase in this structure and that the pattern of cell adhesive may change or reorganize to stabilize the 3D structure.

In the present study, we addressed this question by evaluating the expression of cell adhesion molecules, such as integrins, at both the messenger RNA (mRNA) and the protein levels in periosteal cells under three distinct culture conditions: (i) periosteal cells in 3D genuine periosteal sheets, (ii) dispersed periosteal cells in two-dimensional cultures and (iii) periosteal cells dispersed onto collagen sponges as a 3D hybrid culture mock-up of periosteal sheets.

## Methods

### *Isolation and culture of periosteal tissue segment*

Twenty-two healthy, non-smoking volunteers ages 16–45 years (eight male and 14 female) donated periosteum tissue samples. The periosteum tissue was aseptically dissected from the buccal side of the retromolar region in the mandible, washed three times in $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (PBS) (12), cut into small pieces (1 × 1 mm), plated on 35- or 60-mm plastic dishes and cultured in growth medium [Medium 199 supplemented with 10% fetal bovine serum (FBS), 25 μg/mL L-ascorbic acid, 100 U/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B] (13) or the stem cell medium [MesenPRO-RS (Life Technologies, Carlsbad, CA, USA) supplemented with 2% FBS, 25 μg/mL ascorbic acid 2-phosphate and antibiotics] (9).

After the periosteum-derived cell sheets achieved a diameter of 40–50 mm, the sheets were detached with a solution of 0.05% trypsin in 0.53 mmol/L ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and dispersed for subculture (14). The dispersed periosteal cells were maintained in the growth medium until the cells reached sub-confluence.

For collection of periosteum tissue, all of the subjects enrolled in this study provided written informed consent after the nature of the study had been fully explained. All of the details of this study were approved by the ethics committee for human subject use at the Niigata University School of Dentistry, in accordance with the *Helsinki Declaration* of 1975 (revised in 2008) (9).

### *Preparation of periosteal cell cultures combined with collagen sponges*

Dispersed periosteal cells were suspended in the growth medium at a density of $0.5–2 \times 10^6$ cells/mL and allowed to adsorb to pieces of collagen sponge (2 × 2 × 2 mm) (Terudermis; Olympus Terumo Biomaterials, Tokyo, Japan). After incubation for 2–3 h in a $CO_2$ incubator, the cell-sponge combinations were placed on dishes or plates and cultured, as were freshly excised periosteum tissue segments.

### *Real-time quantitative polymerase chain reactions*

Total cellular RNA was extracted with the use of an RNeasy mini purification kit (Qiagen, Germantown, MD, USA) as described previously (15). Samples of the RNA were converted to complementary DNA with the use of a Transcriptor Universal complementary DNA Master kit (Roche Diagnostic, Basel Schweiz, Switzerland). The polymerase chain reactions (PCR) were performed in a LightCycler Nano System (Roche Diagnostic) with the use of a LightCycler TaqMan Master kit (Roche Diagnostic). The reaction products were quantified with the use of *GAPDH* as the reference gene and further normalized with the use of the control for each experiment. The oligonucleotide sequences of the primers are shown in Table I.

### *Flow cytometric analyses*

Cells were dispersed from cultured periosteal sheets with a solution of 0.05% trypsin in 0.53 mmol/L EDTA, washed twice in PBS and suspended in 0.1 mL of PBS at a density of $1 \times 10^6$ cells/mL. The cells were then probed for 40 min at 4°C with 10–20 μL of mouse monoclonal antibodies against cell surface antigens (Table II). After being washed twice with PBS, the cells were analyzed by flow cytometry (Cell Lab Quanta; Beckman Coulter, Miami, FL, USA) (9). The cells probed with anti-active configurations of CD29 were additionally probed with Alexa Fluor 488–conjugated goat anti-mouse immunoglobulin (Ig)G (1:100 dilution; Life Technologies), washed