รายงานวิจัย

อิทธิพลของคอลลาเจนชนิดที่ 1 ต่อระดับการแสดงออกของโปรตีนออสทีโอพอนทีนในเซลล์เอ็นยึดปริทันต์ที่ถูกกระตุ้นด้วยอินซูลินไลค์โกร๊ธแฟกเตอร์-1

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เรื่อง

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บทคัดย่อ

การสร้างเนื้อเยื่อปริทันต์ขึ้นใหม่ทดแทนส่วนที่ถูกทำลายไป (periodontal tissue regeneration) นั้นต้องอาศัยสัญญาณจากสิ่งแวดล้อมที่อยู่รอบตัวเซลล์ ได้แก่ เมทริกซ์นอกเซลล์ (Extracellular matrix) รวมทั้งโกร๊ธแฟกเตอร์ต่างๆ   ซึ่งจะมีบทบาทต่อพฤติกรรมของเซลล์   วัตถุประสงค์ในการทดลองนี้ต้องการศึกษาถึงอิทธิพลของคอลลาเจนชนิดที่ 1 ที่มีต่อการตอบสนองของ อินซูลินไลค์โกร๊ธแฟกเตอร์-1 (Insulin-like growth factor, IGF-I) ในเซลล์เอ็นยึดปริทันต์ ในแง่ของการดิฟเฟอเรนชิเอทไปเป็นเซลล์สร้างกระดูก โดยดูจากระดับการแสดงออกของออสทีโอพอนทีน (Osteopontin) โดยผลการทดลองพบว่าคอลลาเจนชนิดที่ 1 มีผลในการยับยั้งการแสดงออกของออสทีโอพอนทีน การเติมอินซูลินไลค์โกร๊ธแฟกเตอร์-1 สามารถเพิ่มระดับการแสดงออกของออสทีโอพอนทีนได้ในภาวะที่มีคอลลาเจนชนิดที่ 1 เท่านั้น ซึ่งอาจแสดงถึงการรบกวนสัญญาณในการยับยั้งการแสดงออกของออสทีโอพอนทีนของคอลลาเจนชนิดที่ 1 นอกจากนี้การยับยั้งสัญญาณจากคอลลาเจนโดยใช้แอนตีบอดีที่จำเพาะต่อเบต้า1 อินทีกรินรีเซบเตอร์ (beta1 integrin receptor) ร่วมกับอินซูลินไลค์โกร๊ธแฟกเตอร์-1 ไม่ให้ผลแตกต่างจากภาวะที่มีการเติมแอนตีบอดีหรืออินซูลินไลค์โกร๊ธแฟกเตอร์-1 เพียงอย่างเดียว จากผลการทดลองนี้สรุปได้ว่าอินซูลินไลค์โกร๊ธแฟกเตอร์-1สามารถขัดขวางผลของคอลลาเจนในการยับยั้งการแสดงออกของออสทีโอพอนทีนโดยผ่านทางเบต้า1 อินทีกรินรีเซบเตอร์ ซึ่งการทราบกลไกที่ควบคุมการซ่อมแซมเนื้อเยื่อปริทันต์จะเป็นแนวทางในการพัฒนาและส่งเสริมให้เกิดการสร้างเนื้อเยื่อปริทันต์ขึ้นใหม่ให้มีประสิทธิภาพมากขึ้น

Project Title   The influence of collagen I on insulin-like growth factor-I induced

                        osteopontin expression in human periodontal ligament fibroblasts.

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ABSTRACT

Extracellular matrix and growth factors are the crucial factors that regulate healing and regenerating process in human periodontal ligament (HPDL) cells. The purpose of this study was to examine the effects of type I collagen and insulin-like growth factor-I (IGF-I) on osteopontin (OPN) expression. The data showed that OPN expression was significantly decreased when cells were cultured on collagen-coated plates. Addition of IGF-I obviously induced OPN expression only in a collagen-coated condition, suggesting an attenuation effect of IGF-I on OPN expression inhibited by type I collagen. Cells treated with a combination of inhibitory antibody to $\beta1$ integrin and IGF-I, showed the same level of OPN expression as those of the group treated with either inhibitory antibody to $\beta1$ integrin or IGF-I alone. These results indicate that IGF-I counteracts with the inhibitory signal from type I collagen through a $\beta1$ integrin receptor.

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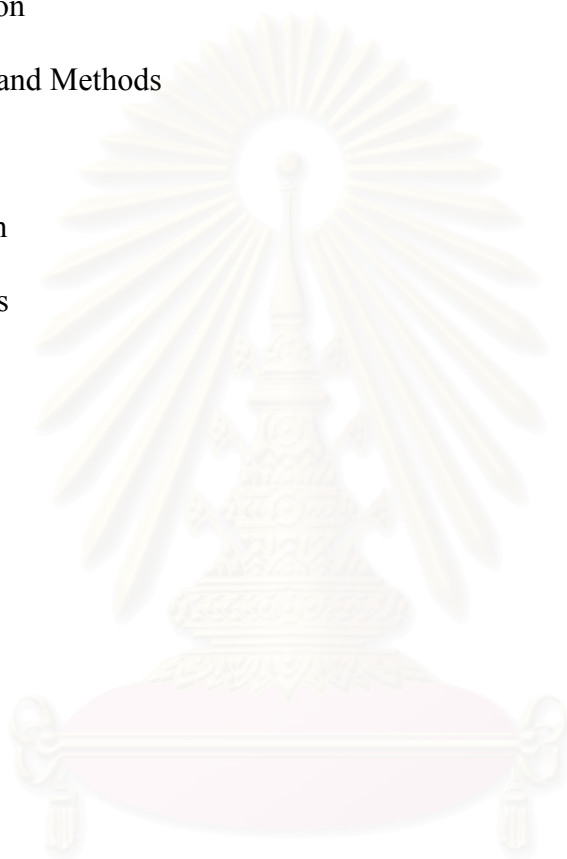
INTRODUCTION

Regeneration of the lost tissues due to periodontal disease is the ultimate goal of the treatment. To achieve the goal, polypeptide growth factors have been applied in addition to the treatment. Both in vivo and in vitro studies have revealed that some growth factors such as insulin-like growth factor-I (IGF-I) and platelet-derived growth factor (PDGF) have the potential to aid in periodontal regeneration [1,2].

Most tissues express IGF-I and the local production of this growth factor is thought to be important for the regulation of growth and differentiation of epithelial and mesenchymal cells. The biological action of IGF-I is mediated by IGF-I receptor (IGF-IR), a transmembrane heterotetramer with tyrosine kinase activity [3,4]. It is well documented that IGF-I associated with the regulation of inflammation and bone formation [5,6]. In the inflamed rat cartilage, the increase of IGF-I plays a role in early acute inflammatory event and later in the formation and remodeling of bone-bridge [7]. Similar result was found in bone fracture healing, IGF-I level increased after the fracture and reached a maximal level in the middle to late stage of callus formation which suggest a role of IGF-I in the process of bone regeneration [8]. Regarding to periodontal tissue, IGF-I has been shown to have mitogenic and chemotactic effects in vitro [9,10] and enhance periodontal wound healing in vivo [1, 11]

Extracellular matrix (ECM) also plays an important role in the regulation of cell dynamic and cell behavior by providing extracellular signal via receptor mediated signaling such as integrin receptors. Integrin receptors are heterodimeric receptors consisting of α and β subunits mediating cell-ECM interactions [12]. Type

I collagen is the major component of ECM which provides tensile strength and support, as well as modulates various cellular functions. There are evidences showing an association between type I collagen and growth factors during the process of wound healing. An application of basic fibroblast growth factor (bFGF) to type I collagen sponge could enhance dermal wound healing [13]. An induction of angiogenesis was detected after embedding type I collagen sponge soaked with several growth factors including IGF-I in the mouse subcutaneous [14]. However, the correlated mechanism between IGF-I and type I collagen for periodontal wound healing was still unknown.

In mineralized tissue, osteopontin (OPN) is one of the predominant RGD-containing ECM proteins [15] and is ubiquitously expressed in all skeletal tissues during embryogenesis [16]. The increase of OPN expression, one of osteogenic differentiation markers, was detected when rat bone marrow cells were exposed to dexamethasone in order to promote an osteoblast phenotype [17]. OPN has been shown to be up-regulated by IGF-I and facilitate wound healing in the initial stage of mineralization of rat periodontal tissue [18,19]. In addition, it was reported that selective adhesion to fibronectin, laminin and type I collagen could induce OPN gene expression in osteoblastic cells [20]. Since ECM, including collagen, involves in maintaining a pool of growth factors to regulate cellular function and homeostasis [21], it is interesting to investigate the effect of IGF-I on OPN expression in the collagen-rich condition. We showed here that in HPDL cells, type I collagen inhibited the OPN expression and IGF-I could attenuate this inhibitory effect via β1 integrin receptors.

MATERIALS AND METHODS

Cell culture

Human periodontal ligament (HPDL) cells were obtained from explants that obtained from periodontal ligament tissue attached to non-carious, freshly extracted third molars, or teeth removed for orthodontic reasons as previous described [22]. All patients gave informed consent. Briefly, teeth were rinsed with sterilized phosphate buffer saline (PBS) several times and the PDL tissues were scraped from the middle third of the roots. The explants were cultured on a 35-mm culture plate (Nunclon Surface, NUNC A/S, Roskilde, Denmark) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-Glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 5 µg/ml of amphotericin B at 37˚C in humidified atmosphere of 95% air, 5% CO_2. The medium and supplements are from Gibco BRL (Carlsbad, CA, USA). The medium was replaced every other day until cells grew out from the explants. After reaching confluence, cells were subcultured with a ratio of 1:3. For this study, cells from the third to the fifth passages were used. All experiments were performed in triplicate using cells prepared from three different donors.

Preparation of type I collagen-coated plate

Type I collagen (Vitrogen100, Cohesion, Palo Alto, CA, USA) was dissolved in 0.012 N HCl in a concentration of 3 mg/ml. Stock solution was diluted in 0.025% acetic acid to a final concentration of 40 µg/cm^2. Before use, 6-well culture plates (Nunclon Surface, NUNC A/S, Roskilde, Denmark) were coated with a film of type I

collagen by a modification technique. Briefly, aliquots of 1ml per well were dispensed and the plates were shaken overnight at 4˚C. The plates were then washed twice with sterilized PBS and air-dried in a lamina flow cabinet at ambient temperature. Normal plastic culture plates were used as control in this study.

Activation of HPDL cells with IGF-I

HPDL cells were seeded in 6-well plates, coated and uncoated with type I collagen, and allowed to attach for 24 hours. Cells were then starved in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO, USA) overnight before further treatments. Cells were treated with 0, 1, 5, 10 ng/ml of IGF-I (Sigma Chemical Co., St. Louis, MO, USA) in serum-free condition for 48 hours.

In the inhibitory experiment, cells were treated with 1 ng/ml of inhibitory antibody to β1 integrin (Chemicon International, Temecula, CA, USA) for 30 minutes prior to application of 5 ng/ml of IGF-I for another 48 hours.

Reverse-transcription polymerase chain reaction (RT-PCR)

Cells were treated with IGF-I as described above for 48 hours, total RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to manufacturer's instructions. The concentration of purified RNA in each sample was determined by the absorption at 260/280 nm using a spectrophotometer (Thermospectronic, Roche, NY, USA). One µg of total RNA per sample was used to generate cDNA by a reverse transcription reaction using AMV (Avian myeloblastosis virus) reverse transcriptase (Promega Corporation, Madison,

WI, USA) for 1.5 hours at 42˚C. Subsequent by a polymerase-chain reaction was performed to detect of OPN cDNA. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as internal control. The primers were designed based on the reported sequences from Genbank. The oligonucleotide sequences of the primers are

OPN   forward   5'TACCCTGATGCTACAGACG3'

                  reverse   5'CAACCAGCATATCTTCATGGCTG3'

GAPDH   forward   5'TGAAGGTCGGAGTCAACGGAT3'

                  reverse   5'TCACACCCATGACGAACATGG3'

After denaturation for 1 minute at 94˚C, 30 cycles of amplification followed by final extension for 10 minutes at 72˚C were performed using Taq polymerase (Qiagen, Hilden, Germany) by a thermocycler (Tpersonal, Whatman Biometra, Goettingen, Germany). The cycling parameters were denatured for 1 minute at 94˚C, annealing for 1 minute at 60˚C and extension for 1.45 minutes at 72˚C. The PCR products were separated electrophoretically on 2% agarose gel and visualized by ethidium bromide fluorostaining.

Western blot analysis

Cells were treated for 48 hours and extracted using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing cocktail protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). The amount of protein was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Protein extraction from each sample was separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a PVDF membrane (Immobilon-P,

Millipore Corporation, Bedford, MA, USA). The membrane was incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at room temperature for 1 hour. Then, the membrane was stained overnight with primary antibody for OPN (Chemicon International, Temecula, CA, USA) or β-actin (Chemicon international, Temecula, CA, USA) at the dilution of 1:1000 in blocking buffer at 4˚C. After extensively washed with PBS, the membrane was incubated with biotinylated-secondary antibody (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 minutes, respectively. The bands of protein were detected using a commercial chemiluminescence system (Pierce, Rockford, IL, USA) and were exposed on CL-X Posture film (Pierce, Rockford, IL, USA). The band intensity was determined by Scion Image analysis software (Scion Corporation, Frederick, Maryland, USA).

Statistical analysis

Statistical analysis of independent t -tests of triplicate determinations was performed. Differences at $p < 0.05$ were considered statistically significant.

RESULTS

IGF-I induces OPN expression in the presence of type I collagen

We first determined the effect of IGF-I on OPN expression. HPDL cells were cultured in the presence of 0, 1, 5, 10 ng/ml of IGF-I for 48 hours and the cell extracts were collected. RT-PCR analysis in figure 1A demonstrated that OPN expression was not altered after treated with various doses of IGF-I. Figure 1B showed that IGF-I had no effect on the level of the OPN protein, which concurred with the result from RT-PCR.

It had been shown that the signal from ECM may affect the response of the cells to cytokines and growth factors [23], we therefore investigated the effect of various concentration (0, 1, 5, 10 ng/ml) of IGF-I in the presence of type I collagen on the OPN expression in HPDL cells. Figure 1C revealed that in the presence of type I collagen, IGF-I could activate OPN mRNA expression as compared to the control. The same result was obtained from Western blot analysis (figure 1D).

Because there was no marked difference on OPN expression when treated with 5 and 10 ng/ml of IGF-I, the minimal effective dose (5 ng/ml) was selected and used throughout the rest of the experiments.

Type I collagen inhibits OPN expression in HPDL cells

To study the effect of type I collagen on the expression of OPN, HPDL cells were cultured on collagen-coated or uncoated plates. The mRNA expression of OPN in the presence and absence of type I collagen was shown in figure 2A. Interestingly, a decrease of OPN expression was observed in the type I collagen-coated condition

as compared to the uncoated one. A similar result was also found in Western blot analysis as shown in figure 2C. Quantitative analysis of the data confirmed that the level of OPN mRNA (figure 2B) and protein (figure 2D) expression were significantly reduced by type I collagen.

We further determined the effect of type I collagen on OPN expression in HPDL cells at different time point. HPDL cells were harvested at 12, 24, 48 hours and the OPN expression was analyzed by RT-PCR. The decrease of the OPN expression in the presence of type I collagen was observed at 24 hours and was obvious at 48 hours (figure 3A, lane 2).

IGF-I attenuates the inhibitory effect of type I collagen on the OPN expression in HPDL cells

We next determined whether IGF-I could affect the OPN expression inhibited by type I collagen in HPDL cells. Five ng/ml of IGF-I was added to the cultures in the presence of type I collagen, cell extracts were collected at different time points (12, 24, 48 hours). RT-PCR analysis was performed.

The results in figure 3A (lane 3) indicated that the inhibitory effect of type I collagen on the OPN expression was attenuated by IGF-I. The quantification of the data (figure 3B) showed that IGF-I significantly restored the OPN expression in HPDL cells on collagen-coated condition at 48 hour. These results indicated that the inhibitory effect of type I collagen on the OPN expression was abolished by IGF-I.

The inhibitory effect of type I collagen on the OPN expression is mediated by β1 integrin receptor

In order to investigate the mechanism of the effect of type I collagen on OPN expression, an inhibitory antibody specific to β1 integrin was used to block type I collagen signaling through this integrin. Interestingly, the expression of OPN was strongly increased at 48 hours after treated with an inhibitory antibody to β1 integrin as compared to the control. The result in figure 4A, lane 4 showed that inhibitory antibody to β1 integrin could abolish the inhibitory effect of type I collagen. A similar result was also obtained from Western blot analysis (figure 4C, lane 4). Figure 4B and 4D, lane 2 and 4 showed that inhibitory antibody to β1 integrin significantly restored OPN expression.

We finally examined the interaction between the IGF-I and type I collagen pathway on the regulation of OPN expression in HPDL cells. RT-PCR analysis of the OPN expression was shown in figure 4A. The lane 5 in figure 4A exhibited the increase of OPN expression after treated with the combination of IGF-I and inhibitory antibody to β1 integrin. However, no difference of OPN expression was observed between the groups treated with IGF-I (lane 3) and those treated with inhibitory antibody to β1 integrin (lane 4) alone. It is noted that the increased level of the OPN expression in all treated groups in collagen-coated condition (lane 3-5) were not higher than those in uncoated condition (lane 1). The Western blot analysis (figure 4C) was corresponding to the result revealed by RT-PCR. The quantitative data showed no significant difference among these 3 groups (figure 4B and 4D, lane 3-5). This result suggested that the enhancing effect of IGF-I on OPN expression in the presence of type I collagen is mediated via β1 integrin receptor signaling pathway.

DISCUSSION

In this study, we determined the effect of type I collagen and IGF-I on the OPN expression in HPDL cells. We showed that IGF-I had no effect on the OPN expression. It is interesting that the level of OPN expression was markedly decreased when cells were cultured on the collagen-coated plates and this effect could be eliminated by an inhibitory antibody that specific to β1 integrin receptor. This result suggested that the inhibitory effect on the OPN expression exerted by type I collagen is signaling through β1 integrin receptor. Moreover, IGF-I could counteract the inhibitory effect caused by type I collagen. This finding shows the direct evidence of the interaction between IGF-I and integrin signaling in HPDL cells.

The effect of type I collagen on the expression of OPN has been reported. Liu and colleagues showed that bone marrow-derived mesenchymal stem cells cultured on type I collagen coated PLGA membrane increased the expression of OPN [24]. The contrasting result of this study might stem from various factors, such as different cell types or different integrins involved in the signaling pathways. It is well documented that the major integrin receptors for type I collagen are α1β1 and α2β1 [25,26]. In addition, non-integrin receptor for type I collagen, such as discoidin domain receptor, has been reported [27]. Therefore, we cannot exclude the effect of such receptor in this study. However, in this experiment, the counteracting effect of the inhibitory antibody to β1 integrin indicated that type I collagen signaling via β1 integrin is involved in the regulation of OPN expression in HPDL cells. This result is corroborated by a previous study showing that type I collagen regulated alkaline phosphatase activity via activation of α2β1 integrin receptor in HPDL cells [28].

Further investigation is necessary to clarify the more specific pathway for type I collagen in the regulation of OPN expression.

Our result demonstrated the association between type I collagen and IGF-I on the expression of OPN in HPDL cells. The result form figure 1 revealed that IGF-I increased the expression of OPN in cells grown under collagen-coated condition but not in the uncoated one. This finding may imply that IGF-I had no direct effect on the OPN expression. We hypothesize that IGF-I attenuates the inhibitory effect of type I collagen on the OPN expression in HPDL cells. Our result corresponds to the previous immunohistochemical report in rat PDL cells. In the area which was mainly composed of type I collagen, OPN-positive staining PDL cells were found in greater number in growth hormone-induced IGF-I-treated group when compared to control [19]. In addition, the result from figure 5 showed that application of both IGF-I and inhibitory antibody to β1 integrin had no additional or synergistic effect on the OPN expression when compared to the condition of adding either one alone. This evidence supports our hypothesis that IGF-I restores the inhibitory effect exerted by type I collagen through β1 integrin.

The upregulation of IGF-IR level by type I collagen may be one possibility to explain the interaction between type I collagen and IGF-I on the OPN expression. This could make cells more susceptible to IGF-I and respond to IGF-I even in the dose that has no effect on plastic plates. There are evidences of the IGF-IR alteration during inflammatory and regenerating process. From a previous study, an increase of IGF-IR and tissue IGF-I levels was detected in pancreatitis, reflecting the correlation of IGF-IR, IGF-I and inflammation [29]. A recent study has suggested the involvement of IGF-IR signaling during mineralized nodule formation by HPDL

cells [30]. Normally, IGF-IR mRNA is expressed at a higher level in HPDL cells than in gingival fibroblasts [31]. Parkar and colleagues revealed that IGF-IR was not detected by immunohistochemical study in HPDL cells but it was occasionally stained weakly in regenerating tissue [32]. However, we found that the level of IGF-IR mRNA was not changed when the cells were cultured on the collagen-coated plate compare to the uncoated condition (data not shown).

The signal from growth factors that can interact with the signal of integrin has been reported. For example, the signals from type I collagen via α2β1 integrin and PDGF synergistically enhance cell proliferation in smooth muscle cells [23]. Rousselle and colleagues also indicated the collaboration among type I collagen, αvβ3 integrin and IGF-I in the regulation of metalloproteinase activity in rabbit bone cells [33]. There was a report showed that signaling from β1 integrin receptor regulated cell adhesion and proliferation in response to IGF-I [34]. Differently, the present study demonstrates that the signal from β1 integrin was modulated by IGF-I. Further study is required to elucidate the role of type I collagen and IGF-I association as well as their downstream signaling pathways in the regulation of OPN expression.

From this study, we conclude that type I collagen inhibits OPN expression in HPDL cells via β1 integrin and IGF-I counteracts this inhibitory signal. This basic knowledge will provide a better understanding of the mechanisms that contribute to the periodontal wound healing and regeneration.

REFERENCES

[1] S. E. Lynch, R. C. Williams, A. M. Polson, T. H. Howell, M. S. Reddy, U. E. Zappa, and H. N. Antoniades, A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration, J. Clin. Periodontol. 16 (1989) 545-548.

[2] W. V. Giannobile, R. A. Hernandez, R. D. Finkelman, S. Ryan, C. P. Kiritsy, M. D'Andrea, and S. E. Lynch, Comparative effects of platelet-derived growth factor-BB and insulin-like growth factor-I, individually and in combination, on periodontal regeneration in Macaca fascicularis, J. Periodontal Res. 31 (1996) 301-312.

[3] D. LeRoith, H. Werner, D. Beitner-Johnson, and C. T. Roberts, Jr., Molecular and cellular aspects of the insulin-like growth factor I receptor, Endocr. Rev. 16 (1995) 143-163.

[4] W. S. Cohick, and D. R. Clemmons, The insulin-like growth factors, Annu. Rev. Physiol. 55 (1993) 131-153.

[5] S. R. Thaller, J. Hoyt, H. Tesluk, and R. Holmes, Effect of insulin-like growth factor-1 on zygomatic arch bone regeneration: a preliminary histological and histometric study, Ann. Plast. Surg. 31 (1993) 421-428.

[6] L. Meinel, E. Zoidis, J. Zapf, P. Hassa, M. O. Hottiger, J. A. Auer, R. Schneider, B. Gander, V. Luginbuehl, R. Bettschart-Wolfisberger, O. E. Illi, H. P. Merkle, and B. von Rechenberg, Localized insulin-like growth factor I delivery to enhance new bone formation, Bone 33 (2003) 660-672.

[7] F. H. Zhou, B. K. Foster, G. Sander, and C. J. Xian, Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats, Bone 35 (2004) 1307-1315.

[8] T. Taniguchi, T. Matsumoto, and H. Shindo, Changes of serum levels of osteocalcin, alkaline phosphatase, IGF-I and IGF-binding protein-3 during fracture healing, Injury 34 (2003) 477-479.

[9] N. Matsuda, W. L. Lin, N. M. Kumar, M. I. Cho, and R. J. Genco, Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors in vitro, J. Periodontol. 63 (1992) 515-525.

[10] S. Ivanovski, H. R. Haase, and P. M. Bartold, Isolation and characterization of fibroblasts derived from regenerating human periodontal defects, Arch. Oral Biol. 46 (2001) 679-688.

[11] R. B. Rutherford, C. E. Niekrash, J. E. Kennedy, and M. F. Charette, Platelet-derived and insulin-like growth factors stimulate regeneration of periodontal attachment in monkeys, J. Periodontal Res. 27 (1992) 285-290.

[12] R. O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673-687.

[13] M. G. Marks, C. Doillon, and F. H. Silver, Effects of fibroblasts and basic fibroblast growth factor on facilitation of dermal wound healing by type I collagen matrices, J. Biomed. Mater. Res. 25 (1991) 683-696.

[14] A. Kanematsu, S. Yamamoto, M. Ozeki, T. Noguchi, I. Kanatani, O. Ogawa, and Y. Tabata, Collagenous matrices as release carriers of exogenous growth factors, Biomaterials 25 (2004) 4513-4520.

[15] Y. Gotoh, E. Salih, M. J. Glimcher, and L. C. Gerstenfeld, Characterization of the major non-collagenous proteins of chicken bone: identification of a novel 60 kDa non-collagenous phosphoprotein, Biochem. Biophys. Res. Commun. 208 (1995) 863-870.

[16] L. C. Gerstenfeld, Osteopontin in skeletal tissue homeostasis: An emerging picture of the autocrine/paracrine functions of the extracellular matrix, J. Bone Miner. Res. 14 (1999) 850-855.

[17] K. L. Yao, R. Todescan, Jr., and J. Sodek, Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture, J. Bone Miner. Res. 9 (1994) 231-240.

[18] P. C. Lekic, D. Rajshankar, H. Chen, H. Tenenbaum, and C. A. McCulloch, Transplantation of labeled periodontal ligament cells promotes regeneration of alveolar bone, Anat. Rec. 262 (2001) 193-202.

[19] H. Li, P. M. Bartold, W. G. Young, Y. Xiao, and M. J. Waters, Growth hormone induces bone morphogenetic proteins and bone-related proteins in the developing rat periodontium, J. Bone Miner. Res. 16 (2001) 1068-1076.

[20] R. S. Carvalho, P. J. Kostenuik, E. Salih, A. Bumann, and L. C. Gerstenfeld, Selective adhesion of osteoblastic cells to different integrin ligands induces osteopontin gene expression, Matrix Biol. 22 (2003) 241-249.

[21] F. Rosso, A. Giordano, M. Barbarisi, and A. Barbarisi, From cell-ECM interactions to tissue engineering, J. Cell Physiol. 199 (2004) 174-180.

[22] K. Pattamapun, S. Tiranathanagul, T. Yongchaitrakul, J. Kuwatanasuchat, and P. Pavasant, Activation of MMP-2 by Porphyromonas gingivalis in human periodontal ligament cells, J. Periodontal Res. 38 (2003) 115-121.

[23] S. T. Hollenbeck, H. Itoh, O. Louie, P. L. Faries, B. Liu, and K. C. Kent, Type I collagen synergistically enhances PDGF-induced smooth muscle cell proliferation through pp60src-dependent crosstalk between the alpha2beta1 integrin and PDGFbeta receptor, Biochem. Biophys. Res. Commun. 325 (2004) 328-337.

[24] G. Liu, Y. Y. Hu, J. N. Zhao, S. J. Wu, Z. Xiong, and R. Lu, Effect of type I collagen on the adhesion, proliferation, and osteoblastic gene expression of bone marrow-derived mesenchymal stem cells, Chin. J. Traumatol. 7 (2004) 358-362.

[25] S. K. Akiyama, K. Nagata, and K. M. Yamada, Cell surface receptors for extracellular matrix components, Biochim. Biophys. Acta 1031 (1990) 91-110.

[26] J. A. Schiro, B. M. Chan, W. T. Roswit, P. D. Kassner, A. P. Pentland, M. E. Hemler, A. Z. Eisen, and T. S. Kupper, Integrin alpha 2 beta 1 (VLA-2) mediates reorganization and contraction of collagen matrices by human cells, Cell 67 (1991) 403-410.

[27] W. F. Vogel, Collagen-receptor signaling in health and disease, Eur. J. Dermatol. 11 (2001) 506-514.

[28] S. Ishikawa, K. Iwasaki, M. Komaki, and I. Ishikawa, Role of ascorbic acid in periodontal ligament cell differentiation, J. Periodontol. 75 (2004) 709-716.

[29] E. Karna, A. Surazynski, K. Orlowski, J. Laszkiewicz, Z. Puchalski, P. Nawrat, and J. Palka, Serum and tissue level of insulin-like growth factor-I (IGF-I) and IGF-I binding proteins as an index of pancreatitis and pancreatic cancer, Int. J. Exp. Pathol. 83 (2002) 239-245.

[30] E. Nemoto, M. Shimonishi, Y. Nitta, and H. Shimauchi, The involvement of platelet-derived growth factor receptors and insulin-like growth factor-I receptors

signaling during mineralized nodule formation by human periodontal ligament cells, J. Periodontal Res. 39 (2004) 388-397.

[31] X. Han, and S. Amar, IGF-1 signaling enhances cell survival in periodontal ligament fibroblasts vs. gingival fibroblasts, J. Dent. Res. 82 (2003) 454-459.

[32] M. H. Parkar, L. Kuru, M. Giouzeli, and I. Olsen, Expression of growth-factor receptors in normal and regenerating human periodontal cells, Arch. Oral Biol. 46 (2001) 275-284.

[33] A. V. Rousselle, C. Damiens, E. Grimaud, Y. Fortun, M. Padrines, N. Passuti, and D. Heymann, Potential synergies between matrix proteins and soluble factors on resorption and proteinase activities of rabbit bone cells, Histol. Histopathol. 16 (2001) 727-734.

[34] H. L. Goel, M. Fornaro, L. Moro, N. Teider, J. S. Rhim, M. King, and L. R. Languino, Selective modulation of type 1 insulin-like growth factor receptor signaling and functions by beta1 integrins, J. Cell Biol. 166 (2004) 407-418.
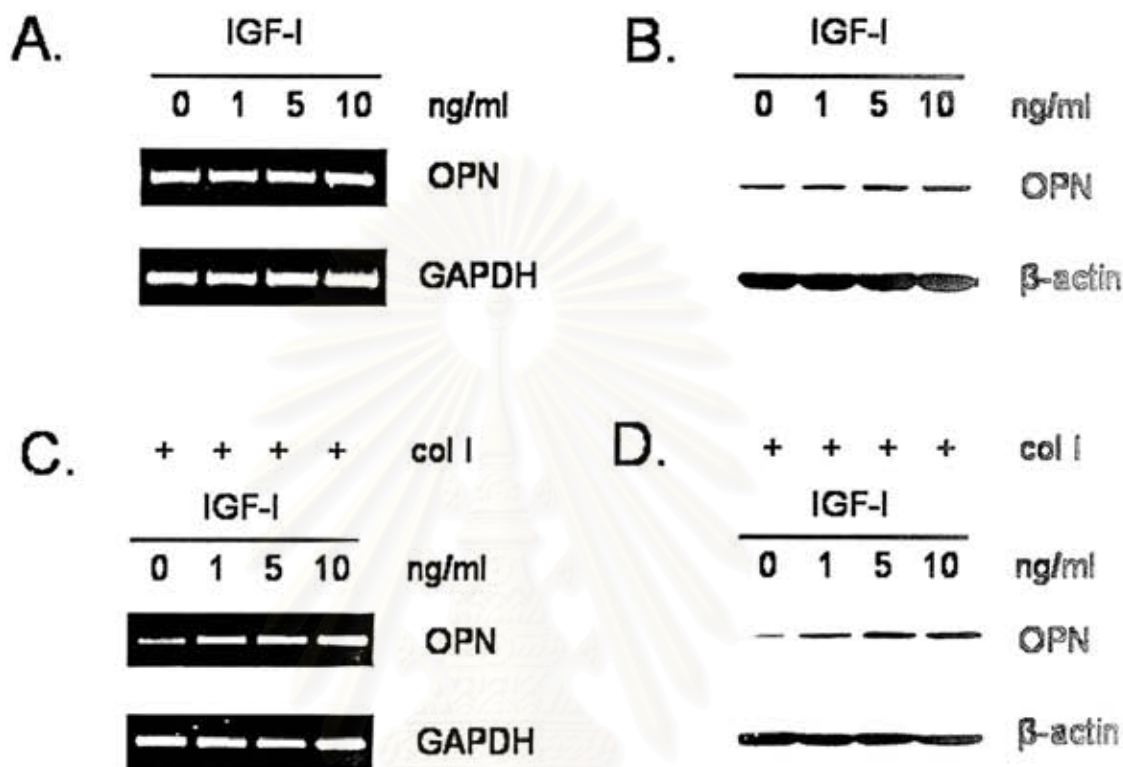
Figure 1 The effect of IGF-I and type I collagen on OPN expression in HPDL cells.
HPDL cells were plated on the plastic (A and B) or type I collagen-coated plate (C and D) for 24 hours. The cells were treated with 1, 5, 10 ng/ml of IGF-I for 48 hours. RT-PCR (A and C) and Western blot analysis (B and D) were performed using OPN primer and monoclonal antibody against OPN, respectively. GAPDH and β-actin were used as internal control. The experiment was performed triplicate using HPDL cells from three donors.
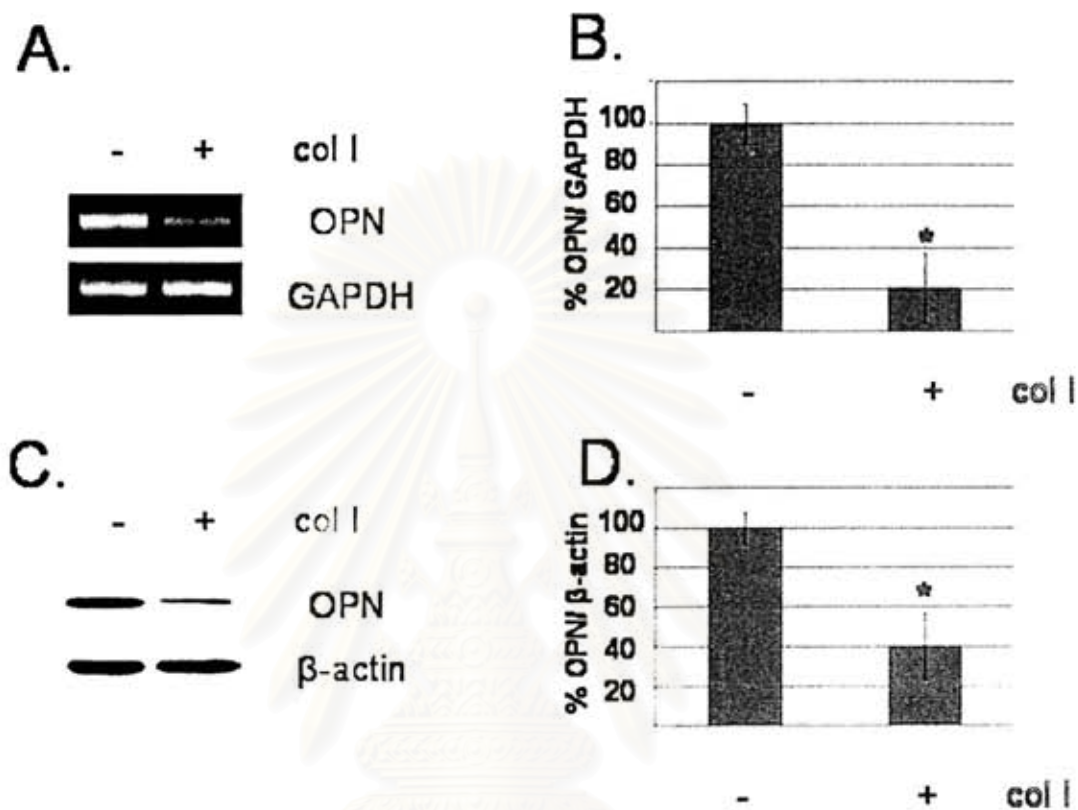
Figure 2 The effect of type I collagen on OPN expression in HPDL cells.

RT-PCR (A) and Western blot analysis (C) of HPDL cells cultured on the plastic or type I collagen-coated plate for 48 hrs. (B) and (D) Graphs show the quantification of OPN expression which normalized to the GAPDH or β-actin. All values are indicated by the average $\pm$ SD relative to the control from three independent experiments. *, significantly different from control ($p < 0.05$).
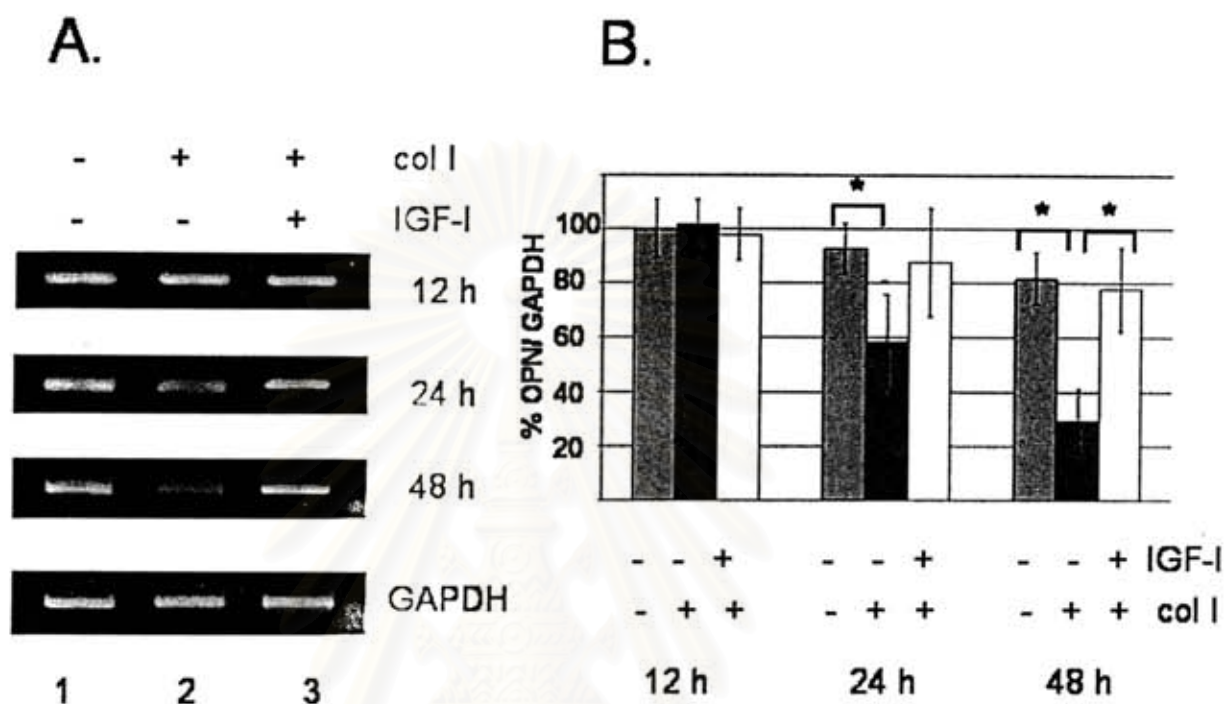
A.

| - | + | + | col I |
| - | - | + | IGF-I |

12 h

24 h

48 h

GAPDH

1　　2　　3

B.

% OPN/ GAPDH

100
80
60
40
20

| - | - | + | | - | - | + | | - | - | + | IGF-I |
| - | + | + | | - | + | + | | - | + | + | col I |

12 h　　　　24 h　　　　48 h

Figure 3 IGF-I stimulates OPN expression in the presence of type I collagen.

(A) HPDL cells were cultured in the absence or presence of type I collagen and then 5 ng/ml of IGF-I was added to the cultured media. The cell extracts were collected at 12, 24 and 48 hours and then RT-PCR analysis was performed.

(B) The mRNA levels of OPN were quantitated and normalized to GAPDH. The data are represented the mean $\pm$ SD relative to the control from three independent experiments. *, statistical significance ($p < 0.05$).
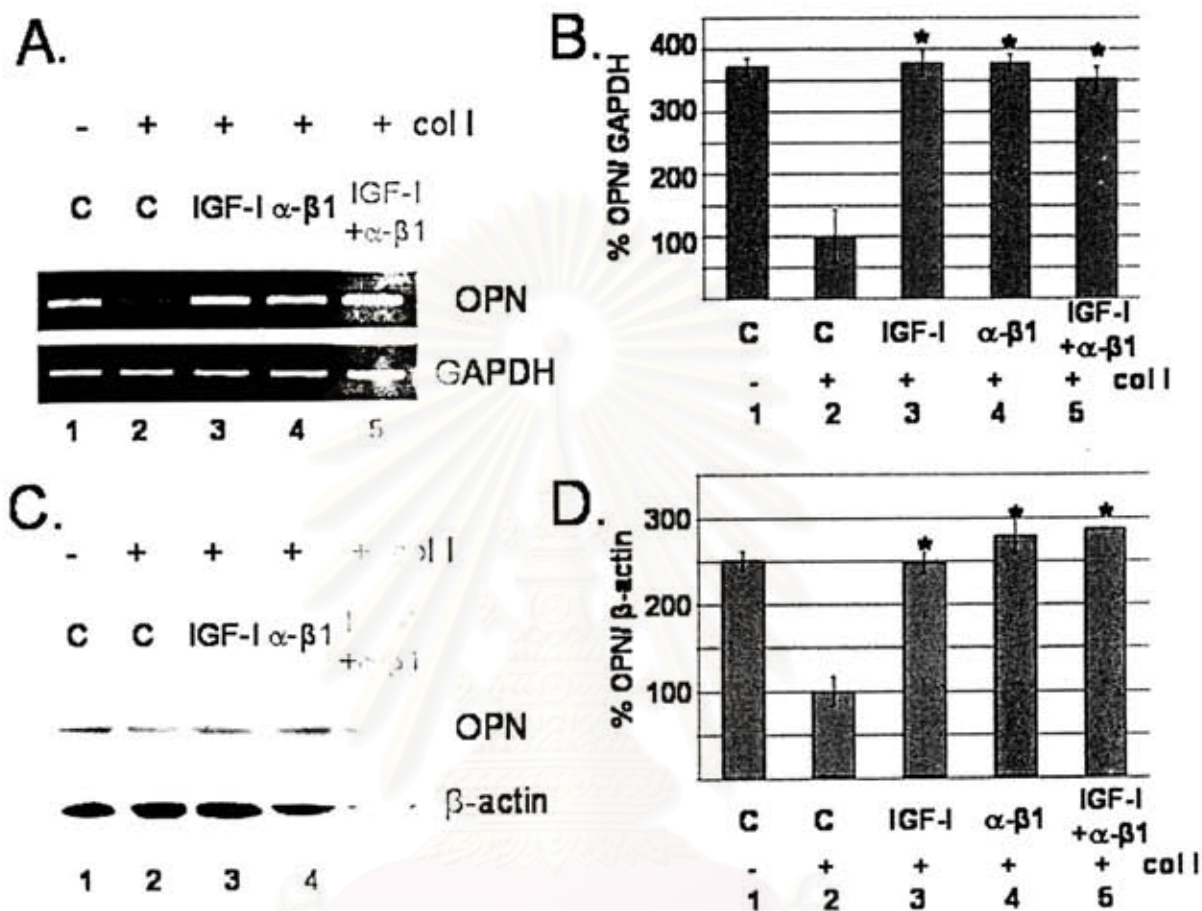
Figure 4 The combination effect of type I collagen, IGF-I and inhibitory antibody to β1 integrin on OPN expression HPDL cells.

HPDL cells were plated on type I collagen-coated plate for 24 hours before treated with 1 ng/ml inhibitory antibody to β1 integrin and/or 5 ng/ml IGF-I. The cell extracts were collected 48 hours after treatment and assessed for RT-PCR (A) and Western blot analysis (C). (B) and (D) Graphs show the quantification of OPN expression which normalized to the GAPDH or β-actin. The results are indicated by the mean ± SD relative to the control from three independent experiments. *, significantly different from control (p < 0.05).