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ORIGINAL ARTICLE

Proliferation and adhesion of periodontal ligament cells on synthetic biominerals

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OBJECTIVE: Hydroxiapatite (HA) has been suggested as a useful biomaterial to support the regeneration of tissues. In this study, we investigated the adhesion of periodontal ligament (PDL) cells on octacalcium phosphate (OCP) and its hydrolyzed apatitic product (HL), which are known precursors of HA.

METHODS: Rat PDL cells were cultured on OCP or HL-coated dishes. Cell proliferation and adhesion and mRNA expression of collagen I, fibronectin integrin subunits were examined. Cell adhesion inhibition assays were carried out by GRGDSPK (Gly-Arg-Gly-Asp-Ser-Pro-Lys).

RESULTS: In early culture period, the cell number of PDL cells was lower on OCP and HL than that on control without any coating. However, the cell number on OCP or HL caught up with control later period. mRNA expression level of collagen I and fibronectin on OCP and HL were similar among OCP HL and control, although they differed early in the culture period. Integrin subunits were expressed on both OCP and HL as well as on control. Cell adhesion was inhibited by RGD inhibitor peptide.

CONCLUSION: Our findings indicated that rat PDL cells produce collagen I and fibronectin on OCP and HL, and then show increased cell numbers depending on adhesion to the matrices through integrins.
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Keywords: periodontal ligament; synthetic biominerals; cell adhesion; integrin; RGD peptide; cell proliferation

Introduction

The periodontium comprises the attachment apparatus of the tooth and consists of cementum, periodontal ligaments (PDL), alveolar bone, cementum, and gingiva. In order to play a critical role in the attachment between cementum and alveolar bone, the PDL contains heterogeneous cell populations that include cementum-forming cells (cementoblasts), bone-forming cells (osteoblasts), fibroblasts, osteoclasts, odontoclasts, and epithelial rests of Malassez's (Bartold *et al*, 2000; Hatakeyama *et al*, 2003a,b, 2006; Seo *et al*, 2004), and these cells maintain self-renewal of the PDL by harmonic synthesis.

Biosynthetic minerals have been used clinically as bone fillers for defects and as a coating on endosseous implants. In tissue engineering applications, hydroxyapatite (HA) has been suggested as a useful biomaterial to support the regeneration of tissues. The prototype for minerals in bone and teeth is considered to be basic calcium phosphate HA, containing impurities, such as carbonate and structural defects. It has been proposed that biologic apatite is formed via precursor phases, such as octacalcium phosphate (OCP) (Brown *et al*, 1962). OCP is not the most stable phase, and can hydrolyze to basic calcium phosphate under physiologic conditions (Brown *et al*, 1962; Suzuki *et al*, 1995a). OCP is more resorbable than other calcium phosphate compounds commonly used as bone graft substitutes, such as HA and β-tricalcium phosphate (β-TCP), and also enhances bone formation more than HA and β-TCP (Kamakura *et al*, 2002).

It has been suggested that synthetic OCP promotes osteoblast differentiation and enhances bone formation when implanted in the subperiosteal region in calvaria (Suzuki *et al*, 1993; Sasano *et al*, 1999), mandibles (Kamakura *et al*, 1996), or in bone defects experimentally (Kamakura *et al*, 1999; Imaizumi *et al*, 2006; Suzuki *et al*, 2006). In contrast, there is no information available about the biologic response of PDL cells to applications of OCP. This study investigated the proliferation and expression of adhesion and extracellular matrix molecules in PDL cells cultured on synthetic calcium phosphate, OCP and its hydrolyzed apatitic product (HL) in comparison with those of cells cultured on plastic substrates. We demonstrated that PDL cells

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attached to OCP and HL through integrin, then produced extracellular matrices related to the periodontium by increasing the cell numbers.

Materials and methods

Synthetic biominerals
The synthetic biominerals used were described in previous reports (Suzuki *et al*, 1991, 1993; Kamakura *et al*, 1996, 1999, 2004; Sasano *et al*, 1999). Briefly, the OCP original (OCP-Ca/P molar ratio 1.26) was treated for 48 h at 70°C to synthesise the OCP hydrolysis (HL-Ca/P molar ratio 1.47) (Suzuki *et al*, 1995b). After 0.5 ml of OCP and HL gel was coated on 48-well plastic cell culture dishes (BD Bio Sciences, Bedford, MA, USA), the plates were baked at 80°C for 1 h to harden. Before using for cell culture, dishes were sterilized by 70% ethanol.

Periodontal ligament cell purification and culture
Periodontal ligament cells were isolated from the surface of the maxillary molars (Hatakeyama *et al*, 2003a,b). Briefly, molars were placed in a 1.5 ml centrifuge tube containing phosphate-buffered saline (PBS) with 1 mg ml^{-1} collagenase (collagenase type II; Invitrogen, Carlsbad, CA, USA) and 0.25% Trypsin-EDTA (Invitrogen Life Technology), and incubated for 1 h at 37°C. Cells were cultured in α-MEM (Sigma, St Louis, MO, USA) containing 10% foetal calf serum (FCS) and antibiotics. Culture media were changed every 3 days. PDL cells were used for three to five passages.

Cell adhesion assay
Cell adhesion assays were carried out as described previously (Hoang *et al*, 2002). Forty eight well plastic plates (control dish; Becton Dickinson, Franklin Lakes, NJ, USA), coated by OCP (OCP-coated dish) or HL (HL-coated dish) were used for the following assays. In cell adhesion assays, 7.25×10^3 cells were seeded in each well of 48-well dishes, respectively, in α-minimum essential medium (MEM) containing 10% fetal bovine serum. Ninety minutes after seeding, plates were washed thrice with PBS to remove unattached cells, and attached cells were collected by treating the samples with 0.25 mg ml^{-1} trypsin in PBS. The number of the cells was counted using a hemocytometer.

Cell adhesion inhibition assay
Cell adhesion inhibition assays were carried out using synthetic peptides, GRGDSPK (Gly-Arg-Gly-Asp-Ser-Pro-Lys) as an inhibitory peptide for RGD-mediated adhesion between integrin and extracellular matrix molecules and GRADSPK (Gly-Arg-Gly-Ala-Ser-Pro-Lys) peptide (Sigma) as a control peptide. PDL cells were re-suspended in 0.5 ml of α-MEM and seeded onto OCP, HL in the presence or absence of 0, 5, 50, 500 μM of each peptide. After culturing for 24 h, wells were washed twice with PBS to remove unattached cells. The attached cells were removed and collected by treating dishes with 0.25 mg ml^{-1} of trypsin and 0.25 mg ml^{-1} of collagenase. The number of cells was counted using a hematocytometer. These experiments were performed in triplicate, using cells obtained from three different subjects.

Cell proliferation assay
Proliferation assays were carried out in 48-well cell culture dishes coated with OCP or HL. In proliferation assays, 7.25×10^3 cells in 0.5 ml of culture medium were seeded in a 48-well dish. Culture medium was changed every 3 days. After 1, 6, 12, and 18 days of culture, plates were washed thrice with PBS to remove unattached cells, and attached cells were collected by trypsinization as described above. The number of cells was counted using a hemocytometer.

RNA isolation and gene expression analysis by RT-PCR
Total RNA was isolated from PDL cells cultured on control cell culture dish, OCP, or HL-coated dishes using a total RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. The RNA samples (1 μg each) were subjected to first-strand cDNA synthesis using the SuperScript™ First-strand Synthesis System for RT-PCR kit (Invitrogen). RT-PCR was performed using gene-specific primers as described in Table 1. PCR amplifications were carried out using DNA polymerase *Taq* (Invitrogen) and a Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA). Thermal cycling for semi-quantitative PCR consisted of 5 min at 95°C for denaturation, 45 s at optimized temperature for annealing, and 45 s at 72°C for extension as the first cycle, followed by similar cycles with the denaturation step changed to 45 s and a final extension cycle of 10 min. Cycle numbers were empirically determined as described in Table 1 to optimize signal and amplification linearity. These findings were confirmed by three independent experiments.

Statistical analysis
All values are reported as mean ± standard deviation (s.d.). The data were analyzed by standard Student's *t*-test. Differences were considered significant at $P < 0.05$ (two-tailed).

Results

Cell adhesion assay and proliferation assay
To investigate whether synthetic biominerals play any potential role in PDL cell adhesion or proliferation, we performed cell adhesion and proliferation assays using PDL cells on control culture dishes, and OCP- or HL-coated dishes. PDL cells on OCP and HL displayed significantly reduced cell adhesion compared with those on the control culture dishes (control dishes = 5.166×10^3 cells/well, OCP = 2.5×10^3 cells/well, HL = 2.5×10^3 cells/well; Figure 1a). One day later, PDL cells showed increased proliferation on the control dishes (Figure 1b). On control dishes, PDL cell numbers reached confluency at 12 days. In contrast, the numbers of PDL cells on OCP- and HL-coated dishes remained small compared with that on control dishes. At 18 days,

Table 1 Amplimer for semi-quantitative RT-PCR

	GenBank accession no.	*Position*	*Upstream*	*Downstream*	*Product size*	*Annealing (°C)*	*Cycle number*
Integrin α2	AB067445	172–883	CAGTGAGAGCCAAGAAACAA	AACCATAGCCAACAGCAAA	712	60	35
Integrin α5	X79003	2427–2934	CTTCGGTTCACTGTTCCTC	TGGCTTCAGGGCATTT	508	53	35
Integrin β1	U12309	1398–2057	ACAGAAGAAGTAGAGGTGGTC	GAGGTTGAAATGGGAGC	660	60	36
Collagen I α1	Z78279	4185–4669	CACCTACAGCACGCTTGTG	AGTGGGCAGAAAGGGACTTAT	484	60	35
Collagen I α2	AF121217	3329–4036	GCGGAGGAGGCTATGACTT	GCAGGCGAGATGGCTTATT	708	56	33
Fibronectin	NM_019143	7697–8289	CCAGTGATGTTAGCAGA	CAGTAGTAAAGTGTTGGC	592	54	33
GAPDH	NM-017008	1232–1716	TGTTTGTGATGGGTGTGAA	ATGGGAGTTGCTGTTGAAG	485	60	30

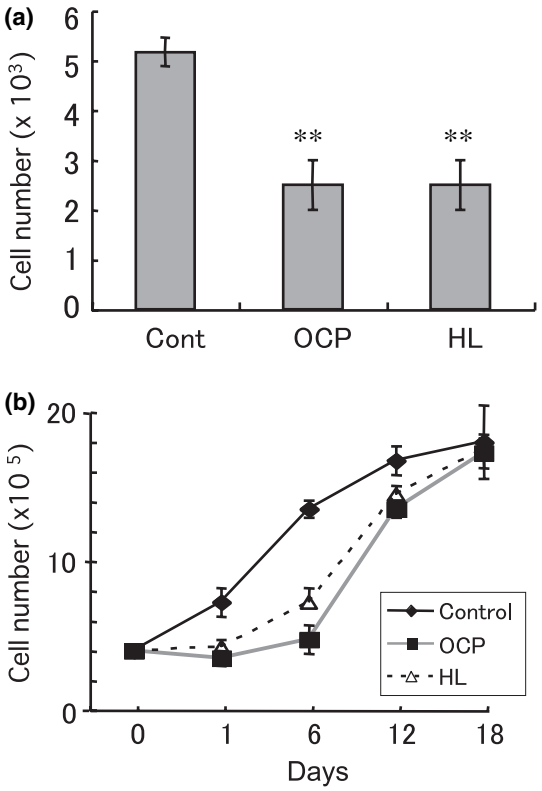

Figure 1 Cell adhesion (**a**) and proliferation (**b**). 7.25×10^3 PDL cells were seeded on each well of 48-well dishes coated with synthetic biomaterials, octacalcium phosphate (OCP) or its hydrolyzed material (HL) and on uncoated control dishes. Adhesion cells on each well were counted 90 min after seeding. $^{**}P < 0.01$ *vs* control dishes. Proliferation assays were carried out in 48-well cell culture dishes, coated with OCP or HL. In proliferation assays, 7.25×10^3 cells in 0.5 ml of culture medium were seeded in a 48-well dish. Culture medium was changed every 3 days. The number of cells was counted after 1, 6, 12, and 18 days of culture. Data are expressed as mean ± s.d. ($n = 3$)

cell numbers on all dishes were equal. There was no significant difference between the OCP- and HL-coated dishes throughout the experiment.

PDL cells expressed extracellular matrix and adhesion molecules mRNA on synthetic biomaterials

To define how PDL cells adhere to synthetic biominerals, the expressions of extracellular matrix and cell adhesion molecules integrins were analyzed. Using RT-PCR, expressions of collagen I α1 (Figure 2a), collagen I α2 (Figure 2b) and fibronectin (Figure 2c) on OCP and HL were found to be lower than those on control culture dish at 1 and 6 days. However, at 12 days, the mRNA expression of collagen I α1, collagen I α2 and fibronectin on OCP and HL reached the same level as that on the control dishes. The expression of mRNAs did not show any significant difference between the OCP and HL.

Expressions of integrin α2 (Figure 3a), α5 (Figure 3b), and β1 (Figure 3c) were examined. PDL cells expressed these integrins only weakly before seeding on plates for culture. In contrast, their expressions increased after 1 day of culture on control, OCP- or

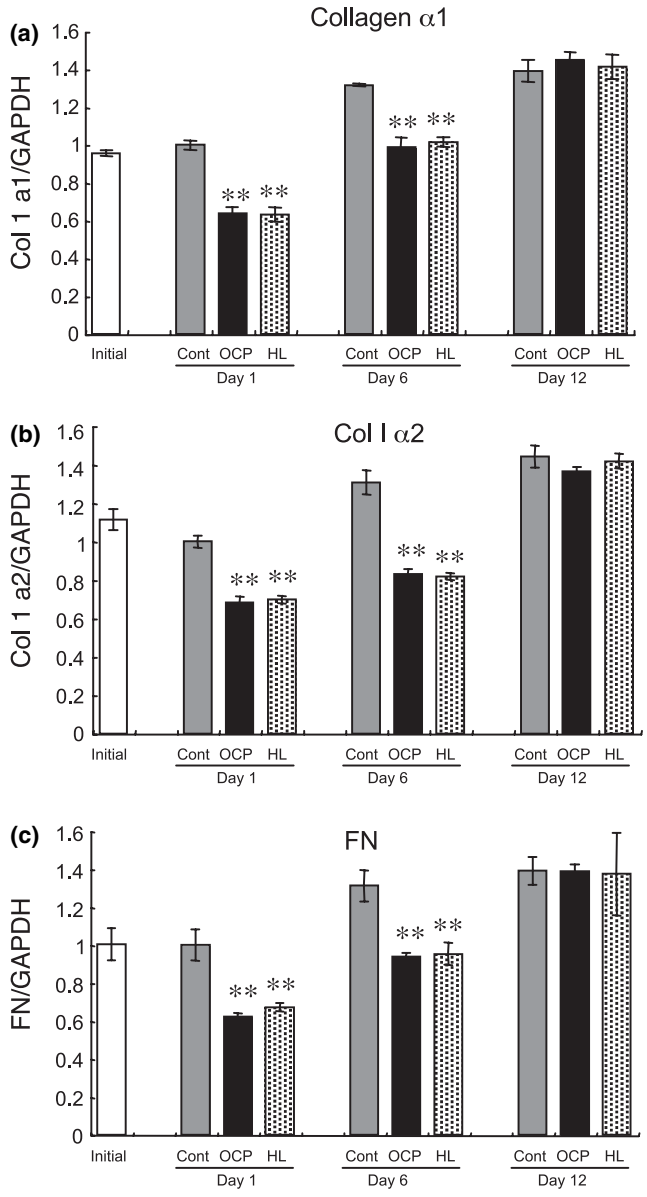

Figure 2 Expression of extracellular matrix molecules mRNA on synthetic biominerals. Collagen α1 (**a**), α2 (**b**), and fibronectin (**c**) mRNA level as measured by semi-quantitative RT-PCR analysis of total RNA extracted from PDL cells cultured on control, octacalcium phosphate (OCP)- or hydrolyzed material (HL)-coated dishes for 1, 6, and 12 days. $^{\#}P < 0.05$ *vs* control and $^{\#\#}P < 0.01$ *vs* control. Data are expressed as mean ± s.d. ($n = 3$)

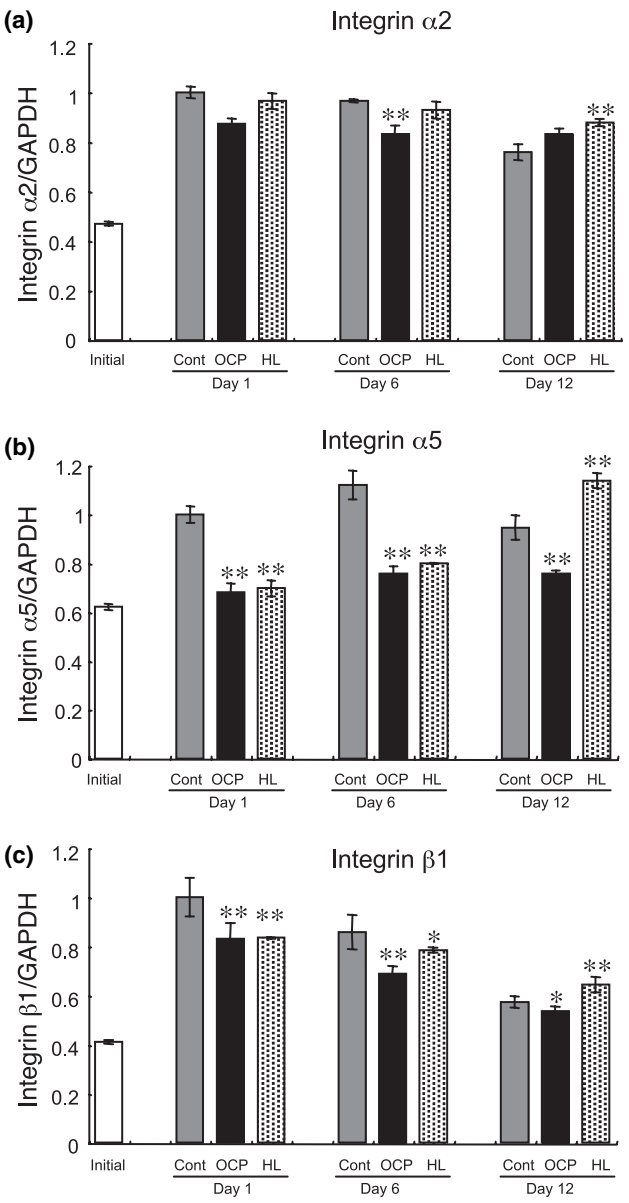

Figure 3 Expression of integrin mRNA on synthetic biominerals. Integrin α2 (**a**), α5 (**b**), and β1 (**c**) mRNA levels as measured by RT-PCR analysis of total RNA extracted from PDL cells cultured on control, octacalcium phosphate (OCP)- or hydrolyzed material (HL)-coated dishes for 1, 6, and 12 days. $^{\#}P < 0.05$ *vs* control and $^{\#\#}P < 0.01$ *vs* control. Data are expressed as mean ± s.d. ($n = 3$)

HL-coated dishes, and were maintained with some fluctuations during the culture period.

RGD sequences were involved in attachment and spreading on synthetic biomaterials

The RGD-containing peptide was used in an attempt to inhibit PDL cell attachment to the control (Figure 4a), OCP- (Figure 4b), or HL- (Figure 4c) coated dishes. RGD peptide inhibitors dose dependently blocked cell adhesion of PDL on control, OCP- and HL-coated dishes. In contrast, RAD peptide inhibitor did not inhibit adhesion.

Discussion

Character of PDL

The PDL is a soft connective tissue and its most important role is supporting teeth by thick bundles of collagen fibers embedded between the cementum and the inner wall of the alveolar bone socket. Furthermore, PDL contributes to tooth nutrition, homoeostasis, and repair of damaged periodontal tissue (Bartold *et al*, 2000; Hatakeyama *et al*, 2003a,b, 2006; Shimono *et al*, 2003).

The PDL includes osteoblasts, cementoblasts, osteoclasts, odontoclasts, fibroblasts, and epithelial rests of Malassez (Lekic *et al*, 2001; Azzaroli *et al*, 2002; Hoang

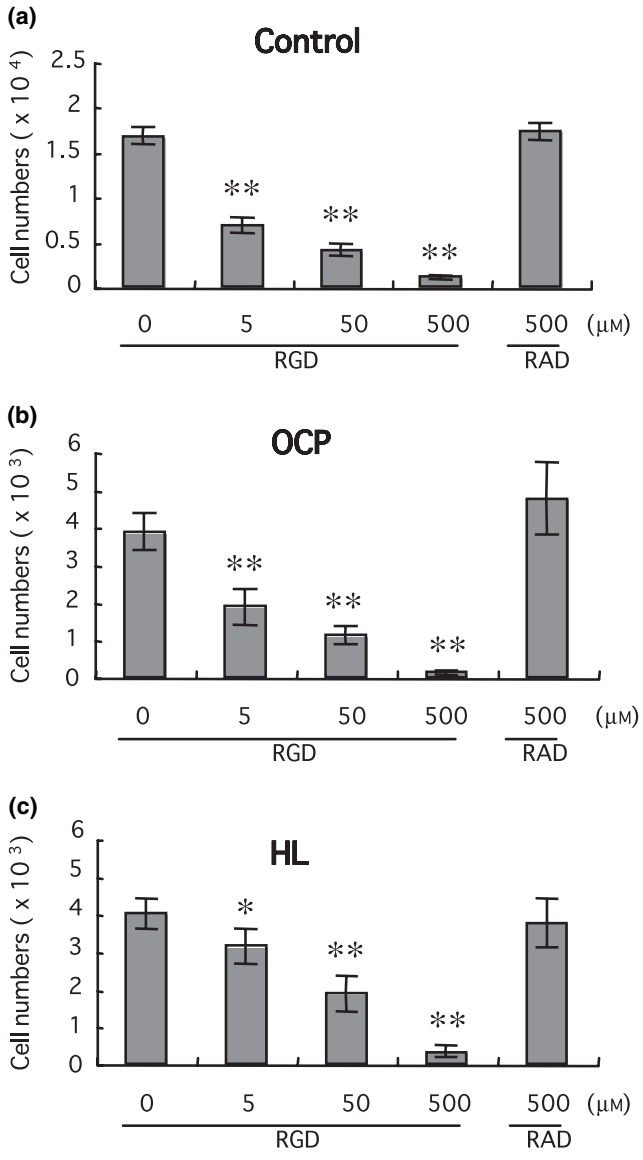

Figure 4 RGD-containing peptide dose dependently inhibited cell adhesion of periodontal ligament cells on control, octacalcium phosphate (OCP)- or hydrolyzed material (HL)-coated dishes for 24 h. RAD-containing peptide inhibitor was used as a negative control. The bars on top of each column show the standard deviation. Significant differences are indicated by $^*P < 0.05$, ($n = 3$) *vs* 0 μM. Data are expressed as mean ± s.d. ($n = 3$)

et al, 2002; Murakami *et al*, 2003; Shimono *et al*, 2003), and it has been suggested that these cells are involved in the repair of damaged periodontal tissues. When PDL is damaged as a result of rupture in bundles of collagen fibers, fibroblasts regenerate these collagen fibers. Along with collagen fiber regeneration, precursor cells of osteoblasts or cementoblasts migrate, attach, proliferate, differentiate, and produce mineralized tissues like bone or cementum, and the newly formed collagen fiber is embedded in these mineralized tissues.

OCP and HL

The prototype for minerals in bone and teeth, which includes alveolar bone and cementum, is usually considered to be basic calcium phosphate HA, containing impurities, such as carbonate and structural defects.

It has been suggested that HA is a useful biomaterial to support tissue regeneration in tissue engineering applications. OCP is known as a precursor of HA, but is not the most stable phase and can hydrolyze to basic calcium phosphate under physiologic conditions. This indicates that OCP is more resorbable and enhances bone formation more than HA. We previously reported that OCP promotes osteoblast differentiation and bone formation when implanted in the subperiosteal region (Suzuki *et al*, 1993; Sasano *et al*, 1999) or in bone defects experimentally (Kamakura *et al*, 1996, 1999).

Recently, OCP has been suggested to be a precursor of biologic apatite crystals and actually detected in human dentin (Bodier-Houlle *et al*, 1998), porcine enamel (Tohda *et al*, 1997), and premature suture closure in mouse calvaria (Crane *et al*, 2006). HL, used in the present study, had non-stoichiometric composition with a low Ca/P 1.46, compared with the stoichiometric value of HA (Ca/P 1.67) (Suzuki *et al*, 2006). The biologic apatite crystals are constituted of poorly crystalline HA with low Ca/P ratio, i.e., Ca-deficient HA. Thus, HL, prepared via OCP, would probably be analogous to biologic apatite (Suzuki *et al*, 2006).

However, no information was available about the biologic response of PDL cells following application of OCP. The present study investigated the proliferation and expression of adhesion and extracellular matrix molecules in PDL cells cultured on synthetic calcium phosphate, OCP and its hydrolyzed apatitic product (HL) in comparison with those of cells cultured on plastic substrates.

Cell adhesion to OCP/HL

The number of cells adhering to the OCP and HL after 1.5 h was lower than that of cells adhering to the plastic substrates. The cell adhesion to OCP or HL showed no significant difference. Adhesion of PDL cells to HA has been reported to be weak initially (Matsumura *et al*, 2004). The present study revealed that the adhesion of PDL cells to OCP or HL might be comparable with that of HA.

Molecules involved in adhesion between PDL cells and OCP/HL

Extracellular matrices and integrins have been suggested to play an important role in adhesion of PDL cells (Grzesik *et al*, 1998; Kapila *et al*, 1998; van der Pauw *et al*, 2002). Integrin $\alpha1\beta1$ and $\alpha2\beta1$ are known as receptors of collagen (White *et al*, 2004) and integrin $\alpha5\beta1$ mediated adhesion to fibronectin (Milliano and Luxon, 2003; Han and Roman, 2006). However, it is not known how PDL cells adhere to OCP/HL.

Our data showed that PDL cells initially expressed mRNA of type I collagen, fibronectin, and integrins. PDL cells may adhere to the extracellular scaffold made of collagens and fibronectin using integrins.

The production of type I collagen and integrins increased as the number of adhering cells increased. Type I collagen and fibronectin have been suggested to

accumulate on HA and OCP (Gilbert *et al*, 2000, 2003; Matsumura *et al*, 2004). These extracellular matrix molecules could be accumulated by PDL cells on OCP/HL, which may promote adhesion and proliferation of the cells, although it cannot be ruled out that fibronectin present in the serum in cell culture is involved in adhesion.

Involvement of integrins

The RGD peptide inhibited cell adhesion of PDL cells to OCP/HL as well as that to plastic substrates dose-dependently. Osteoblast precursors and osteoblasts have been known to adhere to HA through integrins (Okamoto *et al*, 1998). Our study demonstrated, for the first time, that PDL cells adhere to OCP and HL through integrins and the RGD motif.

Selective adhesion of stem cells to OCP/HL

The cultured PDL cells are composed of various cell types and it is not known which cell adheres to OCP/HL. Mesenchymal stem cells (Sawyer *et al*, 2005) and PDL stem cells (Seo *et al*, 2004) are capable of adhering to HA, and adhesion has been suggested to contribute to cell proliferation. PDL stem cells may also adhere to OCP/HL to proliferate. It has been reported that PDL cells differentiate to osteoblastic cells on HA (Matsumura *et al*, 2004). OCP/HL may also induce differentiation of PDL cells as well as proliferation.

Periodontal ligament cells may produce type I collagen and fibronectin on OCP and HL, then proliferate and differentiate with recognising of the ECM molecules accumulated on OCP and HL using integrins.

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