FORMATION OF GAP JUNCTIONS AND EXPRESSION OF CONNEXIN 43 IN CULTURED MALASSEZ’S EPITHELIAL REST CELLS

ABSTRACT

Background and Aim: The purpose of this study was to investigate the formation of gap junctions and to characterize the expression of Connexin (CX) 43 during the proliferation of Malassez’s epithelial rest (MER) cells.

Materials and Methods: MER cells derived from the porcine periodontal ligament were cultured in 35 mm dishes as 2D culture. MER cells mixed with type 1 collagen gels were also cultured as 3D culture. The 2D culture cells were classified into 3 groups; group 1 was the single cell stage, group 2 was the cell to cell contact stage, and group 3 was the multiple cell contact stage. All the cells were observed using immunofluorescence and immuno-electron microscopy with a CX43 antibody. RT-PCR and quantitative RT-PCR were also used to analyze CX43 mRNA levels.

Results: CX43-positive cells were not observed in group 1. A few CX43-positive spots were observed on the cell membranes between cells in group 2 and many CX43-positive spots were observed on the cell membranes in group 3. A few immuno-gold particles were observed between cell membranes. CX43 expression of group 3 had the highest mRNA levels. In the 3D culture, no CX43-positive spots were observed at any of the attached areas, and the expression of CX43 mRNA in cells of the 3D culture were much lower than in the 2D culture.

Conclusion: These results suggest that the 2D culture is better than the 3D culture to investigate epithelial cell morphology and that CX43 expression is important for MER cells to proliferate.

Key words: Connexin 43, Gap Junction, In Vitro, Malassez’s Epithelial Rest, 3D culture

Submitted for Publication: 03.19.2012
Accepted for Publication: 06.04.2012
CLINICAL DENTISTRY AND RESEARCH

INTRODUCTION

Epithelial cells found in the periodontal ligament are remnants of Hertwig’s epithelial root sheath and are known as Malassez’s epithelial rest (MER) cells. They occur as lacy strands close to the cementum surface and in the cementum.1 They appear to be unique with respect to their ability to persist throughout life in the periodontal ligament. MER cells are characterized by condensed rounded nuclei with a high nuclear/cytoplasm ratio, Golgi complexes accompanied by vesicles and a poorly developed rough endoplasmic reticulum. Cells of the MER are tightly connected with desmosomes and gap junctions.2 MER cells usually do not show mitotic activity and have a number of distinct functions, such as to prevent resorption of the tooth root, to maintain the homeostasis of the periodontal ligament, and to induce cementum formation.3 Furthermore, when inflammation occurs in the gingival connective tissue or in the periodontal ligament, MER cells can be stimulated to proliferate and participate in elongation of the periodontal pocket and the lining epithelium of odontogenic cysts.3 In such cases, it is thought that epidermal growth factor may play an important role in regulating MER cell proliferation.4 However, the details of the mechanism of MER cell proliferation related to intercellular junctions, particularly gap junctions, is still not clear.

Gap junctions are widely distributed in the tissues of all animals, and they are considered to be communication junctions because they allow small, water-soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the other, thereby coupling the cells both electrically and metabolically. Intercellular communications through gap junctions are considered to play a role in the regulation of homeostasis because they regulate important cellular processes, such as proliferation and differentiation. Connexins are gap junction proteins that are in a family of structurally-related transmembrane proteins that assemble to form gap junctions in vertebrates. Gap junctions are essential for many physiological processes, such as the coordinated depolarization of cardiac muscle and proper embryonic development. Recently, many studies have suggested a relationship between gap junctions and cell proliferation in salivary glands,5 in the ovary and ovarian tumors,6 in lens epithelial cells,7 in thyroid cells,8 in oral tumor cells,9 in rat liver carcinogenesis,10,11 and also in cell migration of the mouse neural crest.12 Several studies have reported a special relationship between CX43 and proliferation in mouse hepatocytes,13 and in the rat esophagus epithelium.14

The purpose of this study was to investigate the expression of CX43 and gap junction formation by MER cells during proliferation in vitro using 2D and 3D cultures. Immunofluorescence, immuno-electron microscopy and RT-PCR were used to evaluate those characteristics.

MATERIALS AND METHODS

Cell Culture

Porcine MER cells were provided by Professor Abiko, Department of Dental Science, Institute of Personalized Medical Science, Health Sciences University of Hokkaido. MER cells were cultured in 75 cm² tissue culture flasks (Corning, Tokyo, Japan) with α-MEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich®), and 40µg/ml gentamycin (Sigma-Aldrich®) and were cultured by incubation in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After the cells became confluent, they were detached using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% w/v trypsin/0.02% EDTA, pH7.2), and were resuspended in the supplemented culture medium described above. To identify epithelial cells, immunofluorescence staining was carried out using a primary antibody against cytokeratin 19 (1:100 dilution; Abcam, Cambridge, UK) and a secondary antibody labeled with FITC (1:100 dilution; Invitrogen, Grand Island, NY, USA) and were observed using a laser scanning microscope (LSM5 DUO, Carl Zeiss, Oberkochen, Germany) (data not shown).

Observation Period

Approximately 5,000 cells were seeded in 35mm cell culture dishes and MER cells were observed every day using an ECLIPSE TS100 phase microscope (Nikon, Tokyo, Japan). Experimental groups were classified into 3 groups depending on the manner of cell growth.

Group 1: Single cells, none at the cell contact stage
Group 2: Cell to cell contact, only 2 cells at the contact stage
Group 3: Multiple cell contact, at least 3 cells at the contact stage

Immuno-Electron Microscopy

For ultrathin sections, cells collected from group 3 were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 0.5 hr at 4°C. After washing for 30 min in phosphate buffer (pH 7.3), small pieces of each sample were dehydrated in a graded series
of ethanol and were then embedded in LR white resin under UV at 4°C. Silver-to-gold ultrathin sections were cut using an ultramicrotome (Reichert- Nissei, Japan), and the sections were transferred to formvar-coated nickel grids. They then were processed for immunostaining as follows: The samples were floated on a drop of the anti-CX43 polyclonal antibody (1:100) and were incubated at 37°C for 2 hr and then rinsed with PBS. Subsequently, the sections were transferred onto a drop of 10nm colloidal gold-conjugated anti-rabbit IgG (1:50) and then were incubated at 37°C for 1 hr. The bound antibodies were fixed with 1% glutaraldehyde in 120 mM phosphate buffer (pH 7.2) for 10 min, then osmicated and dehydrated. These ultrathin sections were stained with uranyl acetate and lead citrate, and then were examined by transmission electron microscopy (H-7100/ H-7650: Hitachi, Co., Tokyo, Japan).

**Immunofluorescence Observation**

For standard fluorescence microscopic observations, cells in dishes of each group were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature and were then permeabilized in 0.1% Triton X-100 solution for 10 min. The cells were then washed twice with PBS, after which nonspecific binding was blocked with 1% BSA. Filamentous actin (F-actin) stress fibers were visualized using FITC-conjugated phalloidin (1:100; Molecular Probes, Carlsbad, CA, USA). Immunofluorescence staining was carried out using a primary antibody against CX43 for 24 hours and a secondary antibody labeled with rhodamine (1:100 dilution; Invitrogen) and were observed using a laser scanning microscope (LSM5 DUO, Carl Zeiss).

**Three-Dimensional and Layered Cultures**

MER cells (15,000 cells in 10% concentrated culture medium described above) were seeded onto 13.5 mm cell disks in 24 well plates (Sumitomo Bakelite, Tokyo, Japan) using 200 µl Type I collagen gels (Cellmatrix®; Nitta-gelatin, Osaka, Japan). After 7 days of culture, cells were washed with phosphate buffered saline (PBS; GibCO), and were fixed with 10% buffered formalin. Samples were treated with 1% Triton X-100 (Shelton Scientific, IA, USA), and then were incubated with mouse anti-CX43 antibody (Chemicon, CA, USA) followed by Alexa Fluor® 488 goat anti-mouse IgG antibody, Alexa Fluor® 546 phalloidin and DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Invitrogen) for fluorescent staining. Microscopic observation was performed using a confocal laser scanning microscope.

**RT-PCR**

For RNA extraction, cells at each of the time periods were collected. Total RNAs were extracted from the cells using the acid guanidinium-thiocyanate-phenol-chloroform method with TRIzol (Invitrogen) and cDNA synthesis was performed. For analysis of CX43 gene expression by RT-PCR, RNAs were reverse-transcribed to complementary DNAs (cDNAs) using a Takara RNA PCR kit (Takara, Tokyo, Japan). The sequences of the primers were specific, as confirmed by a computer-assisted search of an updated version of GenBank. Primer sequences used to detect human CX43 and β-actin (the internal control) are given in Table 1. For each cell sample, 100ng total RNA were reverse-transcribed using random primers and the products were subjected to PCR amplification under the same conditions described above. The reaction mixtures were added to the DNA solution, incubated at 42°C for 1 hr, heated at 94°C for 5 min, and then chilled at 4°C. For PCR, each cDNA reaction mixture was diluted with 40 µL PCR buffer and mixed with 50 pmol of the ‘S’ and ‘3’ primers. Reactions were carried out in a DNA thermal cycler (MJ Research Inc., Watertown, MA, USA) under the following conditions: 94°C for 30sec, 60°C for 30sec, and 72°C for 1min. The amplified products were then analyzed by 1.7% agarose gel electrophoresis and were visualized by UV illumination after staining with ethidium bromide.

**Quantitative-RT-PCR**

RT-PCR products were analyzed using quantitative real-time RT-PCR in TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the target gene CX43 (F5′- AAAAGCGTTAAGGATCGGTG-3′R5′-GTCATACGGCGAAGCTT-3′). The TaqMan Endogenous Control (Applied Biosystems) for the target gene β-actin (F5′-TGTATGCCCTCCTGCTGACCAC-3′ R5′- CTCCTCAAGCGAAGTACTCTG-3′) was used as a control. The primer sequences used are shown in Table 2. All PCR reactions were performed using a real-time PCR 7500 fast system. Quantification of gene expression using TaqMan Gene Expression Assays was performed as the second step in a two-step RT-PCR. Assays were performed in 20-IL singleplex reactions containing TaqMan Fast Universal PCR Master Mix, TaqMan Gene Expression Assays, distilled water and cDNA, according to the manufacturer’s instructions (Applied Biosystems). The reaction conditions consisted of a primary denaturation at 95°C for 20 sec, then cycling for 40 cycles at 95°C for 3 sec and 62°C for 30 sec. PCR data are reported compared with the corresponding control.
CLINICAL DENTISTRY AND RESEARCH

Quantitative RT-PCR analyses were reproduced 4 times. 2D cultured cells were used as a control.

Statistical analysis
Data were analyzed using one-way analysis of variance and were compared using Scheffe’s test.

RESULTS

Cultured MER Cells
The cultured MER cells initially grew individually and they were round with central nuclei. After 24 to 48 hr of culture, the cells became spindle-like and began to contact each other’s membranes. After 72 hr of culture, the cells became small and round and were arranged in a pavement-like structure (Figure 1).

Immunohistochemical and Immuno-Electron Microscopic Observations
In the later stage of group 3, many CX43-positive spots were seen between the MER cells (Figure 4). Ultrastructurally, almost all organelles of cells in group 3 were destroyed but the cell membranes were well preserved. A few immuno-gold particles were observed between cells where gap junctions were seen (Figure 2).

Immunofluorescence Observations of Cx43
Group 1: No CX43-positive spots were observed in any of the cells (Figure 3a).
Group 2: A few CX43-positive spots were observed on the cell membranes between cells. However there were no CX43-positive spots in the cytoplasm (Figure 3b).
Group 3: Many CX43-positive spots were observed on each cell membrane. However, there were no CX43-positive spots in the cytoplasm (Figure 3c).

RT-PCR Analysis
β-actin gene expression (used as an internal control) was positive as a band at 804bp and was equivalent in all samples. CX43 gene expression was recognizable as a band at 438bp in all groups, with the highest amount in group 3 and the next highest amount in group 2 (Figure 4).

Immunofluorescence Observations of CX43 in 3D Culture
The cells grew well in the Type 1 collagen gel and the cell shape in the 3D culture was star-like and the cells contacted each other with cell processes. However, the cells were never attached at their membranes at any of the time periods. No CX43-positive spots were observed at any of the attached areas (Figure 5).

Quantitative Real Time RT-PCR Analysis
The expression of CX43 mRNA in the 3D culture was significantly lower than that in the 2D culture at 7 days (P<0.05) (Figure 6).

DISCUSSION
It is known that MER cells normally don’t proliferate and simply exist in the periodontal ligament throughout its life. However, as a result of inflammation occurring due to pulpitis or periodontitis, MER cells in the periapical area begin to proliferate to form a mass of cells that eventually produces either an epithelial nest of radicular granuloma cells or the lining epithelium of a radicular cyst and the
Figure 3. Immunofluorescence images of CX43 in the 2D culture. Group 1: No CX43-positive spots were observed in any of the cells (Figure 3-a). Group 2: A few CX43-positive spots were observed on the cell membranes between adjacent cells; however, there were no positive spots in the cytoplasm (Figure 3-b). Group 3: Many CX43-positive spots were observed on each cell membrane; however, there were no positive spots in the cytoplasm (Figure 3-c).

Figure 4. RT-PCR analysis of the 2D culture β-actin gene expression, used as an internal control, was positive at 804bp and was equivalent in all samples (left). CX43 gene expression was recognizable at 438bp in all groups, however, group 3 was the highest of the 3 groups and next was group 2 (right).

Figure 5. Immunofluorescence observations of CX43 in 3D culture. The cell shape in the 3D culture was star-like with contacts with each other by cell processes at 3 days. The cells were never attached with the cell membranes at any of the time periods. No CX43-positive spots were observed at any of the attached areas.
periodontal pocket epithelium. However, the mechanism by which MER cells proliferate is still unclear. Substrates used in 2D cultures on which cells grow and adapt to a flat surface are considerably limited and don’t reproduce the in vivo 3D environment. In this study, the 3D culture system was also used and a Type 1 collagen gel was used as the scaffold to mimic the environment that exists in situ. However, the 3D culture result of this study showed no CX43-positive reactions and the expression of CX43 mRNA was also much lower than that of the 2D culture.

It is known that the proliferation of epithelial cells in situ occur just the same as in the 2D culture, because epithelial cells attach and contact each other with their membranes. In contrast, mesenchymal cells grow in 3D culture similarly to the environment in situ, and cells contact each other via cell processes and also with the extracellular matrix. Therefore, the 3D culture system may prove to be a tremendous advantage to simulate the in situ environment. From this point of view however, in this 3D culture study, the epithelial cells grew in the same manner as mesenchymal cells and connected to each other with the extracellular matrix and with their cell processes, but not via the cell membrane which is not a normal environment for epithelial cell growth patterns. This is probably the reason why the proliferating epithelial cells in the 3D culture did not express CX43 and contacts via the cell membrane might be necessary for epithelial cells to proliferate.

Gap junctions were first demonstrated in 1958, by inserting microelectrodes into each of two interacting nerve cells in the nerve cord of crayfish. When a voltage gradient was applied between the two electrodes, the current readily passed, indicating that inorganic ions could pass freely from one cell interior to the other. Each gap junction is composed of two hemichannels which are themselves each constructed of 6 connexin molecules. Connexins are four-pass transmembrane proteins with C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extracellular loops (EL-1) and (EL-2). Connexins are assembled in groups of 6 to form hemichannels and 2 hemichannels then combine to form each gap junction. The connexin gene family is diverse, with 21 members identified in the human genome, and 20 in the mouse genome. They range between 26 and 60 kDa, and have an average length of 380 amino acids. The various connexins have been observed to combine into both homomeric and heteromeric gap junctions, each of which may exhibit different functional properties, including pore conductance, size selectivity, charge selectivity, voltage gating, and chemical gating. In the recent literature, connexins are most commonly named according to their molecular weights, e.g. CX43 is the connexin protein of 43 kDa. CX43 was initially identified in rat cardiac myocytes. Recently, it has been reported that the levels of CX43 expression in young rat bone marrow cells is higher than in older ones. Furthermore, the expression and localization of CX43 in the rat incisor, which is a continuously erupting tooth, is high in young odontoblasts. Muramatsu et al. reported that the reduction of CX43 expression may be associated with a loss of viability in human dental pulp. Schwab et al. reported that the number of CX43-immunoreactive osteogenic cells is higher than in fibroblastic cells in vitro, and this higher incidence of CX43 expression is probably related to the growth and cytodifferentiation programmed in the developing tissue.

White et al. reported that gap junctions between epithelial cells are essential for normal lens growth. In mice, the knockout of CX50 caused smaller lenses because of decreased epithelial cell proliferation. Generally, CX43 is known as the principal epithelial cell connexin, but CX50 also influences epithelial cell proliferation. Livny et al. reported that lycopene significantly up-regulates the expression of CX43, a key protein in the formation of gap junctional communication during human oral tumor cell proliferation. However, Yamasaki et al. suggested that different stimuli may affect cell proliferation and gap junctional intercellular communication differentially by distinct mechanisms and that gap junctions formed by CX32 are involved in the regulation of the growth of hepatocytes.

In this study, the expression of CX43 increased with cell proliferation at the morphological and molecular levels and this may suggest that the expression of CX43 is important for MER cells to proliferate. Our results further suggest that
the 2D culture system is better than the 3D culture system for investigating epithelial cell morphology and function. In the clinic, when inflammatory stimuli from either gingival connective tissues or the periodontal ligament affect the MER, the cells start to proliferate and increase the expression of CX43 and they eventually make a lining epithelium of the odontogenic cyst or the periodontal pocket epithelium.

CONCLUSION

The 2D culture is better than the 3D culture to investigate epithelial cell morphology and that CX43 expression is important for cell proliferation.

ACKNOWLEDGEMENTS

The author would like to thank members of the Department of Clinical Pathophysiology for their assistance. This research was supported, in part, by an Oral Health Science Center hrc 7 grant from the Tokyo Dental College and by a “High-Tech Research Center” Project grant for Private Universities, and by a matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2007-2009.

REFERENCES


