ผลของคอลลาเจนชนิดที่หนึ่งร่วมกับอินซูลินไลค์โกรทแฟคเตอร์ชนิดที่หนึ่ง ต่อระดับการแสดงออกของออสทิโอพอนทินในเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์

นางสาว นิราภร ชมพูทวีป

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THE EFFECT OF TYPE I COLLAGEN AND INSULIN-LIKE GROWTH FACTOR-I ON OSTEOPONTIN EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS

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Filed of study	Oral Biology
Thesis Advisor	Assistant Professor Piyamas Sumrejkanchanakij
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ความสัมพันธ์ระหว่างเมทริกส์นอกเซลล์ กับโกรทแฟคเตอร์มีบทบาทในการหายและการซ่อมแซมของเนื้อเยื่อปริทันต์ การศึกษาครั้งนี้มีวัตถุประสงค์ที่จะศึกษาผลของคอลลาเจนชนิดที่หนึ่งร่วมกับอินซูลินไลค์โกรทแฟคเตอร์ ต่อระดับการแสดงออกของออสซิโอพอนทิน รวมทั้งกลไกการทำงานที่เกิดขึ้นในเซลล์ เอ็นยึดปริทันต์ของมนุษย์ การศึกษาทำโดยเลี้ยงเซลล์ที่ได้จากเอ็นยึดปริทันต์ของมนุษย์ ในสภาวะที่มีและไม่มีคอลลาเจนชนิดที่หนึ่ง เป็นเวลา 48 ชั่วโมง วิเคราะห์การแสดงออกของยีนด้วยเทคนิค RT-PCR และตรวจสอบการสร้างโปรตีนด้วยวิธีเวสเทิร์น (Western blot analysis) ผลการศึกษาพบว่าระดับการแสดงออกของออสซิโอพอนทินลดลงเมื่อเปรียบเทียบกับสภาวะที่ไม่มีคอลลาเจนชนิดที่หนึ่ง และเมื่อยับยั้งเบต้า-1 อินทิกรินด้วยแอนติบอดียับยั้งต่อเบต้า-1 อินทิกริน พบว่าระดับการแสดงออกของออสซิโอพอนทินเพิ่มขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุม จากผลการศึกษาแสดงถึงคอลลาเจนชนิดที่หนึ่งสามารถยับยั้งระดับการแสดงออกของออสซิโอพอนทิน ทั้งในระดับของอาร์เอ็นเอนำรหัสและโปรตีน ในเซลล์เอ็นยึดปริทันต์ของมนุษย์โดยผ่านทางเบต้า-1 อินทิกริน และเมื่อกระตุ้นด้วยอินซูลินไลค์โกรทแฟคเตอร์ที่ความเข้มข้น 5 นาโนกรัมต่อมิลลิลิตรในสภาวะที่มีคอลลาเจนชนิดที่หนึ่ง พบว่าอินซูลินไลค์โกรทแฟคเตอร์สามารถเหนี่ยวนำให้การแสดงออกและการสร้างโปรตีนของออสซิโอพอนทินเพิ่มขึ้น จากผลการศึกษาแสดงถึงผลของอินซูลินไลค์โกรทแฟคเตอร์ในการยับยั้งสัญญาณจากเบต้า-1 อินทิกริน นอกจากนี้เมื่อทำการยับยั้งเบต้า-1 อินทิกรินร่วมกับกระตุ้นด้วยอินซูลินไลค์โกรทแฟคเตอร์พบว่าระดับการแสดงออกของออสซิโอพอนทินเพิ่มสูงขึ้นในระดับเดียวกับที่ทำการยับยั้งเบต้า-1 อินทิกริน หรือกระตุ้นด้วยอินซูลินไลค์โกรทแฟคเตอร์เพียงอย่างเดียว จากผลการศึกษาแสดงถึงบทบาทในการยับยั้งสัญญาณที่ระดับเดียวกันของสัญญาณจากอินซูลินไลค์โกรทแฟคเตอร์และสัญญาณจากคอลลาเจนชนิดที่หนึ่งซึ่งส่งผ่านทางเบต้า-1 อินทิกริน ผลของการศึกษาครั้งนี้แสดงให้เห็นถึงบทบาทของคอลลาเจนชนิดที่หนึ่งในการยับยั้งระดับการแสดงออกของออสซิโอพอนทินโดยผ่านทางเบต้า-1 อินทิกริน และอินซูลินไลค์โกรทแฟคเตอร์สามารถเหนี่ยวนำให้การแสดงออกของออสซิโอพอนทินเพิ่มขึ้นในเซลล์เอ็นยึดปริทันต์ของมนุษย์

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KEY WORDS: HUMAN PERIODONTAL LIGAMENT CELL / INSULIN-LIKE GROWTH FACTOR-I / TYPE I COLLAGEN / OSTEOPONTIN / DIFFERENTIATION / REGENERATION

NIRAPORN CHOMPOOTAWEEP: THE EFFECT OF TYPE I COLLAGEN AND INSULIN-LIKE GROWTH FACTOR-I ON OSTEOPONTIN EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS. THESIS ADVISOR: ASSIST. PROF. PIYAMAS SUMREJKANCHANAKIJ, Ph.D, THESIS COADVISOR: ASSOC. PROF. TUSSANEE YONGCHAITRAKUL, 57 PP. ISBN 974-53-1996-1

Extracellular matrix and growth factors might be 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 IGF-I on OPN expression in HPDL cells. Cells were cultured on plastic or type I collagen-coated plates for 48 hours, then cell extracts were collected. RT-PCR and Western blot analysis were used to analyze OPN expression at transcriptional and translational level, respectively. It was found that OPN expression was significantly decreased when cells were cultured on collagen-coated plates and this effect could be diminished by blocking with an inhibitory antibody to β1 integrin. This result indicates that the inhibitory effect on the OPN expression exerted by type I collagen is signaled through β1 integrin receptor. We also found that after HPDL cells were treated with 5 ng/ml of IGF-I, OPN expression was increased in cells grown on collagen-coated plates but not in uncoated condition. This result implies that IGF-I can attenuate the inhibitory effect of type I collagen on OPN expression in HPDL cells. In the last experiment, the cells were treated with the combination of inhibitory antibody to β1 integrin and IGF-I. The result showed that OPN expression was increased to the same level as in the group treated with the inhibitory antibody to β1 integrin or IGF-I alone. This result suggests that the effect of IGF-I associated in counteracting the inhibitory signal from type I collagen mediated by β1 integrin. In conclusion, type I collagen inhibited OPN expression in HPDL cells via β1 integrin and IGF-I can attenuate this inhibitory effect. It is possible that type I collagen and IGF-I play a role in periodontal regeneration by regulating the expression of the OPN in HPDL cells.

Field of study	Oral Biology	Student's signature.....
Academic year	2004	Advisor's signature.....
		Co-Advisor'signature .....

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ABBREVIATIONS

ALP	alkaline phosphatase
AMV	Avian myeloblastosis virus
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BSP	bone sialoprotein
CDC	cell division cycle
DMEM	Dulbecco's Modified Eagle medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
Eta	early T-lymphocyte activation
FACIT	fibril associated collagen with interrupted triple helic
FAK	focal adhesion kinase

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gly	glycine
Grb	growth factor receptor-bound protein
HPDL	human periodontal ligament cells
HCl	hydrochloric acid
Hyp	hydroxyproline
I	insert
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGFR	Insulin-like growth factor receptor
IRS	insulin receptor substrate
JNK	c-jun kinase
MAPK	mitogen-activated protein kinase
OC	osteocalcin
OPN	Osteopontin

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3 phosphate
Pro	proline
rER	rough endoplasmic reticulum
RGD	arginine-glycine-aspartate
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
Sos	son of sevenless
TNF	tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Periodontitis is an inflammatory disease of the supporting tissues of teeth including gingiva, cementum, periodontal ligament and alveolar bone. The pathogenesis of the disease involves both connective tissue and bone destruction leading to tooth loss. The disease progresses as antigens from plaque activate monocytes to produce high concentrations of cytokines, locally resulting in the activation of metalloproteinases, which leads to subsequent tissue degradation and loss of ligament attachment. Moreover, alveolar bone destruction was induced by stimulation of osteoclastic activity (Reynold and Meikle 1997, Kinane 2001).

The goal of periodontal therapy is to induce not only healing but also periodontal regeneration of the affected tissues in order to restore their original architecture and function. The elementary procedures are usually performed by root surface debridement and removal and control of soft tissue infection (Karing 1988). Nonetheless, an alternative treatment to achieve satisfactory results have included advanced surgical procedures (Barrington 1981), use of autologous and synthetic bone grafting materials

(Brunsvold and Mellonig 2000), barrier membranes (Gottlow et al.1984), and root surface treatment such as acid etching and growth factor application (Ririe et al. 1980, Daryebgi et al. 1981, Terranova et al.1987, Bartold and Raben 1996, King et al. 1997).

Periodontal wound healing is a complex process involving both temporal and spatial coordination of cellular and extracellular events. Many types of cells in the periodontal tissue can play an important role in the regeneration of the periodontal attachment apparatus (Meyer 1986, Polson 1986, Egelberg 1987, Aukhil 1990). Biologically, regeneration would succeed when cells that reside in the periodontal tissue migrate, proliferate and differentiate into osteoblast-like or cementoblast-like cells (Davidson et al. 1986, Cho et al. 1992, Mariotti et al. 1990). The interaction between cells and many biological factors including systemic hormones, extracellular matrix (ECM) proteins and growth factors is required for the healing process. Various growth factors are believed to play roles during the early event of periodontal healing and periodontal tissue regeneration. One of the growth factors that has been implicated in these processes and regeneration is insulin-like growth factor (IGF)-I. It is well-documented that IGF-I is a key mediator of wound healing by its ability to induce epithelial and mesenchymal cell proliferation. In periodontal tissues, IGF-I can stimulate periodontal fibroblast proliferation in vitro (Daniel et al. 2004). It has been recently reported that IGF-I and platelet-derived growth factor (PDGF) enhance periodontal tissue regeneration in

the artificially induced periodontal disease in beagle dogs and monkeys (Lynch et al. 1989, Rutherford et al. 1992).

ECM proteins also play an important role during matrix synthesis phase of periodontal wound healing. The structural integrity is improved since fibrin-fibronectin provisional matrix framework is replaced by a fibronectin-rich granulation tissue. This replacement provides a vascularized network for collagen deposition and also supports collagen bundle formation and fibril crosslinking (Bornstein and Sage 2002). In addition, ECM can regulate cell dynamics and cell behavior by providing extracellular signals via receptor-mediated signaling such as integrin receptors. ECM also involves in maintaining a pool of cytokines and growth factors to affect cellular functions and ECM homeostasis (Rosso et al. 2004). For instance, disruption of ECM function by using an antibody to collagen, or the use of tumor necrosis factor α (TNFα) to perturb the homeostasis of ECM proteins can disrupt the Sertoli cell function in testis (Sui et al. 2004). ECM consists of two major groups of molecules,i.e., (i) structural proteins such as fibrin, fibronectin , laminin and collagen. (ii) matrixcellular proteins, proteins that play roles in regulating cell behaviors, such as galectin, SPARC, tenascins, thrombospondins, vitronectin and osteopontin (OPN) (Bornstein and Sage 2002).

Type I collagen is the major component of ECM which provides tensile strength and support, as well as modulates various cellular function. There are evidences showing the association between type I collagen and growth factors during the process

of wound healing. An application of basic Fibroblast Growth Factor (bFGF) in type I collagen sponge could enhance dermal wound healing (Marks 1991). An induction of angiogenesis in the mouse subcutis was detected after treated with several growth factors including IGF-I in the type I collagen sponge (Kanematsu 2004). However, the association of type I collagen in the mechanism of periodontal wound healing is still unknown. In this study, we evaluate OPN expression induced by IGF-I in the presence of type I collagen. We detect the level of OPN expression which is a differentiation marker for osteblast-like cell. This result may be useful for clinical application in periodontal regeneration procedure.

1.2 Research questions

1.2.1 Whether IGF-I can affect OPN expression in human periodontal ligament (HPDL) cells.

1.2.2 Whether type I collagen can affect OPN expression in HPDL cells.

1.2.3 Whether combination of type I collagen and IGF-I can affect OPN expression in HPDL cells.

1.2.4 Whether the effect of type I collagen is mediated by β_1 integrin.

1.2.5 Whether IGF-I affect OPN expression in the presence of type I collagen via $\beta 1$ integrin.

1.3 Objectives

1.3.1 To determine the level of OPN expression in HPDL cells modulated by IGF-I.

1.3.2 To determine the level of OPN expression in HPDL cells modulated by type I collagen.

1.3.3 To determine the level of OPN expression in HPDL cells modulated by the combination of IGF-I and type I collagen.

1.3.4 To determine the level of OPN expression in HPDL cells modulated by type I collagen and inhibitory antibody to β_1 integrin.

1.3.5 To determine the level of OPN expression in HPDL cells modulated by combination of type I collagen, IGF-I and inhibitory antibody to $\beta_1$integrin.

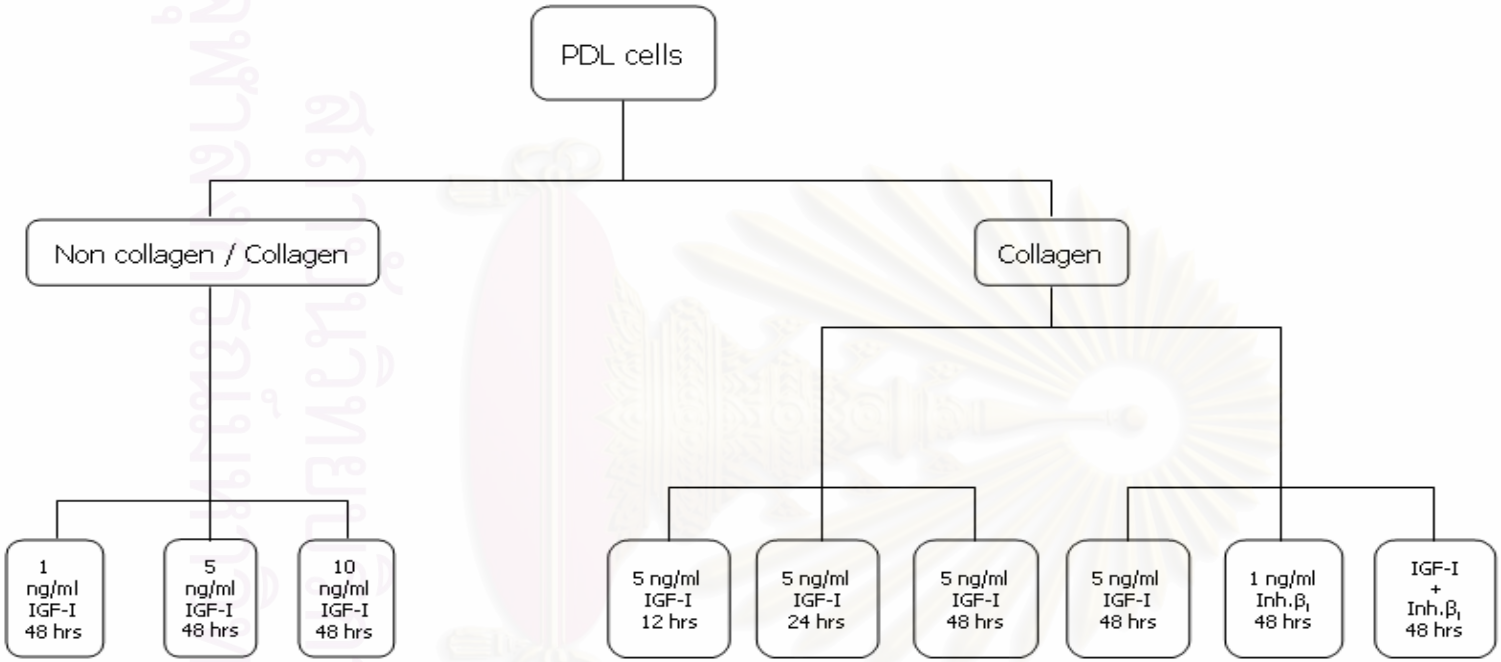
1.4 Hypothesis

Type I collagen and IGF-I could affect OPN expression in HPDL cells.

1.5 Experimental design

mRNA level : RT-PCR analysis

protein level : Western blot analysis



Experimental Design

1.6 Key word

Human periodontal ligament (HPDL) cell

Insulin-like growth factor-I (IGF-I)

Type I collagen

Osteopontin (OPN)

Differentiation

Regeneration

1.7 Research design

Laboratory experimental research

1.8 Limitation of research

In vitro study

1.9 Benefit

We anticipate that the results from this line of investigation would provide a better understanding of the regenerative process of periodontal tissue. With

proper knowledge of the biological processes, manipulation of the response of the cellular network may be possible, thus leading us to new and specific therapies for periodontal disease in the future.

1.10 Ethical consideration

none

CHAPTER 2

LITERATURE REVIEW

2.1 Collagen

The most abundant proteins of the ECM are members of the collagen family of proteins. Collagen is the major structure element of the connective tissue, tendon, ligament, cornea and periodontium. The most commonly occurring collagens are type I, II and III, which form the long recognized characteristic fiber bundles in many tissues. There are 25 different genes that code for at least 19 different collagen molecules. Collagen is a protein with three polypeptide chains. Each chain has 1000 amino acids and contains at least one stretch of the repeating amino acid sequence Gly-X-Y, where X and Y can be any amino acid but are usually proline and hydroxyproline (van der Rest and Garrone 1991). The basic unit of collagen, tropocollagen, is a rigid rod shaped molecule approximately 3000 A° in length and 15 Å in diameter. Collagen has two different types of structural domain: triple helix and globular (Burgeson and Nimni 1992). Most collagen consists of two α-1 chain and one α-2 chain. An individual α chain is a left-handed helix with approximately 3.3 residues per turn. The α chains are twisted together to form a right-handed superhelical structure. Hydrogen bonds form between residues of different chains. The characteristic structure of both chains is a repeating unit of three

amino acids, one third of all amino acids in each collagen chain is glycine (Gly). Proline (Pro) and hydroxyproline (Hyp) follow each other frequently, about 10% of the molecule has the sequence Gly-Pro-Hyp. Procollagen is the precursor of tropocollagen. The procollagen molecule has three pro α-chains arranged in a triple-stranded conformation and differs from tropocollagen in that it contains six extraglobular tails. The N-terminal propeptide contains intrachain disulfide links, where as the polypeptides from the C-terminal region are linked to each other by disulfide bridges. The nonhelical region of procollagen are partially cleaved by procollagen peptidase (Patino et al. 2002). (Figure 1)

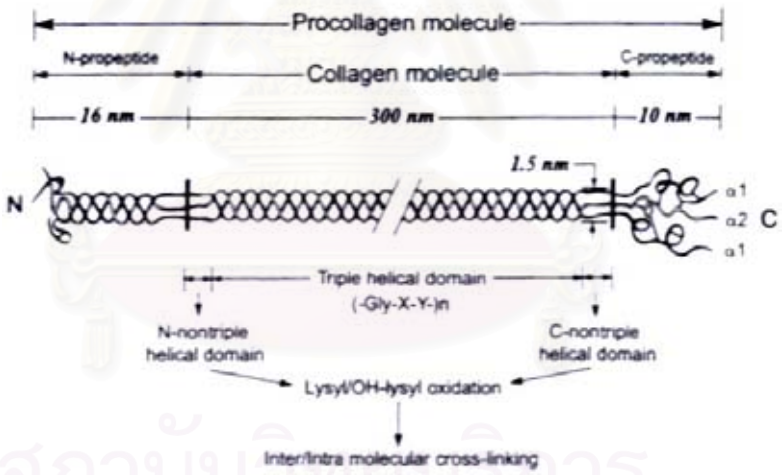


Figure 1 **The type I procollagen molecule structure and its various domain.** After C- and N-propeptide extensions are cleaved off during the extracellular processing, the collagen molecule has three domains: N-non triple helical, triple helical, C-non triple helical domains. (Anderson and Garner, Calcium and phosphorus in health and disease. 1996:131)

At present, at least nineteen collagen types and more than 30 unique α-chains have been identified. Each type has a characteristic tissue distribution and apparently a unique biological function. These different types of collagen could be classified into three groups :

1. fibril-forming : Type I, II, III, V, XI
2. FACIT (Fibril Associated Collagen with Interrupted Triple Helices) : Types IX, XII, XIV, XIX
3. Nonfibrillar collagen, such as, sheet-forming, beaded filament, anchoring fibril, growth plate-specific,etc. :Type IV, VI, VII, VIII, X, XIII

Fibril-forming collagens contained the type I, II, III, V and XI collagens. These collagens form highly organized fibers and fibrils and provide the structural support. Type I collagen is the most abundant type of collagen in many connective tissues, bone, tendon, skin, arteries, uterus, and cornea. It comprises between 80%-99% of total collagen (Burgeson and Nimni 1992). Type I collage is the prototype fibril-forming collagen. The molecules are about 300 nm long and are heterotrimer composed of two α-1 chains and one α-2 chain,[$\alpha_1(I)_2\alpha_2(I)$]. This molecule consists of three domains : the NH_2 terminal nontriple helical, the triple helical, and the COOH terminal nontriple helical domains. The single (uninterrupted) ripple helical domain represents more than 95% of the molecule. The molecule is secreted in a precursor form, procollagen. The collagen

molecule is generated outside the cell by enzymatic cleavage at both the C- and the N-terminal ends of the procollagen molecule (Anderson and Garner 1996).

The biosynthesis of type I collagen is a complicated, multistep process. Translation of the mRNA of pre-procollagen chain is initiated with the synthesis of the signal peptide extensions, and the newly synthesized polypeptides required a number of co- and posttranslational modifications. These modifications that take place intra- and extracellularly require the act of at least 10 different enzymes, many of them which are unique to collagen and other proteins containing collagenous sequence (Anderson and Garner 1996). (Figure 2)

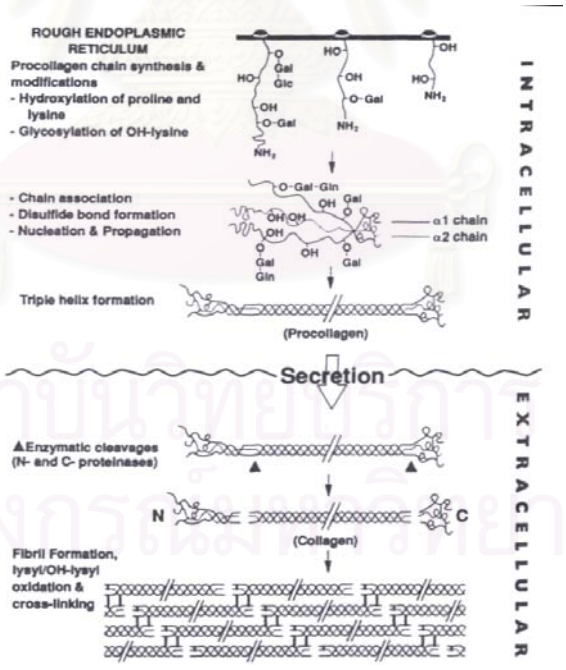


Figure 2 **Sequential events in the biosynthesis of type I collagen** (Anderson and Garner, Calcium and phosphorus in health and disease. 1996:132)

Intracellular events : The first step in the process of collagen synthesis is the formation of collagen specific mRNA. After gene transcription, the gene is spliced yielding a functional mRNA that contains about 3000 bases. Specific mRNA are transported to the cytoplasm and translated on membrane-bound polysomes to the rough endoplasmic recticulum (rER) (Burgeson and Nimni 1992). As the collagen polypeptides are synthesized in the rER, important cotranslational events accompany this process. Prolyl and lysyl hydroxylase mediate the hydroxylation of proline and lysine. Glycosylations occur that are catalyzed by galactosyltransferase and glusosyltransferase (Hulmes 1992). Accompanying these enzymatic events, association of pro-α chains in the correct chain registration and triple-helix assembly occur. During alignment, cysteine residues are juxtaposed for the formation of disulfide bridges that will link the individual pro-α chain at the C-terminal end.

The procollagen molecules travel from the rER toward the Golgi apparatus through the microsomal lumen. In the Golgi, procollagen molecules are packed into secretory vesicles and translocate to the surface of the cell, where they are secreted into the extracellular environment by exocytosis (Hulmes 1992).

Extracellular event : Once in the extracellular matrix, the newly synthesized procollagen molecules interact with processing enzymes and undergo fibril formation and cross-linking (Seyedin and Rosen 1990, Sandell and Boyd 1990). The enzymes are procollagen N-proteinase (removal of N-propeptide), procollagen C-proteinase (removal

of C-propeptide) and lysyl oxidase. Lysyl oxidase, an extracellular amine oxidase, initiates cross-linking of collagen by oxidative deamination of certain lysine and hydroxylysine residual located in the short N- and C-terminal nonhelical regions (telopeptides) that remain after the removal of the procollagen propeptides. Bifunctional cross-link undergoes further intra- and intermolecular reactions to form a variety of trifunctional cross-links (Kagan and Trackman 1991).

Type I collagen has biochemical and biophysical properties. They provided high tensile strength that depends on number of cross-links and the interaction with glycoproteins and proteoglycans. Collagen also modulates various biological functions of cells via cell surface receptors such as integrin receptor.

The integrins represent a collection of cell surface proteins that mediate the binding of cells to extracellular proteins and to one another. Integrins are heterodimeric noncovalent proteins composed of α and β subunits, with long amino-terminal in extracellular domain binding to ECM, a hydrophobic transmembrane domain, and a short carboxyl-terminal cytoplasmic domain associating with the actin cytoskeleton and affiliated proteins (Lee and Juliano 2004). (Figure 3) α subunits are more heterogenous than the β subunits, and many α subunits contain a 206 amino acid sequence known as the insert (I) domain. Although not all α subunits contain this region, integrins with the I domain interact with diverse macromolecules in the extracellular matrix and may be involved in its assembly and organization (Ignatius et al. 1990, Milam et al. 1991). The

extracellular portion of the β subunit contains the arginine-glycine-aspartate (RGD) binding region near the amino terminus as well as up to 4 cysteine-rich regions that contribute to its rigidity and tertiary structure (Milam et al. 1991). The amino acid tripeptide, arginine-glycine-aspartate, has been shown to be a common recognition sequence on growth factors that binds to integrins. At least 18 distinct α subunits and 8 or more β subunits of the integrin receptors leading to 24 or so distinct αβ heterodimeric receptors. Many integrin α and β subunits have the ability to associate with more than one partners, resulting in a redundancy of integrin subtype heterodimerization. Most integrin heterodimers recognize several ECM proteins, whereas integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind only type I collagen. Integrins are important component not only for the structure and architecture of tissue but also for signal transduction leading to many biological functions in a cell (Lee and Juliano 2004). Interaction of integrins with ECM at focal adhesion leads to clustering of integrin subunits and the recruitment of signaling molecules ,including focal adhesion kinase (FAK), extracellular signal regulated kinase (ERK) and c-Jun kinase (JNKs), mitogen-activated protein kinase (MAPK) and Rho GTPase family members including Rho A , Rac A , cell division cycle (CDC) 42. These molecules have been shown to regulate cell adhesion, spreading, proliferation, differentiation, cell survival and morphological change (Juliano 2002). (Figure 4)

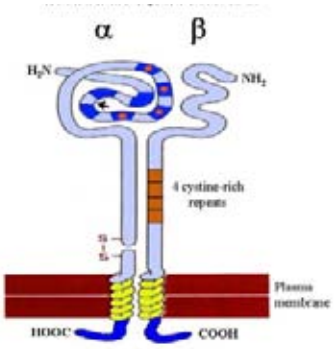


Figure 3 The integrin receptor, is a glycoprotein that consists of α and β subunits. These two subunits are integral components of the cell membrane and bind to cytoskeletal elements in the cell.

(www.medicine.ox.ac.uk/ndog/mardon/integrin.htm)

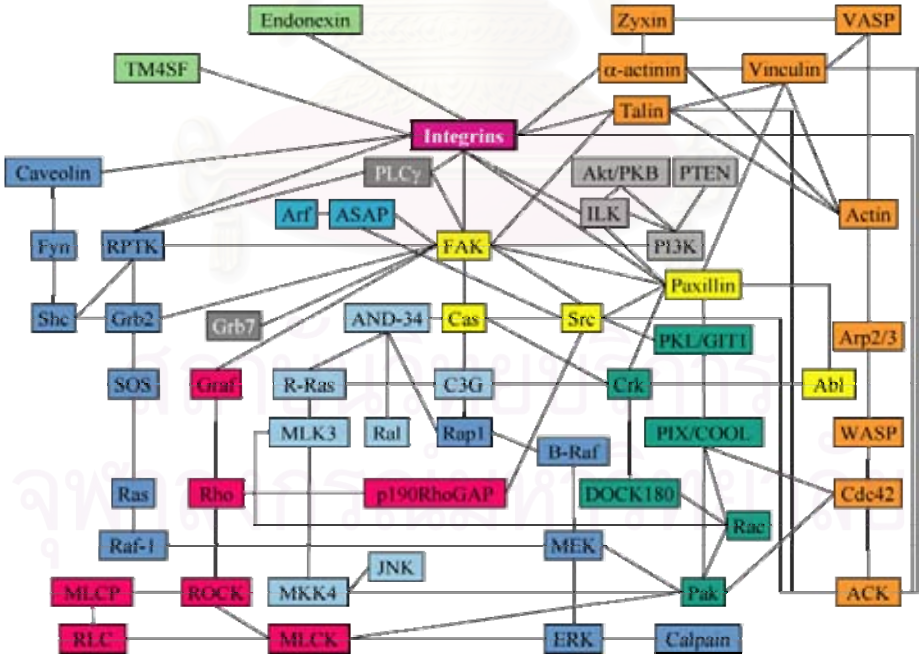


Figure 4 Integrin signaling pathway

(http://wrpx00.bioch.virginia.edu/cellmig_mol/integrin_pagef.html)

2.2 Insulin-like growth factor-I (IGF-I)

Insulin-like growth factor was discovered initially as " sulfation factor" , serum factor that stimulates cartilage sulfation (Salmon and Daughaday 1991). It was renamed "somatomedin" after it was shown to stimulate other cellular processes (Jones and Clemmons 1995). Other studies identified a serum factor that exhibited insulin-like activity but that could not be suppressed by anti-insulin antibodies and therefore was termed "nonsuppressible insulin-like activity" (Froesch et al 1966). Finally, these factors were cloned and found to be identical, and then was renamed "IGF-I". The IGF family consists of insulin, IGF-I, and IGF-II, which share 50% amino acid sequence homology (Hsu et al. 2004). IGF-I is a 7.6 kDa single chain and its gene locates on chromosome 12q22-q24.1 (Meinel et al. 2003). IGF-I binds to its specific cell surface receptor, the IGF-I receptor (IGF-IR), a transmembrane heterotetramer with tyrosine kinase activity. The IGF/IGFR interaction is modulated by IGF binding proteins (IGFBPs) which bind to IGF-I with high affinity. IGFBPs regulate ligand availability and prolong its half life in serum and interstitial compartment (Edmondson et al. 2003). IGF-I can either act on local tissue in an autocrine/paracrine fashion or enter the circulation and exert distance endocrine effects. Locally acting IGF-I is thought to be important in the regulation of growth and differentiation. Local IGF-I is produced in a variety of cells such as keratinocytes, osteoblasts, fibroblasts or other cells where it is regulated by growth

hormone or parathyroid hormone. Locally acting IGF-I can bind directly to its receptor to modulate cellular function, circulating IGF-I largely is produced by the liver, where it is induced by growth hormone. They were bound in the serum to 1 of 6 different IGF binding proteins (IGFBP-1–6), predominantly IGFBP-3. When IGFBP-3 is cleaved, the circulating IGF-I is aided by other binding proteins to leave the intravascular space and enter target tissues, where it then binds to cell membrane-bound IGF-I receptor activates intracellular signal transduction pathway (Hsu et al. 2004).

The biological activity of IGF-I is transduced by the IGF-IR. The IGF-IR possesses tyrosine kinase activity and autophosphorylates after binding to IGF-I ligand. The initial postreceptor signal transduction event is the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). This is followed by the initiation of two signaling cascades involving phosphatidylinositol-3 kinase (PI-3K) and Ras/Raf/MAPK. Activation of PI-3 kinase results in the formation of phosphatidylinositol-3 phosphate, which can serve as a signal for cell growth and differentiation. The Ras/Raf/MAPK pathway is activated by the tyrosine phosphorylation of IRS-1, which results in the formation of the IRS-1-growth factor receptor-bound protein-2 (Grb2)-Son of sevenless (Sos) complex. These complexes activate Ras, which in turn binds to and activates Raf. Then, Raf protein subsequently phosphorylates and activates MAPK. Phosphorylated MAPK transmits a signal to the nucleus leading to cell cycle progression and cell proliferation (Phan et al. 2003). (Figure 5)

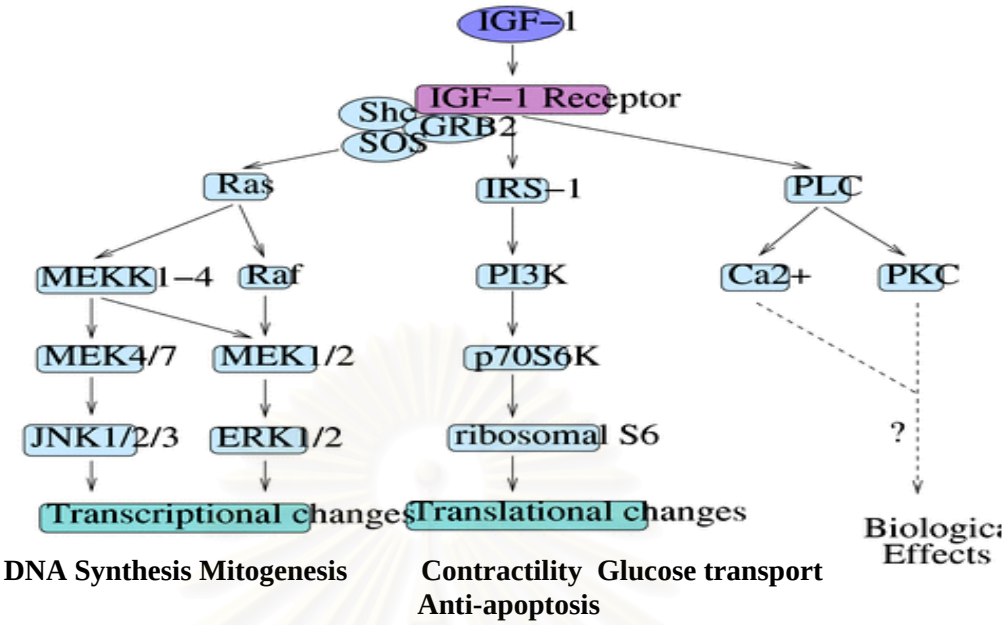


Figure 5 IGF-I signaling pathway

(http://www.cellml.org/examples/repository/qualitative/IGFI_cascade_doc.html)

IGF-I has widespread physiologic and pathologic effects. On the tissue level, it is essential in embryonic development and plays a crucial role in regulating growth after birth (Baker et al. 1993). On the cellular level, circulating IGF-I has been shown to have effects by stimulating DNA and protein synthesis, matrix synthesis, peripheral glucose uptake, glycogen synthesis, neuronal survival, myelin synthesis, and bone formation. Locally acting IGF-I also is involved in wound healing process (Le Roith 1997). In bone, IGF-I stimulates osteoblastic cell proliferation and metaboilism on fracture healing in rats (Schmidmaier et al. 2003). In tendon, exogenous IGF-I improves healing by stimulating DNA synthesis, cell proliferation, collagen content and increasing mechanical stiffness

in flexor tenditis (Abrahamsson et al. 1991). Treatment of wound with IGF-I has been shown to stimulate collagen synthesis in fibroblasts of skin (Guler et al. 1988). In periodontium, IGF-I stimulates periodontal ligament cell proliferation (Palioto et al. 2004). Recently studies have shown the involvement of IGF-IR signaling during mineralized nodule formation in HPDL cells (Nemoto 2004). Furthermore, it has been reported that IGF-I and PDGF enhance periodontal regeneration in artificially induced periodontal disease in beagle dogs and monkeys (Lynch et al. 1989, Rutherford et al. 1992).

2.3 Osteopontin (OPN)

OPN is also known as early T-lymphocyte activation-1 (Eta-1), bone sialoprotein-1 (BSP-1), 2ar or bone-bridge protein. It is a sialic acid-rich secreted phosphoglycosylate noncollagenous matrix protein. This protein is composed of 297 amino acid residues with molecular mass between 44 and 75 kDa (O'Regan and Berman 2000). Its gene maps to human chromosome 4q13. OPN contains several interesting structural domains, including the arginine-glycine-aspartate acid (RGD) tripeptide integrin binding domain. There are calcium binding site and two heparin binding domains, these two domains may co-interact with RGD domain (Patarca et al. 1993, O'Regan and Berman 2000) (Figure 6). OPN is synthesized by a variety of immune and non-immune cells. During pathological process, it is expressed by

activated T cells, activated macrophages and natural killer cells. Non-immune sources of OPN include epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, osteoclast/osteoblast and tumor cells (O'Regan and Berman 2000). In normal tissues, OPN is expressed in a variety of human tissues, including kidney, thyroid, gastrointestinal tract, breast, testes, endometrium, uterine residual cells, placenta, bone and teeth. It is also present in secretions such as milk, bile and urine (Senger et al. 1989, Shiraga et al. 1992, Daiter et al. 1996). OPN expresses at high level during development but not in the normal adult and is up-regulated at sites of tissue remodeling. OPN associates with ECM but does not perform a structural role within tissues as other ECM proteins. Instead, it acts to provide signals that trigger specific cell activities via its receptor. OPN receptors consist of both RGD-dependent and –independent ones. The best characterized OPN receptor is $\alpha v\beta 3$ integrin. OPN can also interact with $\alpha v\beta 1$, $\alpha v\beta 5$, $\alpha 9\beta 1$, $\alpha 8\beta 1$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins. All integrins bind to the N-terminal thrombin cleaved fragment of OPN which contains the RGD domain (Liaw et al. 1995b). OPN also interacts with RGD-independent receptor, CD44, or directly interact with ECM proteins such as collagen and fibronectin (O'Regan and Berman 2000, Weiss et al. 2001). OPN plays an important role in cell physiology including cell adhesion and signaling, differentiation, regulation of intracellular calcium level, modulation of the immune response to infection and neoplasia (Denhardt and Guo 1993). In the immune system during inflammation, OPN acts as a cytokine in T cell and a chemoattractant in

macrophage which leaded to inhibition of production of nitric oxide during responses to infection, pathogen resistance and wound healing (Weber and Canton 1996). OPN can act as an adhesion substrate and migration stimulus by interacting with several integrins. The interaction leads to downstream signaling events that include calcium mobilization, protein phosphorylation, regulation of gene expression and cell differentiation (Denhardt et al. 1995). Function of OPN in differentiation of primary osteoblast indicates that binding of OPN to integrin regulates intracellular signal transduction, and upregulates alkaline phosphatase (ALP) and osteocalcin (OC) expression via focal adhesion kinase (FAK) phosphorylation in osteoblasts (Liu et al. 1997, Yabe et al. 1997). They are also involved in angiogenesis, apoptosis, tumor metastasis, inflammation, wound healing process and tissue remodeling (Omigbodun et al. 1997).

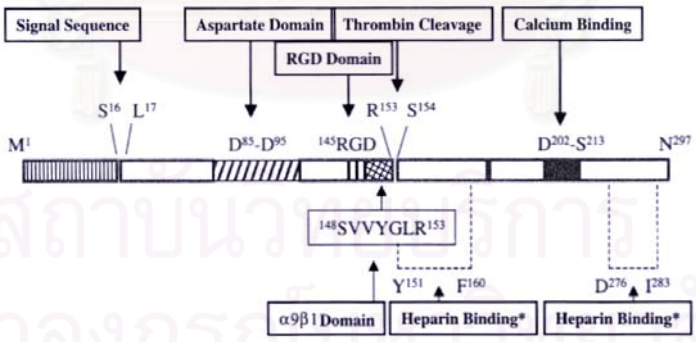


Figure 6 **structure of osteopontin indicating known functional domain.** M, methionine; S, serine; L, leucine; D, aspartate; R, arginine; G, glycine; N, asparagines; V, valine; Y, tyrosine; F,phenylalanine; I, isoleucine

(***Int. J. Exp. Path.*** 2000;81:375)

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell culture

Human periodontal ligament (HPDL) cells were established from explants obtained from periodontal ligament tissue attached to non-carious, freshly extracted third molars, or teeth removed for orthodontic reasons as previous described (Pattamapun et al. 2003). 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, Nalge Nunc International, Naperville, IL, USA) 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 grow out from the explants. After reaching confluence, cells were subcultured with 1:3 ratio. 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.

3.2 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 $\mu g/cm^2$. Before use, six-well cultured plates (35 mm diameter wells: Nunclon Surface, Nalge Nunc International, Naperville, IL, USA) were coated with a film of type I collagen by a modification technique. Briefly, aliquots of 1ml/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 culture plates were used as control in this study.

3.3 Activation of HPDL cells with IGF-I

HPDL cells were seeded in 6-well plates in either normal or type I collagen-coated condition in 10% DMEM and were allowed to attach for 24 hours. Cells were starved in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO, USA) overnight before further treatment. 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 β_1 integrin (Chemicon International, Temecula, CA, USA) for 30 minutes before the 5 ng/ml of IGF-I application for 48 hours.

3.4 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 were used to generate cDNA by a reverse transcription reaction using AMV (Avian myeloblastosis virus) reverse transcriptase (Promega, 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.

3.5 Western blot analysis

Cells were treated for 48 hours and the cell layer was extracted using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50Mm Tris PH 8.0) containing cocktail protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). The amount of proteins was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Protein extraction from each sample was subjected to SDS-polyacrylamide gel

electrophoresis (PAGE) under a non-reducing condition on 12% polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred onto a nitrocellulose 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 protein bands 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.

3.6 Statistical analysis

Statistical analysis of independent *t* -tests and one-way ANOVA of triplicate determinations were performed. Differences at $p < 0.05$ were considered statistically significant.

CHAPTER 4

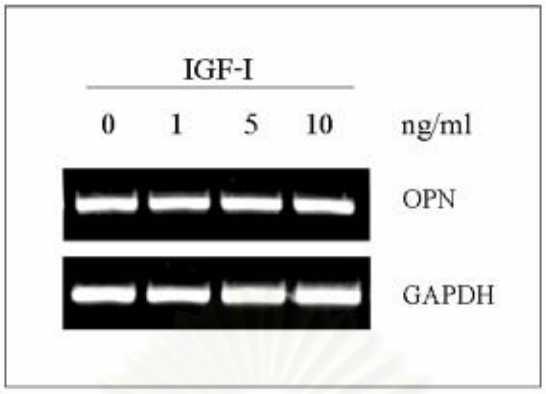
RESULT

4.1 The effect of IGF-I on OPN expression in HPDL cells

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. The levels of gene expression and proteins were detected by RT-PCR and Western blot analysis, respectively.

RT-PCR analysis in figure 1A demonstrated that OPN expression was not altered after treated with various doses of IGF-I. Figure 1B shows that IGF-I has no effect on the level of OPN protein expression, which concurs with the RT-PCR result.

A



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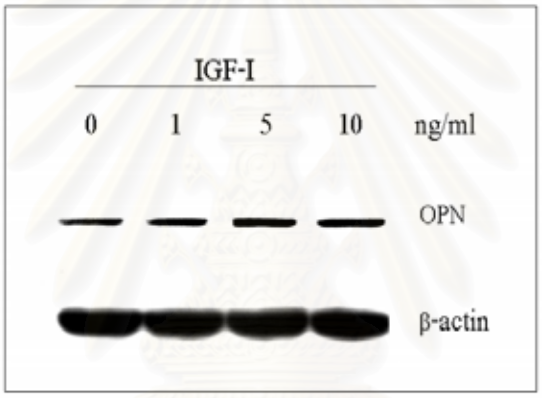


Figure 1 The effect of IGF-I on OPN expression in HPDL cells

(A) RT-PCR analysis of the levels of OPN mRNA expression in HPDL cells after treatment with 0, 1, 5, 10 ng/ml of IGF-I in uncoated-collagen condition for 48 hrs. (B) Western blot analysis of OPN expression in the same condition as (A). GAPDH and β-actin were used as internal control. The experiments were performed in triplicate using HPDL cells from three donors.

4.2 The combination effect of type I collagen and IGF-I on OPN expression in HPDL cells

It had been shown that the signal from ECM may affect the response of the cells to cytokines and growth factors (Hollenbeck et al. 2004), 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 OPN expression in HPDL cells.

RT-PCR analysis (figure 2A) revealed that in the presence of type I collagen, IGF-I could activate OPN expression as compared to the control. The expression of OPN was increased in a dose-dependent manner. The same result was obtained from Western blot analysis (figure 2B), IGF-I could increase OPN expression corresponding to the result revealed by RT-PCR.

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

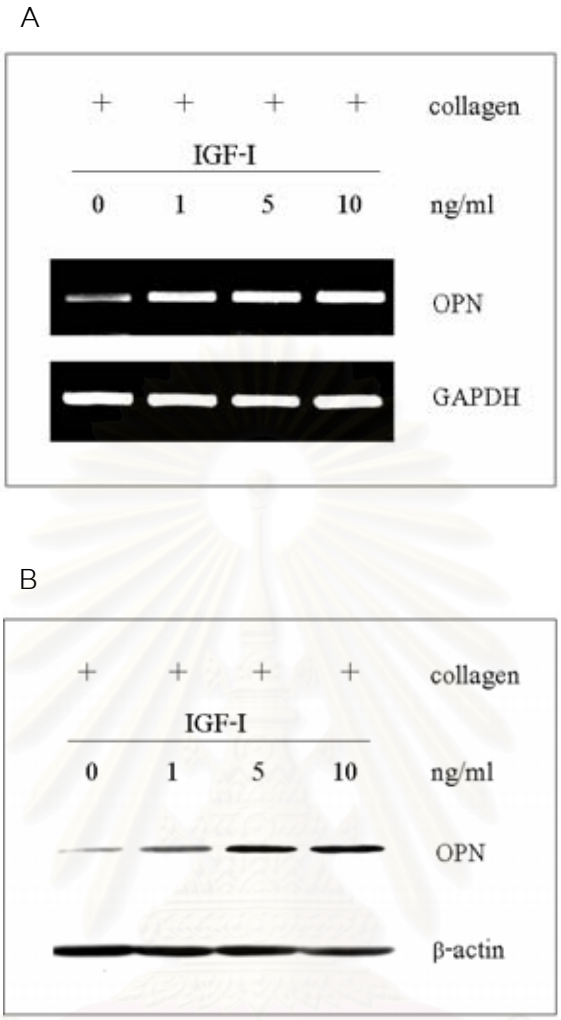


Figure 2 The effect of type I collagen and IGF-I on OPN expression in HPDL cells

HPDL cells were seeded on type I collagen-coated plates, and then treated with 0, 1, 5, 10 ng/ml of IGF-I for 48 hrs. RT-PCR (A) and Western blot analyses (B) were performed using OPN primers and a monoclonal antibody against OPN, respectively. GAPDH and β-actin were used as internal control. The experiments were performed in triplicate using HPDL cells from three different donors.

4.3 The effect of type I collagen on OPN expression in HPDL cells

To study the effect of type I collagen alone, OPN expression of HPDL cells cultured on collagen-coated plates was compared to that of cells cultured on uncoated plates for 48 hours.

The mRNA expression of OPN in the presence and absence of type I collagen was shown in figure 3A. Interestingly, a decrease of OPN expression was observed in the presence of type I collagen compared to the uncoated condition. Similar result was also found in Western blot analysis as shown in figure 3C. Quantification of the data confirmed that the level of OPN mRNA (figure 3B) and protein (figure 3D) expression were markedly reduced by type I collagen.

We further determined the effect of type I collagen on OPN expression in HPDL cells at different time points. HPDL cells were harvested at 12, 24, 48 hours and OPN expression was analyzed by RT-PCR.

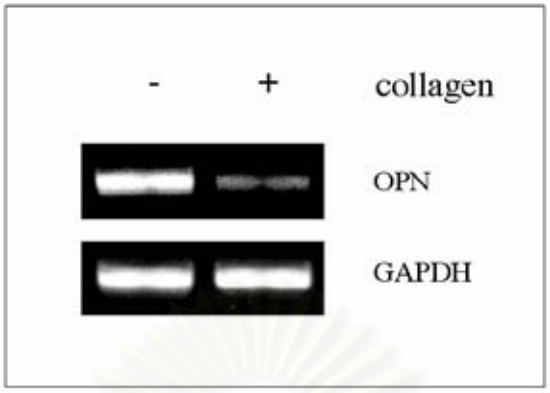
Figure 4 revealed that OPN expression was decreased in the presence of type I collagen in a time-dependent manner. The quantitative data was also shown in figure 4B. The inhibitory effect of type I collagen was detected by 24 hours and was strongly detected at 48 hours, so we continue to use this timing throughout the rest of the experiments.

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

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

Comparing to the control, the expression of OPN was increased by time after treated with 5 ng/ml of IGF-I in the presence of type I collagen. The results in figure 4 indicated that the inhibitory effect of type I collagen on OPN expression was attenuated by IGF-I. The quantitation of the data (figure 4B) show that IGF-I significantly increased the OPN expression in HPDL cells on collagen-coated plates.

A



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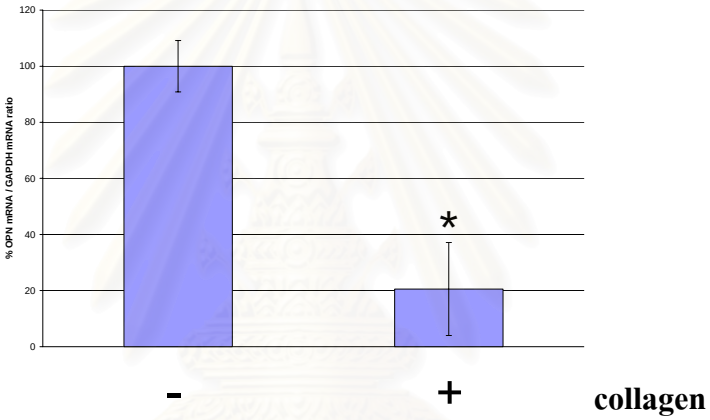


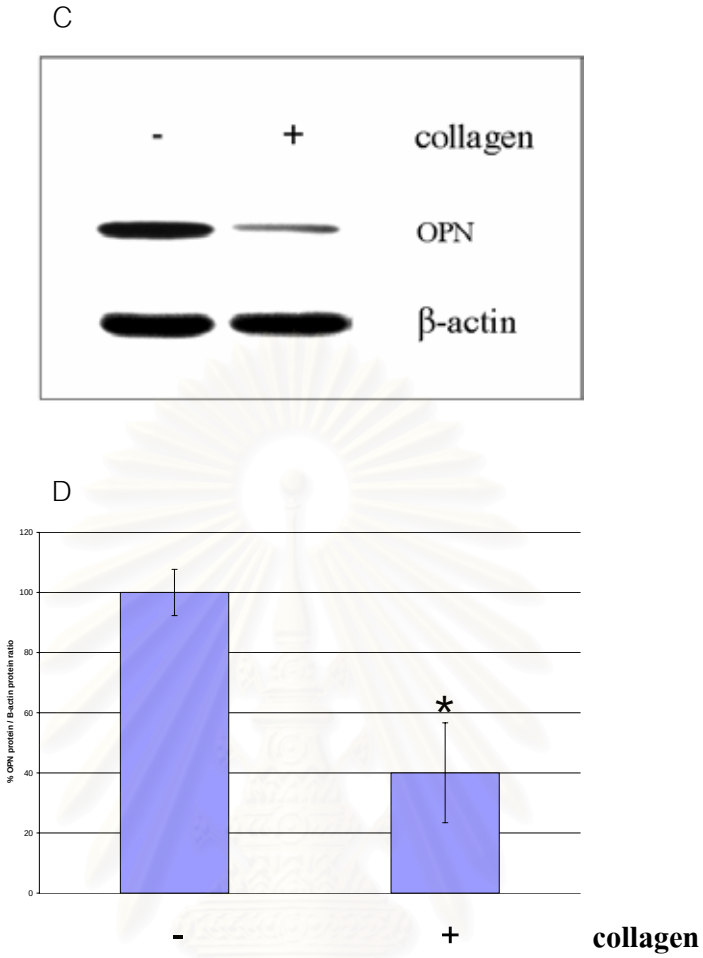


Figure 3 The effect of type I collagen on OPN expression in HPDL cells

HPDL cells were cultured on uncoated plastic or type I collagen-coated plates for 48 hours, then the cells extracts were collected. The levels of the gene expression and proteins were detected by RT-PCR (A) and Western blot analysis using anti-OPN antibody (C), respectively. (B) and (D) graphs show the quantitation of OPN expression normalized to the GAPDH or β-actin. All values are indicated by the mean±SD relative to the control from three independent experiments, * $p < 0.05$ versus control, determined by independent *t* -test.

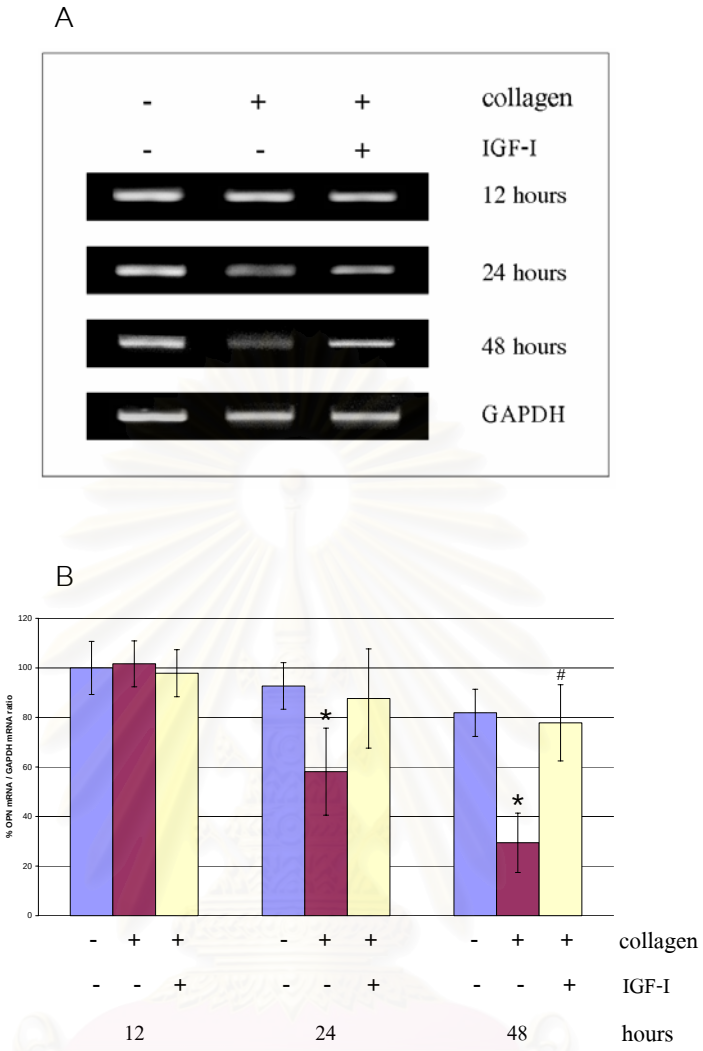


Figure 4 **The effect of type I collagen and IGF-I on OPN expression at various time points**

(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 medium. 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 represent the mean±SD relative to control from three independent experiments. * $p < 0.05$ versus uncoated plastic plate, # $p < 0.05$ versus collagen-coated plate determined by independent *t* -test.

4.5 The effect of inhibitory antibody to β1 integrin on OPN expression in the presence of type I collagen

In order to investigate the mechanism of type I collagen's effect 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 results in figure 5A, lane 4 shows that inhibitory antibody to β1 integrin can abolish the inhibitory effect of type I collagen in HPDL cells, similar result is also obtained from Western blot analysis (figure 5C, lane 4). Figure 5B and 5D, lane 2 and 4 showed that inhibitory antibody to β1 integrin restored the OPN expression.

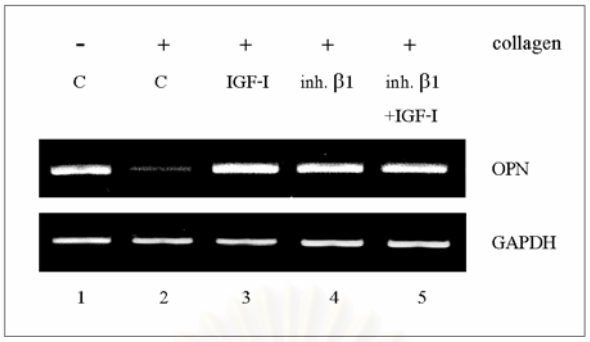
4.6 The combination effect of IGF-I and inhibitory antibody to β1

HPDL cells were treated with IGF-I, the inhibitory antibody to β1 integrin or the combination of IGF-I and inhibitory antibody to β1 integrin in the presence of type I collagen for 48 hours. The levels of the gene expression and proteins were detected by RT-PCR and Western blot analysis, respectively.

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 OPN expression was shown in figure 5A, lane 5 exhibited the increased of OPN expression

after treated with the combination of IGF-I and inhibitory antibody to β1 integrin. However, the level of OPN expression was the same as those of the groups treated with IGF-I (lane 3) or inhibitory antibody to β1 integrin (lane 4) alone. We can observe that the OPN expressions in all treated groups in collagen-coated plate (lane 3-5) were also increased not more than in uncoated plate (lane 1). The similar result was also shown in Western blot analysis (figure 5C). The quantitative data showed no significant difference between these 3 groups (figure 5B and 5D, lane 3-5). This result suggested that the OPN expression enhancing effect of IGF-I in the presence of type I collagen is mediated by β1 integrin receptor signaling pathway.

A



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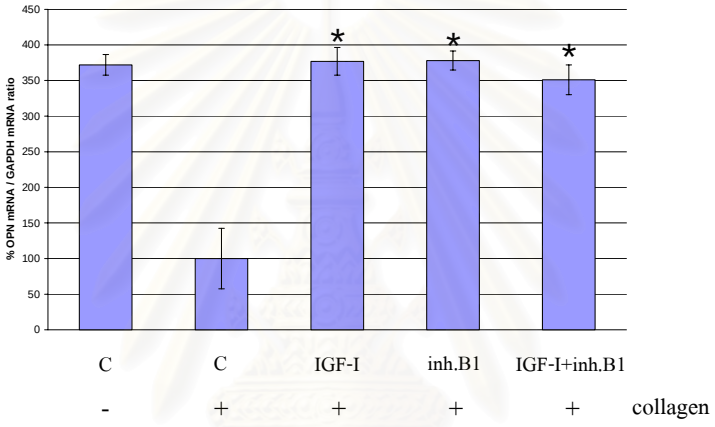

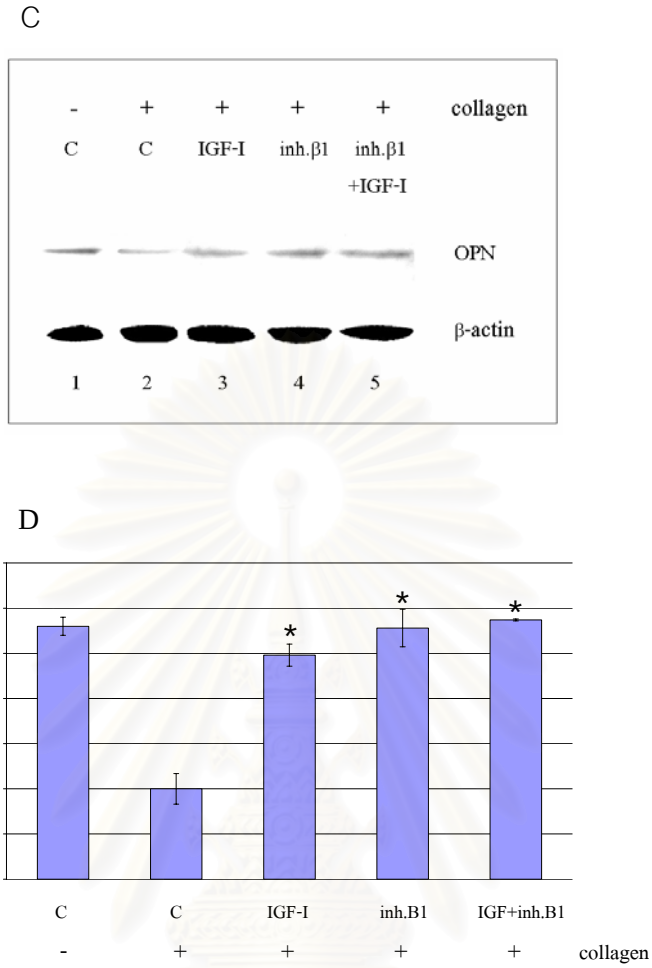

Figure 5 The effect of type I collagen, IGF-I and inhibitory antibody to β1 integrin on OPN expression in HPDL cells

HPDL cells were plated on type I collagen-coated plate for 24 hours before treated with 1 ng/ml of inhibitory antibody to β1 integrin or 5 ng/ml of IGF-I alone or both together. The cell extracts were collected 48 hours after treatment and assessed by RT-PCR (A) and Western blot analyses (C). (B) and (D) graphs show the quantitation of OPN expression normalized to GAPDH or β-actin. All values represent the mean±SD relative to respective control from three independent experiments, * $p < 0.05$ versus collagen-coated control, determined by one-way ANOVA.

CHAPTER 5

DISCUSSION AND CONCLUSION

In this study, we determined the effect of type I collagen and IGF-I on OPN expression in HPDL cells. We found that IGF-I has no effect on 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 eliminate by an inhibitory antibody that specific to β_1 integrin receptor. This result suggested that the inhibitory effect on the OPN expression exerted by type I collagen is signaling through β_1 integrin receptor. Moreover, IGF-I could counteract the inhibitory effect caused by type I collagen. This finding is the first 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 (Liu et al. 2004). The contrasting result of this study might stem from various factors, such as different cell types, or different integrins that used in the signaling pathways. It is well documented that the major integrin receptors for type I collagen are $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Akiyama et al. 1990). In addition, non-integrin receptors for type I collagen, such as

discoidin domain receptor have been reported (Vogel 2001) and we cannot exclude the effect of such receptors in this study. However, in this experiment, the counteracting effect of the inhibitory antibody to $\beta 1$ integrin indicates that the signal via $\beta 1$ integrin is involved in the regulation of OPN expression in HPDL cells. This result is corroborated by a previous study showing that a main signal from type I collagen in alkaline phosphatase activity passed through $\alpha_2\beta_1$ integrin receptor in HPDL cells (Ishikawa et al. 2004). Further investigation is necessary to clarify more specific integrin receptor for type I collagen in the regulation of OPN expression.

We also found the association between type I collagen and IGF-I on the expression of OPN in HPDL cells. IGF-I increased the expression of OPN in the cells grown on collagen-coated plates but not in the uncoated condition. This finding may imply that IGF-I had no direct effect on OPN expression. We hypothesize that IGF-I attenuates the inhibitory effect of type I collagen on OPN expression of HPDL cells. Our results correspond to a previous immunohistochemical report in rat PDL cells. OPN-positive staining PDL cells was found in greater number in growth hormone-induced IGF-I-treated group when compared to the control, in the area which mainly composed of type I collagen (Li et al. 2001). In addition, the result from figure 5 showed that the application of IGF-I and inhibitory antibody to β_1 integrin has no additional or synergistic effect on OPN expression when compared to the condition of adding either one alone.

This evidence supports our hypothesis that IGF-I restores inhibitory effect exerted by type I collagen to the signal transduces through β_1 integrin.

Another possible explanation for the interaction between type I collagen and IGF-I on effects HPDL cell OPN expression is that type I collagen may increase the number of IGF-IR. This could make the 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. Normally, IGF-IR mRNA is expressed at a higher level in HPDL cells than in gingival fibroblasts (Han and Amar 2004). From a previous study, an increase of IGF-IR and tissue IGF-I levels was detected in pancreatitis; this reflects the correlation between levels of IGF-IR, IGF-I and inflammation (Karna et al. 2002). Parkar and colleagues revealed that IGF-IR was not detected by immunohistochemical study in HPDL cells but they sometimes stained weakly in regenerating tissue (Parkar et al. 2001). In addition, the level of IGF-IR was not significantly different after the formation of mineralized nodule induced by dexamethasone in HPDL cells (Nemoto et al. 2004). From these evidences, further experiment in detection of IGF-IR level in the presence of type I collagen is required to elucidate this possibility.

Up-regulation of OPN correlates with the differentiation stages of bone and PDL cells (Lekic et al. 2001). An immunological study showed that the expression of OPN in the central region of periodontal space was lower than the peripheral region, which is closed to the mineralized area (Bosshardt et al. 1998). Since the PDL cells reside within the bundles of periodontal ligaments, it is possible that this environment regulates the behavior or perhaps differentiation of the cells. The increase of certain growth factors, such as IGF-I, may modulate signal and give rise the adjustment of cells to a new environment.

It is well documented that the increase of IGF-I associates with inflammation and healing process (Meinel et al. 2003). A previous study showed the secretion of IGF-I by macrophage in pulmonary fibrosis (Wynes et al. 2004). In 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 (Zhou et al. 2004). Similar result was found in bone fracture healing, IGF-I level increased after the fracture and reached a maximum level in the middle to late stage of callus formation which suggest a role of IGF-I in the process of periodontal regeneration (Taniguchi et al. 2003).

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 $\alpha_2\beta_1$ integrin and PDGF

synergistically enhance cell proliferation in smooth muscle cell (Hollenbeck et al. 2004). Rousselle and colleagues also indicated the collaboration between type I collagen, $\alpha v\beta 3$ integrin and IGF-I in the regulation of rabbit osteoclastic resorption (Rousselle et al. 2001). However, the above reports demonstrated synergistic effects or cross-talk between IGF-I and integrin signaling pathways. Our report here is different since it shows that the interfering interaction between IGF-I and type I collagen occurs at the level of integrin signaling cascade. However, further study is required to elucidate the role of type I collagen and IGF-I association as well as their downstream signaling pathways.

In conclusion, from this study we suggest that type I collagen inhibit OPN expression in HPDL cells via $\beta 1$ 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.

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