doi:10.1111/j.1365-2591.2008.01420.x

Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin

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Abstract

Mizuno M, Banzai Y. Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin. *International Endodontic Journal*, **41**, 933–938, 2008.

Aim The effect of calcium ions on dental pulp cells was examined and the mechanism of dentine bridge formation by calcium hydroxide was investigated.

Methodology Human dental pulp cells were treated with high concentration of calcium or magnesium ions for 24 h and fibronectin gene expression was measured by the quantitative PCR method. Human dental pulp cells were then cultured on fibronecin-coated dishes for 24 h, and osteocalcin and osteopontin gene expression, which are typical phenotypes of mineralized tissue forming cells, were measured by the quantitative PCR method.

Results Fibronectin gene expression was stimulated by calcium ions dose-dependently. On the other hand, magnesium ions did not influence fibronectin gene expression. Furthermore, pulp cells cultured on fibronectin-coated dishes enhanced the expression of phenotypes of mineralized tissue forming cells.

Conclusions Calcium ions released from calcium hydroxide stimulates fibronectin synthesis in dental pulp cells. Fibronectin might induce the differentiation of dental pulp cells to mineralized tissue forming cells that are the main cells to form dentine bridges, via contact with cells.

Keywords: calcium hydroxide, fibronectin, human pulp cells.

Received 10 August 2007; accepted 15 February 2008

Introduction

It has been reported that the dental pulp has an ability to form a dentine bridge after direct capping by calcium hydroxide or following pulpotomy (Tziafas 1994). Calcium hydroxide destroys the original odontoblasts and underlying pulp cells, and then initiates the recruitment of stem or undifferentiated cells that have an ability to differentiate to mineralized tissue forming cells (Schroder 1985, Fitzgerald *et al.* 1990, Goldberg & Smith 2004).

Calcium hydroxide supplies calcium ions which are a potent regulator for cell functions in tissue. Rashid *et al.* (2003) reported that calcium ions stimulate bone-related gene expression in human dental pulp cells. This finding implies that calcium ion might have a key role for the differentiation of dental pulp cells into mineralized tissue forming cells. However, the mechanism of how calcium ions regulate the differentiation of dental pulp cells to mineralized tissue forming cells is unclear.

Fibronectin (FN) is present in blood and in tissue as a component of the extracellular matrix. This

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protein has multifunctions, such as adhesion, migration and differentiation of cells (Ruoslahti 1981). During tooth development, the differentiation of odontoblasts is regulated by specific basement membrane-mediated epithelial–mesenchymal interactions (Ruch 1990). FN is present in the dental basement membrane, and mediates the elongation and polarization of odontoblasts by transmembrane–cytokine interaction (Nakashima 2005). It was also assumed that FN had an important role for the terminal differentiation of odontoblasts from undifferentiated cells present in dental pulp (Ruch 1990, Lesot *et al.* 1992).

These findings raise the possibility that calcium ions released from calcium hydroxide stimulates FN synthesis in dental pulp cells and FN enhances dentine bridge formation by the stimulation of recruitment of mineralized tissue forming cells from dental pulp cells.

In this study, the effect of divalent cations (calcium and magnesium ions) on FN gene expression of pulp cells and the effect of FN on mineralized tissue forming cells-specific gene expressions of pulp cells were investigated to evaluate the above possibility.

Materials and methods

Preparation of cells

Human pulp cells (HP cells) were obtained by explant culture from healthy pulp of premolar teeth from patients receiving orthodontic therapy with their informed consent following permission of the ethical committee of Hokkaido University. HP cells were cultured until the confluent stage in 35-mm-culture dishes with alpha-modified essential medium including 10% foetal calf serum and 100 μg mL^{-1} of streptomycin. Then, cells were dispersed with 0.5% trypsin and 0.02% EDTA, and transferred to 100-mm-culture dishes in the presence of the above medium. HP cells were cultured for six passages and were used for the experiment.

Cell culture condition

Human pulp cells were seeded at a density of 10.5×105 cells in 100-mm-culture dishes coated with type I collagen, and maintained in 7 mL of RPMI 1640 and Dulbecco's minimum essential medium (mixed with equal volume) containing 10% foetal calf serum for 7 days. The medium was changed to the above media containing 0.1% foetal calf serum and various concentrations of additional calcium or magnesium ions (0.2–0.7 mmol L^{-1} of each divalent cations). After cells were cultured for 24 h, they were harvested and total RNA was extracted to determine mRNA level of each gene.

Culture of pulp cells on FN-coated dishes

The 100-mm-culture dishes were coated with purified FN (1 mg mL^{-1}) which was purchased from Sigma Chemical Company (Tokyo, Japan), as previously described by Schaffer *et al.* (1994). Then, pulp cells were plated at a density of 2.5×104 cells cm^{-2}. After cells were cultured for 24 h in alpha-modified minimum essential medium containing 10% foetal calf serum, cells were harvested to analyse gene expression level.

Measurement of mRNA level by quantitative PCR method

Total RNA was extracted using ISOGEN® (WAKO Pure Chemical Industries, Osaka, Japan) and the amount of RNA was quantified by spectrometry at 260 and 280 nm. Messenger RNA present in total RNA was converted to cDNA by avian myeloblastosis virus (AMV)-reverse transcriptase and oligo dT primers. This reaction was performed for 60 min at 42 °C.

The mRNA levels of FN, osteocalcin (OCN) and osteopontin (OPN) were measured using quantitative PCR machine (Smart Cycler System™, Cepheid, CA, USA). The primers for FN, OCN and OPN were designed according to the sequence data of NCBI (Table 1). The PCR reaction tube (25 μL) contained PCR buffer,

Table 1 Primer sequence of human genes

	Forward	Reverse	NCBI accession no.
GAPDH	GGTGAAGGTCGGAGTCAACGG	TCTCGCTCCTGGAAGATGGTG	M33197
FN	AAGGAGAAGACCGGACCAAT	GGCTTGATGGTTCTCTGGAT	NM212482
OCN	ATGAGAGCCCTCACACCAC	CCATTGATACAGGTAGCGCC	X53698
OPN	ACCTGTGCCATACCAG	TTACATCATCAGAGTCG	AB209987

FN, fibronectin; OCN, osteocalcin; OPN, osteopontin.

3 mmol L^{-1} $MgCl_2$, 0.3 mmol L^{-1} dNTP mixture, 0.3 μmol L^{-1} of each primer, and Taq polymerase (1.25 unit per tube). Thermal cycling was performed at 95 °C for 30 s, and 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s. The amount of PCR product was estimated by measurement of the intensity of fluorescence of Syber Green I intercalated into the PCR product. Dividing them by the amount of glyceraldehyde-3 phosphate dehydrogenase (GAPDH), mRNA normalized the mRNA levels of FN, OCN and OPN in each sample.

Data analysis

All analyses were carried out at least three separate preparation of cells, and all data were presented as a fold of increase in expression over that of the controls, which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from three separate analysis. All statistical analysis was performed by Student's *t*-test.

Results

Figure 1 shows the effect of additional calcium or magnesium ions on FN gene expression in human dental pulp cells. The expression of FN increased with increasing calcium ions in a dose-dependent manner (additional concentration: 0.2–0.7 mmol L^{-1}); however, magnesium ions did not influence FN gene expression. This result indicates that calcium ions increase the synthesis of FN in pulp cells.

Osteocalcin and OPN gene expression in dental pulp cells were examined after cells were cultured on FN-coated dishes for 24 h. Both genes are typical phenotypes of mineralized tissue forming cells (Pockwinse *et al.* 1992). Pulp cells showed higher OCN and OPN gene expression compared with that cultured on noncoated dishes (Fig. 2).

Discussion

This study demonstrated that high concentrations of calcium ion enhanced FN gene expression in dental pulp cells. The culture medium originally contained 1.1 mmol L^{-1} of calcium ion, therefore, the range of calcium ions concentration exposed to cells was 1.1–1.8 mmol L^{-1}, and dental pulp cells showed a twofold higher FN gene expression under 1.8 mmol L^{-1} of calcium ions (Fig. 1). Lopez-Cazaux *et al.* (2006) reported that human pulp cells exposed to 1.8 mmol L^{-1} of calcium ions stimulated cell proliferation, alkaline phosphatase activity and odontoblast-specific gene (dentine sialo-phosphoprotein) expression compared with that of cells exposed to 0.4 mmol L^{-1} of

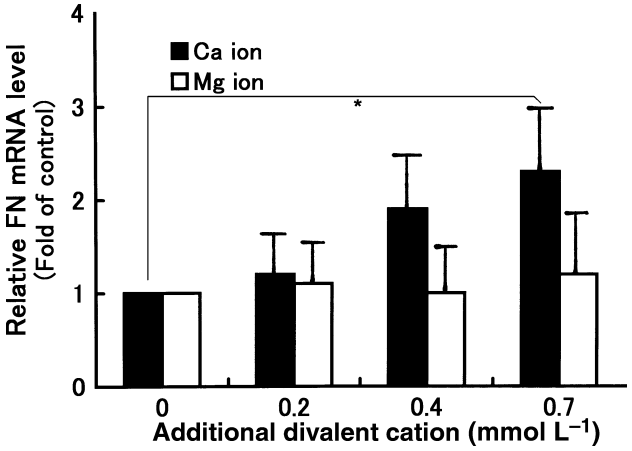

Figure 1 The effect of divalent cations on fibronectin (FN) gene expression in human pulp (HP) cells. Measurement of relative mRNA levels of FN in HP cells exposed to additional calcium or magnesium ions (0.2–0.7 mmol L^{-1}) using the real-time PCR machine. All experiments were triplicated and all analyses were carried out at least three separate preparations of experiments, and all data were presented as a fold of increase in expression that of the control, which was determined from parallel cultures grown under identical conditions without additional divalent cations. All bars show the average of three separate experiments and error bars represent the standard deviation (SD) of the determinations from three separate experiment; *statistically difference ($p < 0.05$).

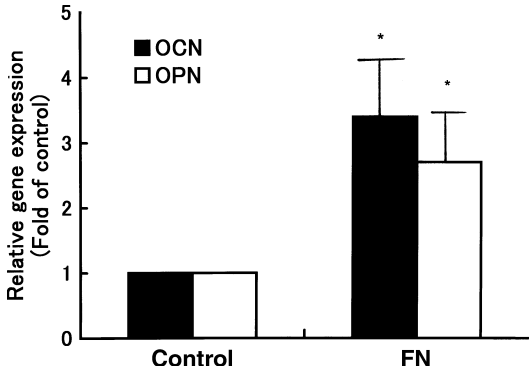

Figure 2 The effect of fibronectin (FN) on osteocalcin (OCN) and osteopontin (OPN) gene expressions in HP cells. Measurement of relative gene expressions of OCN and OPN in HP cells cultured on FN-coated dishes (FN) and noncoated culture dishes (control) by the analysis of quantitative PCR method. All experiments were triplicated and all analyses were carried out at least three separate preparations of experiments, and all data were presented as a fold of increase in expression that of the controls, which were determined from parallel cultures grown under regular culture dishes. All bars show the average of three separate experiments, and error bars represent the standard deviation (SD) of the determinations from three separate experiment; *statistically difference ($p < 0.05$) compared with control group.

calcium ion. In human serum, the calcium concentration is 2.5 mmol L^{-1} with 53% ionized. Therefore, the concentration of calcium ions in tissue is 1.3 mmol L^{-1}. The 1.8-mmol L^{-1} of calcium ions enhanced FN gene expression by pulp cells is obviously higher than that originally present in tissue. On the other hand, magnesium ions did not influence FN gene expression, which implies that the effect of calcium ions on pulp cells is specific, and is not a common feature of divalent cations. These findings indicate that calcium ions released from calcium hydroxide might stimulate the synthesis of FN in dental pulp cells.

Fibronectin is synthesized by fibroblasts, endothelial cells, chondrocytes, osteoblasts, glia cells and myoblasts. FN binds cells via cell membrane receptors including integrin families and transmembrane proteoglycan (Damsky & Werb 1992). The interactions of FN with cell-membrane receptors induce the rearrangement of cytoskeleton, which change the function of cells (Larsen *et al.* 2006). Concerning the effect of FN on osteoblasts, FN facilitates the differentiation and survival of osteoblasts from pre-osteoblasts (Moursi *et al.* 1996, 1997, Globus *et al.* 1998). Furthermore, vascular smooth muscle cells differentiate into osteoblastic cells when cultured on FN-coated dishes (Ding *et al.* 2006).

These findings raise the possibility that FN synthesized by pulp cells, induces the differentiation of dental pulp cells to mineralized tissue forming cells.

Several reports support this possibility. FN is found around the crystalline that had formed on the calcium hydroxide containing cement *ex vivo* (Seux *et al.* 1991) and *in vivo* (Tziafas *et al.* 1995), and pulp cells were also observed in close contact with these crystals. Furthermore, odontoblasts and dentine formation have been found in contact with implants of filters coated with plasma FN (Tziafas *et al.* 1992). It has been reported that the FN-rich layer formed between necrotic tissue and healthy pulp tissue is associated with hard-tissue forming cells (Yoshiba *et al.* 1995, 1996, Piva *et al.* 2006), and a FN-rich matrix may serve as a reservoir of growth factors, which have participated in the differentiation of odontoblasts (Tziafas *et al.* 1995, Murray *et al.* 2001). FN is detected immunohistochemically within interodontoblastic collagen fibres, which means that FN is important for initial dentine bridge formation (Kitasako *et al.* 2002).

Fibronectin has been referred to be crucial for cell migration, adhesion, proliferation and cell differentiation (Nakashima 2005). To form dentine bridge by dental pulp cells, cells should differentiate to mineralized tissue forming cells such as odontoblasts or osteoblasts.

To confirm the possibility that FN facilitates the differentiation of dental pulp cells to mineralized tissue forming cells, dental pulp cells were cultured on FN-coated dishes for 24 h. Thus, the expression of OCN and OPN gene, which are typical phenotypes of mineralized tissue forming cells (Pockwinse *et al.* 1992), was stimulated. These results imply that dental pulp cells expressed the phenotypes of mineralized tissue forming cells via contact with FN. On the other hand, OCN and OPN mRNA levels did not change when cells were cultured on FN-coated dishes for 3 h (data not shown). Carvalho *et al.* (1998) demonstrated that OPN gene expression in osteoblasts was enhanced when cells were cultured on FN-coated dishes for 24 h, but the effect of FN was not recognized by the long-term culture (48 h) on FN-coated dishes. These findings might indicate that the interaction of dental pulp cells with FN for the appropriate time is necessary for differentiation to mineralized tissue forming cells. They also reported that interaction of the arginine-glycine-aspartic acid (RGD) sequence present in FN with integrin receptors is a pre-requisite for the elevation

of OPN gene levels of osteoblasts (Carvalho *et al.* 1998). A similar interaction may be recognized in dental pulp cells cultured on FN-coated dishes.

Osteopontin is a noncollagenous protein belonging to the super family of cell adhesion molecules and performs multi functions (Guo *et al.* 1995). OPN is present in dentine (Butler 1995) and synthesized by pulp cells (Yokota *et al.* 1992, Nagata *et al.* 1994). OPN is known to regulate the mineralization process of dentine bridges (Ninomiya *et al.* 2001). These findings might imply that OPN synthesized by dental pulp cells is crucial for the initial mineralization during dentine bridge formation.

Conclusion

Calcium ions released from calcium hydroxide move to pulp tissue and elevate extracellular calcium ion concentrations. This elevation induces FN synthesis of pulp cells, followed by accumulating FN in the necrotic tissue adjacent to healthy pulp tissue. High amounts of FN might induce differentiation of pulp cells to mineralized tissue forming cells and dentine bridges might be formed by these cells.

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