ORIGINAL ARTICLE

Effect of a Novel Calcium Carbonate-based Hypersensitivity Suppression Material on Integrin $\alpha_v\beta_3$ Expression in Human Deciduous Dental Pulp-derived Fibroblasts

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SYNOPSIS

Dental desensitizing materials are often applied to wedge defects, potentially allowing the material to come into contact with pulp cells. In this study, we aimed to clarify cell proliferation, calcium dynamics, and expression of integrin $\alpha_v\beta_3$ by culturing cells in a medium containing a calcium carbonate-based dental hypersensitivity treatment agent.

In this experiment, we performed a WST assay and observed fluorescently labeled antibodies using a confocal laser scanning microscope. The number of cells increased in the experimental group compared to the control group on day 1 of incubation. On days 4 and 7 of incubation, material-derived calcium was localized in the cytoplasm of the experimental group, overlapping with the Integrin $\alpha_v\beta_3$ expression sites. It was suggested that calcium derived from the material is taken up into the cytoplasm and promotes cell proliferation in the early stages of culture.

***Key words**: calcium, cell proliferation, dentin hypersensitivity, human deciduous dental pulp fibroblast like cells, integrin*

INTRODUCTION

Dentin hypersensitivity (DH) is a common disease in dental practice [1-6], and the incidence of DH tends to increase as the population composition ages [7,8]. Among the various etiologies of DH that have been suggested, the theory that the opening of dentin tubules results in the migration of intraductal dentin fluid is the most likely explanation [9-11]. In this case, it is accompanied by a substantial loss of dentin[12-14].

Materials that close the dentin tubules for the treatment of DH include polymeric materials, calcium phosphate, and aluminum lactate [15,16]. There are also materials that demineralize the dentin at the dentin tubule orifice to remineralize it [17]. The material we used this time is a newly developed material in Japan, mainly composed of calcium carbonate [18]. In previous studies, we observed that this material forms calcium phosphate inside and outside the dentin tubules by adding phosphoric acid supply solution, and that this material does not drop out of the dentin tubules by brushing with a toothbrush and ultrasonic cleaning. When human deciduous dental pulp-derived fibroblasts, human periodontal ligament fibroblasts, and human osteoblasts were cultured in

the material-added medium, the authors reported that the material had affinity for all three cell types and promoted the proliferation of each cell type[19].

Calcium is an inorganic substance[20] involved in signal transduction, muscle contraction, secretion of hormones and chemical transmitters, regulation of gene expression and cell cycle, and cell differentiation, and binds to Integrin. Among integrins, which are cell adhesion molecules, Integrin $\alpha_v\beta_3$ is expressed during the wound healing process. In this study, we cultured human deciduous dental pulp-derived fibroblasts in a medium containing a novel dental hypersensitivity suppression material based on calcium carbonate, and observed cell proliferation, cell morphology, intracellular and extracellular localization of calcium derived from the material, and expression of Integrin $\alpha_v\beta_3$, and discussed their relationships.

MATERIALS AND METHODS

1. Cell culture

Third-generation human deciduous dental pulp-derived fibroblasts (hDDPF; All Cells, Alameda, CA, USA) were seeded in 6 well microplates (AGC Technoglas Corp., Shizuoka, Japan) at a cell density of 2.15 × 10^5 /mL and grown under 37 °C, 5 % CO_2 for 1 week until semi-confluent. A stock solution of antibacterial and antifungal drug mixture (Antibiotic-Antimycotic Mixed Stock Solution (100 x) (stabilized); Nacalai Tesque, Kyoto, Japan) was adjusted to 10 % and added to Dulbecco's Modified Eagle's Medium Low Glucose (Nacalai Tesque) containing 10 % fetal bovine serum (Funakoshi Corp., Tokyo, Japan). During the incubation period, the medium was changed once every three days. Fourth Dentine® (4D: Medibo, Osaka, Japan) solution A (Calcium carbonate) and solution B (Phosphate feed solution) were mixed and added to each well. At this time, 0.5 g of solution A and 0.5 g of solution B were added per well. The experimental group consisted of cells cultured with 4D, and the control group consisted of cells alone. In the control group, the medium was replaced with a new medium that did not contain 4D.

2. WST assay

The hDDPF in proliferation was adjusted to a cell density of 2.15 × 10^5 cells/mL and seeded in 6 well microplates, 1 mL each. After confirming cell adhesion under a phase-contrast microscope (LSM700, Carl Zeiss Co., Ltd., Oberkochen, Germany), the cells were cultured for 1 or 7 days. After each incubation period, Cell Count Reagant SF (Nacalai Tesque) was added at a rate of 160 µL/well and incubated for 60 minutes at 5 % CO^2, 37 ˚C. After pipetting, 110 µL of each well of culture was collected in a 96 well microplate and formazan absorbance was measured in a microplate reader (SpectraMax M50S, Molecukar Devices, Sunnyvale, CA, USA). Statistical processing was used by unpaired *t*-test, and $p < 0.05$ was considered significant (n=8).

3. Conforcal Lazer Scanning Microscorpy (CLSM) images

The hDDPF in proliferation was adjusted to a cell density of 2.15 × 10^5 cells/mL and seeded in 6 well microplates, 1 mL each. After confirming the cell wall attachment, the culture medium was replaced with the same medium as the aforementioned experimental and control groups and cultured for 4 or 7 days. Calcium in solution A of 4D was pre-stained with Alizarin Red S (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and mixed with solution B of 4D. After each incubation period, working solution was prepared by adding 0.5 µL of Calcein AM to 1 mL of phosphate-buffered saline (-) (PBS(-)), and working solution was added to the culture medium at a ratio of 1 mL/well. The culture medium was incubated at 37 °C for 30 minutes in a 5 % CO_2 environment. Cells were then fixed in cold

80 % alcohol and diluted with anti-human integrin $\alpha_v\beta_3$ mouse antibody (Dako REAL Antibody Diluent: Agilent Technologies Japan, Ltd. Santa Cruz Biotechnology, Inc., Germany, Europe) at a dilution ratio of 1:100, and the cells were allowed to react with the antibody diluent for 60 minutes in the dark. Washing before and after antibody treatment was performed three times each in PBS(-). Cell morphology, 4D-derived calcium, and integrin $\alpha_v\beta_3$ localization were observed using LSM700, respectively. Each wavelength was used for cell morphology with Calcein AM (excitation wavelength 495 nm, fluorescence wavelength 515 nm), 4D-derived calcium with Alizarin Red S (excitation wavelength 425 nm, fluorescence wavelength 920 nm) and integrin $\alpha_v\beta_3$ (Alxa488 labeled anti-integrin $\alpha_v\beta_3$ antibody: excitation wavelength 490 nm, fluorescence wavelength 488 nm).

RESULTS

1. WST assay

After 1 day of incubation, the number of cells in the experimental group was significantly higher than in the control group. After 7 days of incubation, there was no significant difference in cell count between the control and experimental groups (Fig. 1).

2. CLSM images

After 4 and 7 days of incubation, the cell morphology of both the control and experimental groups was oval and spindle-shaped. In the experimental group, calcium stained with Alizarin red S was found inside and outside the cells during both incubation periods. In both the control and experimental groups, expression of integrin $\alpha_v\beta_3$ was observed (Fig. 2, 3), and in some of the experimental groups, the calcium in the material overlapped with the integrin $\alpha_v\beta_3$ expression sites (Fig. 3).

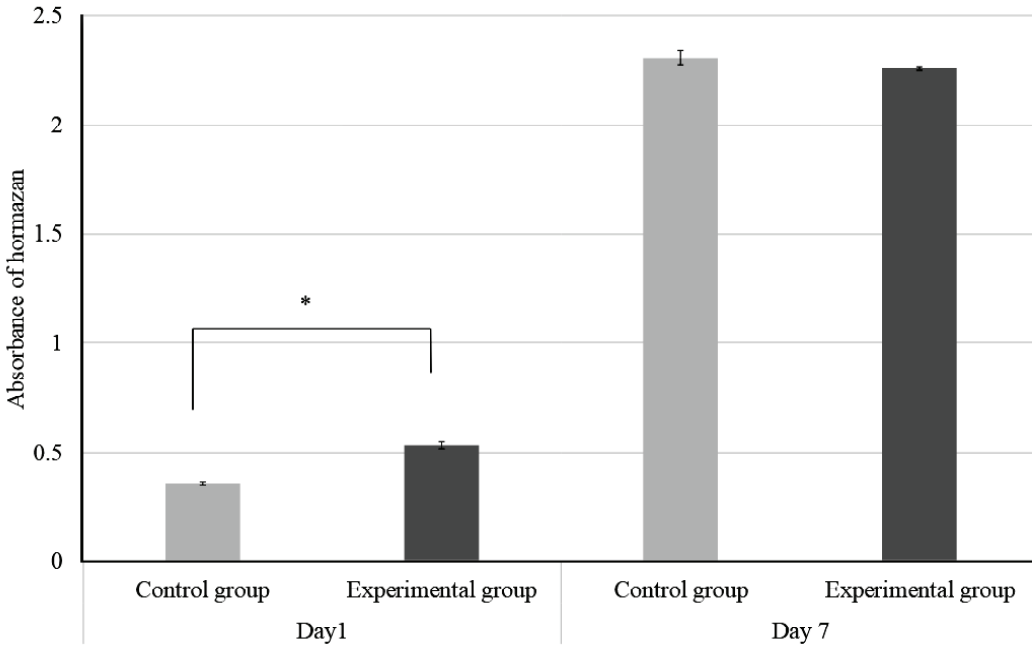

Fig.1 Cell counts after 1 and 7 days of culture. $p<0.05$

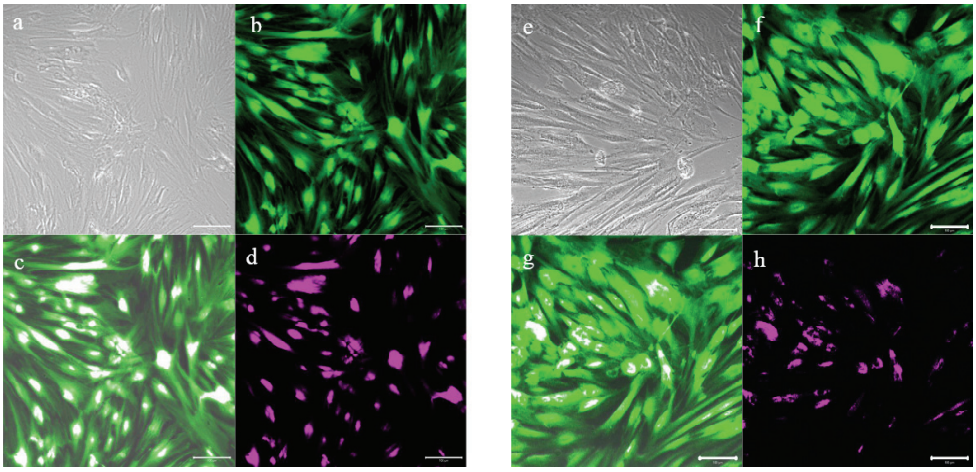

Fig.2 Calcein staining and 4D-derived calcium localization in the control group on days 4 and 7 of culture
The cells were oval and spindle shaped. Additionally, expression of integrin $\alpha_v\beta_3$ was observed.
a, b, c, d: Day 4 of culture, e, f, g, h: Day 7 of culture.
Green: living cell, Magenta: Integlin. The bar was shows 100 μm.

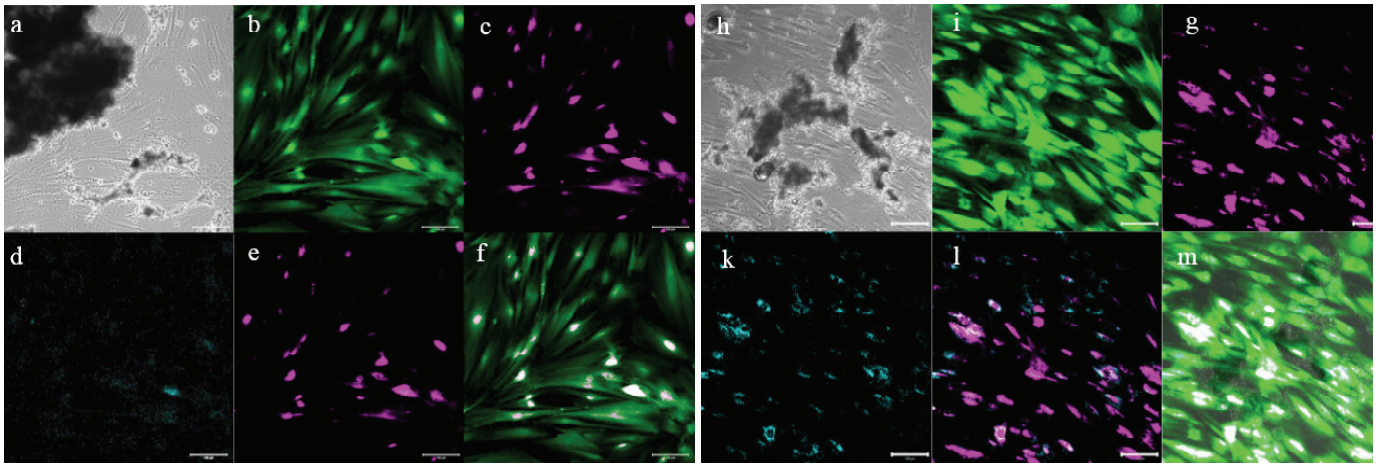

Fig.3 Calcein staining and 4D-derived calcium localization in the experimental group on days 4 and 7 of culture
The cells were oval and spindle shaped. Furthermore, expression of integrin $\alpha_v\beta_3$ was observed, and calcium stained with Alizarin Red S was observed inside and outside the cells. Calcium partially overlapped with the site of integrin $\alpha_v\beta_3$ expression.
a, b, c, d, e, f: Day 4 of culture, h, i, g, k, l, m: Day 7 of culture.
Green: living cells, Magenta: Integlin, Cyan: Alizarin. The bar was shows 100 μm.

DISCUSSION

In this study, in order to clarify the effect of calcium, the main component of the new dental hypersensitivity material, on hDDPF, we stained the hDDPF with calcium in advance and observed the phenomenon that calcium was localized on the cell surface or inside the cell, and cell proliferation was enhanced early in the culture. This suggests that calcium derived from this material is taken up by the cells and used for physiological activities such as cell proliferation within the cells, that is, it may bind to newly produced integrins or be used as a second messenger.

Calcium plays an important role as a signal transmitter within cells and is thought to be involved in a wide range of physiological functions[21-23]. Ca^{2+} is also involved in regulating cellular functions as a signal mediator in eukaryotic cells. For example, cell cycle control by the Ca^{2+} signaling pathway has been eluci-

dated at the molecular level [24, 25]. In cultured human gingival fibroblasts, it has been suggested that voltage-dependent l-type Ca^{2+} channels may be related to cell proliferation [26]. In fibroblasts, Ca^{2+} plays a signaling role and regulates cell proliferation as a second messenger [27]. When normal human skin fibroblasts are cultured with the addition of coral exoskeleton, a calcium carbonate crystal, the calcium carbonate that constitutes the coral exoskeleton moves into the cells, enhancing cell proliferation[28]. Integrins are cell adhesion molecules that connect cells to the extracellular stroma and transmit information from outside the cell[29]. Integrins are heterodimers consisting of α and β chains and are transmembrane glycoproteins present on the cell surface[30]. To date, at least 24 types of integrins have been identified, each consisting of 18 α-chains and 8 β-chains. The domain structure of the α chain contains a divalent metal ion-binding site, and it is thought that calcium binds to the α chain after integrin production and is involved in calcium signaling[31]. A deficiency of calcium ions destabilizes the structure of integrin[32]. In this study, we focused on integrin and considered calcium as a component of integrin.

Integrin $\alpha_v\beta_3$ is expressed during repair processes such as wound healing, angiogenesis, and bone regeneration in vivo[33]. Fibroblasts play an important role in the repair process, and calcium, which affects the function of fibroblasts, is present throughout the cytoplasm and in the nucleus. Thus, calcium for integrin $\alpha_v\beta_3$ and fibroblasts is necessary to promote repair.

CONCLUSION

It is suggested that when hDDPF is cultured with a novel hypersensitivity suppression material containing calcium carbonate, the calcium in the material is taken up by hDDPF and increases cell proliferation in the early stages of culture and may be used as a component of Integrin $\alpha_v\beta_3$, which is expressed by hDDPF.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest related to this study.

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