Dentin phosphophoryn promotes cellular migration of human dental pulp cells

Yoshiyuki Yasuda, DDS, PhD, Masanobu Izumikawa, DDS, PhD,

Kuniaki Okamoto, DDS, PhD,[1] Takayuki Tsukuba, DDS, PhD, [1] Takashi Saito, DDS, PhD

Division of Clinical Cariology and Endodontology, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, 061-0293, Japan

[1]Division of Oral Molecular Pharmacology, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8588, Japan

Address requests for reprints to Dr. Yoshiyuki Yasuda, Division of Clinical Cariology and Endodontology, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido, 061-0293 Japan. TEL: +81-133-23-2841, FAX: +81-133-23-1423, E-mail address: yasuda@hoku-iryo-u.ac.jp.

Acknowledgements

Abstract

  Dentin phosphophoryn (DPP) is a dentin sialophosphoprotein (DSPP) gene product that has

an RGD motif and repeat sequences of aspartic acid and phosphorylated serine. To date, the

function of DPP in the early stage of reparative dentin formation still remains unclear. The

objective of this study was to evaluate the effects of DPP on pulp cell migration and

proliferation. DPP promoted cell migration in a concentration-dependent manner, thus

increasing it by about 3-fold at 1000 ng/ml compared to the control, but it had no effect on cell

proliferation. Dephosphorylated DPP also promoted cell migration, similarly to DPP. However,

cell migration was significantly suppressed by the addition of $\alpha v \beta 3$ integrin antibody to the

medium. Furthermore, porcine DPP-derived RGD peptide, but not its mutant RAD peptide,

significantly promoted cell migration. These results indicate that the RGD motif of DPP plays

an important role in the migration of human dental pulp cells.

Key words: Phosphophoryn; dentin sialophosphoprotein; migration; RGD motif; dental pulp

cells; reparative dentin

Introduction

 Reparative dentin formation on the exposed pulp surface is essential for healing after direct

pulp capping (1-3). Pulp capping agents must have an activity of hard-tissue-formation as well

as good biocompatibility and margin-sealing property. Calcium hydroxide has been used as a

pulp-capping agent, but an inflammatory reaction of pulp tissue and uneven hard tissue

formation have been observed (4, 5). Recently, the use of mineral trioxide aggregate (MTA) in

direct pulp capping has been reported, but a variety of histological responses were still noted (6,

7). Furthermore, the data from long-term clinical results are not yet available.

 Dentin phosphophoryn (DPP) is a dentin sialophosphoprotein (DSPP) gene product and a

highly phosphorylated protein that accounts for 50% of non-collagenous dentinal proteins (8,

9). DSPP is mainly expressed in odontoblasts and it is known to be a marker of the

differentiation of pulp cells into odontoblasts (10-14). DPP has the RGD motif at position 26

from the N-terminal and repeat sequences of aspartic acid and serine as its characteristic

domains (15). Aspartic acid and serine account for at least 75% of all amino acid sequences,

and 85-90% of the serine residues are phosphorylated (15). Porcine DPP consists of 550 amino

acids, while human DPP comprises 839 amino acids. The characteristic motif is almost

identical. We have previously reported that DPP covalently cross-linked to type I collagen

induces the mineralization of the collagen in calcium phosphate solutions and plays an

important role in calcification of dentin (16, 17). Recently, DPP has been reported to induce the

differentiation of human mesenchymal stem cells into osteoblasts as its RGD motif binds with

αvβ3 integrin on the cell surface (18). Reparative dentin formation is considered to be achieved

as the undifferentiated mesenchymal stem cells in the pulp tissue undergo the processes of

migration, proliferation, differentiation into odontoblasts, and calcification (19). However,

little has been reported concerning the role of DPP in the initial process of reparative dentin

formation. This study was carried out to clarify the involvement of DPP in pulp cell migration

and proliferation.

Materials and Methods

Cells and Cell-culture Conditions

Human dental pulp cells were obtained from a healthy third molar of 19 years old male which

were extracted during orthodontic treatment. The protocol for these experiments was reviewed

and approved by the Health Sciences University of Hokkaido Research Ethics Committee, and

informed consent was obtained from all tissue donors. The pulp tissues were separated from the

crown and rinsed once in PBS before being digested with 2 mg/ml collagenase (GIBCO, Grand

Island, NY) at 37°C for 30 min. The resultant suspension was then centrifuged to collect the

released cells. These cells were subsequently cultured in Dulbecco's Modified Eagle Medium

(DMEM, Sigma, St. Louis, MI) supplemented with 10% fetal bovine serum (FBS, Sigma),

10,000 U/ml penicillin (GIBCO), and 10 mg/ml streptomycin (GIBCO) at 37°C in a humidified

atmosphere of 5% CO_2.

Purification of Porcine DPP

The DPP was prepared from molar teeth extracted from porcine jaws obtained from a local

abattoir, as described previously (16). Briefly, the porcine dentin was powdered (400-700 μm)

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under liquid nitrogen. The powdered dentin was decalcified at 4°C with 0.5 M EDTA and 0.05

M Tris-HCl, pH 7.4, including protease inhibitors (100 mM 6-aminohexanoic acid, 5 mM

benzamidine-HCl, and 1 mM phenylmethylsulfonyl fluoride, Sigma). The extract was dialyzed

against distilled water and lyophilized. DPP was then purified using the calcium precipitation

method followed by DEAE-cellulose column chromatography (Amersham Biosciences,

Piscataway, NJ). After performing alkaline hydrolysis, the purified DPP underwent amino acid

and phosphate analyses as described previously (17). Phosphate analyses were based on a

highly colored complex of phosphomolybdate and malachite green (20).

The fragment peptide of porcine DPP-derived RGD peptide (DPP-RGD,

SEGDNNHSSRGDTSYNSDES) and its mutant RAD peptide (DPP-RAD,

SEGDNNHSSRADTSYNSDES) were synthesized by Invitrogen (Carlsbad, CA).

Preparation of Collagen-coated Inserts

Bovine dermal collagen solution (Sigma) was prepared according to the manufacturer's

instructions. Briefly, 8 ml of chilled collagen solution was mixed with 1 ml of 10 x PBS on ice.

The combination was blended with 1 ml of 0.1 M NaOH and the pH was adjusted to 7.2-7.6.

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The collagen gel was dispensed on the membranes of the inserts at 15 µg/50 µl/insert and then

incubated at 37°C overnight to dryness. The inserts were rinsed with sterile water to remove any

salts.

Cell Migration Assay

The migration of dental pulp cells was measured via a modified Boyden-chamber assay using

a transwell polycarbonate membrane insert of 6.4 mm in diameter with an 8 µm pore size. The

transwell inserts were placed in the 24-well plate and precoated with denatured collagen, as

described above. The cells (1 x 10^5/ml) were briefly pretreated in a final volume of 250 µl of

serum-free media (containing 0.1% BSA) with porcine DPP (0, 100, 250, 1000, 2500 ng/ml) ,

DPP-RGD (5 µM), DPP-RAD (5 µM) or fibronectin (Sigma, 1000 ng/ml) in 1.5 ml tubes and

placed in the upper compartments. In some cases, the cells were first treated for 20 min with

αvβ3-integrin antibody (25 µg/ml, Santa Cruz Biotechnology Inc, Santa Cruz, CA) in the tube,

and then were placed immediately in the upper chamber. To induce the migration by

chemoattractants, the lower chambers were filled with 750 µl of serum-free media conditioned

by the mouse NIH-3T3. The cells were incubated at 37°C for 24 h. The cells that had not

migrated through the barrier were removed with a cell scraper, and then the migrated cells were

then stained with Diff-Quik (Sysmex, Kobe, Japan). The number of migrated cells in each well

was counted in four random high power fields.

Dephosphorylation of Purified Porcine DPP

One milligram of porcine DPP was dephosphorylated with potato acid phosphatase (Sigma).

The porcine DPP was suspended in 10 mM sodium acetate, 50 mM EDTA, and 0.5 mU of the

enzyme/mg DPP was added. After digestion at 37°C for 48 h, the enzyme was then removed

by dialysis with distilled water. Phosphate analysis was performed after alkaline hydrolysis as

described previously (20).

Proliferation Assay

The proliferation assays were performed using the Cell Counting Kit-8 (Dojindo, Kumamoto,

Japan) according to the manufacturer's instructions. Briefly, human dental pulp cells were

treated with 1000 ng/ml of porcine DPP or dephosphorylated DPP in 96-well culture plates for 5

days in a 100 μl medium. WST-8 reagents were added to each well and incubated for 30 min at

37°C. Absorbance readings at a wavelength of 450 nm and reference wavelength of 650 nm

were taken using a spectrophotometer (Model 680; Bio-Rad, Hercules, CA).

Statistical Analysis

A statistical analysis was performed with the data obtained from five independent

experiments. The data were expressed as the means ± SD. The data were compared using

one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *P*

values < 0.05 were considered to be significant.

Results

We evaluated the effects of DPP on the cell migration of human pulp cells to clarify its role in

an early stage of reparative dentin formation. A clearly larger number of cells treated with DPP

at 1000 ng/ml migrated than did the control cells (Fig. 1A). Concerning the DPP

concentration-dependence of cell migration, the number of cells per microscopic field did not

differ at 100 ng/ml compared to the control (Fig. 1B). However, it clearly increased at 250

ng/ml, and rose to about 3-fold at 1000 ng/ml, compared to the control cells. On the other hand,

DPP exerted no effect on pulp cell proliferation at the concentration of 1000 ng/ml (Fig. 1C).

Next, we studied whether the RGD motif or phosphate residues of DPP are related to its cell

migration-promoting effect. DPP dephosphorylated with acidic phosphatase significantly

promoted cell migration by 3-fold compared to the controls, in a similar manner as that

observed for the same concentration of phosphorylated DPP (Fig. 2A). Moreover,

dephosphorylated as well as phosphorylated DPP showed no effect on cell proliferation (Fig.

2B). The pretreatment of pulp cells with αvβ3 integrin antibody (25 μg/ml) significantly

suppressed the cell migration-promoting effect of DPP nearly to the control level (Fig. 3A).

We further investigated the interaction RGD motif with αvβ3 integrin. Porcine DPP-RGD

peptide (5 µM) significantly promoted cellular migration as well as did fibronectin as a

positive control, but mutant DPP-RAD peptide did not (Fig. 3B).

Discussion

The DSPP gene has been reported to be expressed in bone as well as teeth (21), but as the level of its expression is very low, it has been used as a marker of odontoblast differentiation in many reports (10-14). DSPP is processed at its central part immediately after synthesis and thereafter becomes dentin sialoprotein (DSP) at the N terminal and DPP at the C terminal (22). We previously showed that DPP covalently cross-linked to type I collagen plays a major role in dentin calcification (16, 17). This role is also suggested by reports that human DSPP gene mutation causes dentinogenesis imperfecta (23) and that DSPP-deficient mice exhibit a pathologic condition resembling human dentinogenesis imperfecta (24). We found in this study that DPP also promotes pulp cell migration. These results suggest that DPP performs various important functions in reparative dentin formation.

DSPP is a member of the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family, showing an RGD motif, phosphorylated parts, and glycosylated parts along with bone sialoprotein (BSP), osteopontin (OPN), and dentin matrix protein 1 (DMP1) (25). These proteins are considered to directly regulate the appearance and growth of the crystalline

components of bone and teeth. In cancer cells, matrix metalloproteinase (MMP) is activated by

binding with SIBLING proteins, and this activated MMP was reported to degrade the matrix

around cells and promote cell migration (26). Recently, Karadag *et al.* found that BSP

promotes the migration of bone marrow stem cells via this RGD motif, but that OPN or DMP1

has no such effect (27). In this study, DPP was found to promote pulp cell migration, as does

BSP. Therefore, these differences in the function of the same RGD motif suggest that the

conformation of the amino acid sequences before and after RGD thus plays an important role

in the cell functions.

The treatment of pulp cells with DPP at 1000 ng/ml increased their migratory capacity by

about 3-fold compared to the control cells. However, DPP at the same concentration had no

effect on proliferation. This means that enhanced cell migration was not simply a result of an

increase in the number of cells. Previous studies clarified the importance of RGD motif in cell

differentiation, and the phosphate group in calcification (11, 16-18). To investigate which of

the characteristic motifs is more important for the cell migratory function, we used

dephosphorylated DPP, DPP-RGD peptide, DPP-RAD peptide and integrin antibody. While

dephosphorylated DPP and DPP-RGD peptide promoted cell migration, DPP in which the

RGD motif was blocked by pretreatment with integrin antibody and DPP-RAD peptide had no

effect on cell migration. These results suggest that the RGD motif rather than the phosphate

esters of DPP plays an important role in cell migration. Our results are supported by the

findings of previous report, in which that integrins were shown to be expressed on the surface

of dental pulp cells (28). However, further studies are needed to clarify precisely how the RGD

motif of DPP controls cell migration and differentiation.

The wound-healing process of injured pulp tissue has recently been investigated, and the

important roles of BMP and FGF2 in the early phase have been clarified. Kaida *et al.* found

that the number of cells expressing BMPs gradually increased in reparative dentin formation

(29). Furthermore, the induction of pulp cell differentiation and calcification by BMP-7

overexpression (30), and induction of reparative dentin formation by the control of FGF-2

release from a carrier have also been reported (31). It is also known that BMP and FGF2

strongly induce DSPP expression (11, 32). These findings suggested that DPP produced by

cells stimulated with BMPs and FGF2 may promote cell migration, while also enhancing

wound healing.

  To summarize, DPP was shown to promote not only differentiation or calcification but also

cell migration by acting on integrins on the surface of pulp cells via its RGD motif. Since DPP

plays an important role in the process of reparative dentin formation, it may therefore be

effective as a dental pulp capping agent.

Figure Legends

Fig. 1 The effect of porcine dentin phosphophoryn (DPP) on the migration and proliferation of dental pulp cells. Representative photographs of migrated cells (Original magnification × 400) (A). The concentration-dependent effect of DPP on cellular migration (B). The effect of DPP (1000 ng/ml) on cell proliferation: control cells (open circles), 1000 ng/ml DPP-treated cells (closed circles) (C). The bar represents the mean ± SD (n = 5, * p < 0.05 vs control, **p < 0.01 vs control).

Fig. 2 The effect of dephosphorylated DPP (De-DPP) on cellular migration (A) and proliferation: control cells (open circles), 1000 ng/ml De-DPP-treated cells (closed circles) (B). The bar represents the mean ± SD (n = 5, **p < 0.01 vs control).

Fig. 3 Interaction of RGD motif with αvβ3 integrin plays an important role in cell migration. The effect of αvβ3 integrin antibody on the cellular migration (A). The effect of porcine

DPP-derived RGD peptide (DPP-RGD) and its mutant peptide (DPP-RAD) on the cellular

migration (B). The bar represents the mean ± SD (n = 5, **$p < 0.01$ vs porcine DPP or

DPP-RGD-treated cells).

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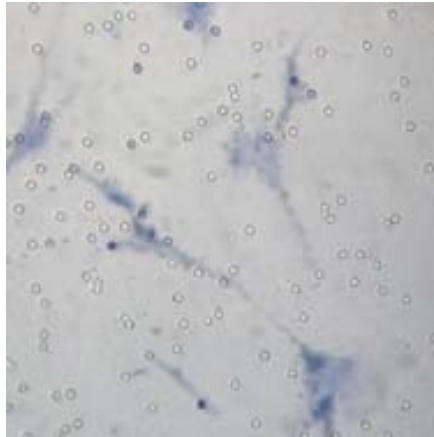
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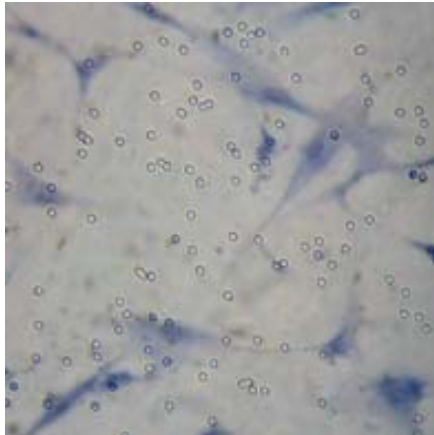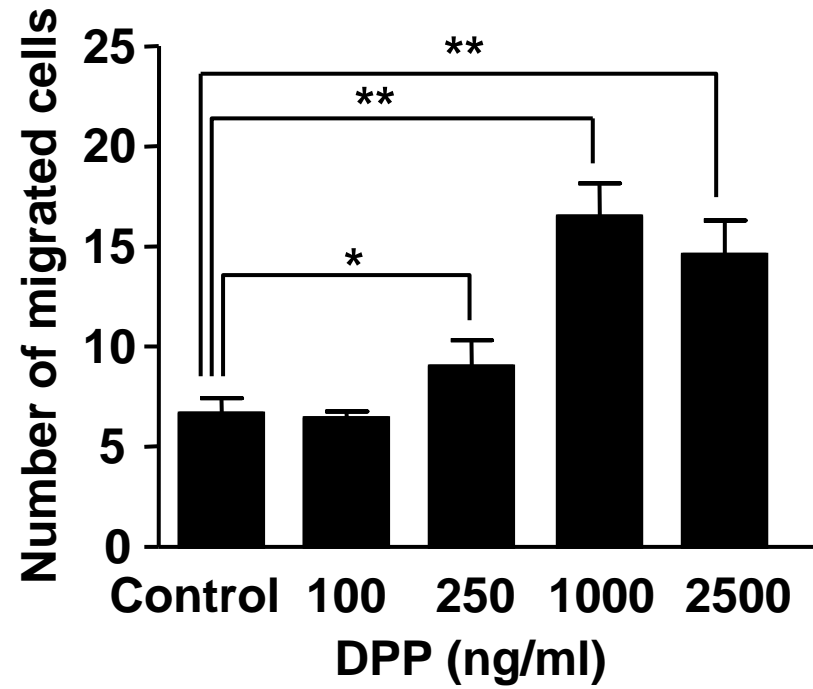
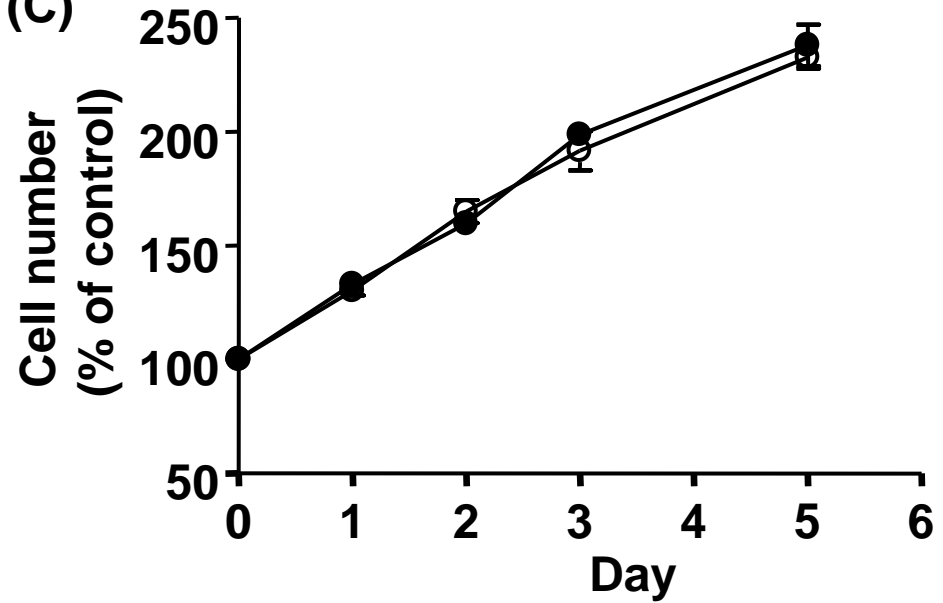# Fig. 1

(A)

Control

DPP 1000 ng/ml

(B)

DPP (ng/ml)

(C)

Day

Fig. 2

(A)

(B)

Fig. 3

(A)

DPP	−	+	−	+
αvβ3	−	−	+	+

(B)

DPP-RGD	−	+	−	−
DPP-RAD	−	−	+	−
Fibronectin	−	−	−	+