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The Effect of the IGF-I treated Gingival and Periodontal Ligament Fibroblast on Osteoblasts

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Insulin-like growth factor I (IGF-I) has the local tissue regulating actions. In bone, IGF-I increases the replication of osteoblastic lineage, probably preosteoblasts, and enhances osteoblastic collagen synthesis and matrix composition rates. The purpose of this study was to investigate the local regulatory effect of IGF-I on periodontium totally, both in an autocrine and paracrine manner. To examine the effect of IGF-I directly on osteoblast (OB) of test rats, and indirectly on OB via periodontal ligament fibroblast (PDLF), and the effect of gingival fibroblast (GF) on OB via cellular paracrine manner for the understanding of humoral action of adjacent tissue, GF and PDLF were obtained from male Sprague-Dawley rats of six to eight weeks of age. OB was obtained from frontal and parietal calvarial bone of Sprague-Dawley 21day-old-fetus. After each cell was incubated 24 hours, for collecting conditioned medium, different concentrations of IGF-I (1,10,100 ng/ml,1ml/well) was adding in the GF, PDLF cells, and the supernatant from these cultures was put into the primary OB culture with $1x10^4$cell/ml/well. The experimental group was divided into six groups - control OB, IGF-I treated OB, OB culture with conditioned medium from PDLF, OB culture with conditioned medium from IGF-I treated PDLF, OB culture with conditioned medium from GF, OB culture with conditioned medium from IGF-I treated GF. After final IGF-I treatment, OB was incubated for 24 hours, and alkaline phosphatase activity assay, BMP expression, cell proliferation measurement using MTT assay, total protein measurement, collagen synthesis assay using western blot, and examination of bone nodule synthesis were done.

Alkaline phosphatase expressions were increased in the group of PDLF-IGF-I supernatant treatment. Direct IGF-I treatment with concentrations of 100ng/ml showed increased viable cell number measured by MTT assay. And IGF-I treatment did not increase total protein amount. The entire experimental group showed BMP2, 4 expression in western blot, and there was no significant difference between control and experimental groups.

These results suggested that supernatant from PDLF effects on increasing cellular activities of OB regardless of IGF-I, and at high concentration, IGF-I increases OB cell proliferation.

Key words : Insulin-like growth factor I, Osteoblast, Periodontal fibroblast

The heperiodontal alveolar bone integrity depends on the continuous remodeling of periodontal osteoblast. It needs a coordinated sequence of cell-mediated events of osteoclastic bone resorption followed by the refilling of each resorption cavity with new bones. Therefore the bone formation and remodeling necessitates stringent control of osteoblast proliferation and differentiation as well as the control of osteocalst. Current research reveals that osteobalstic activity is dependant upon many tissue factors, one of the most important being the growth promoting activity of the insuline-like growth factors (IGFs). The IGF

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system in bone consists of IGF peptide and receptors, IGF binding proteins (IGFBPs), and IGFBP proteases, and is focused by its local regulation of bone formation with an autocrine or paracrine manner. IGF peptides, IGF-I and IGF-II are anabolic peptides structurally and functionally related to insulin, being available to skeletal tissues through de novo synthesis by bone cells and by release of stored peptide from the bone matrix.

IGF-I increases the replication of the osteoblastic lineage[1], probably preosteoblasts, and enhances osteoblastic collagen synthesis and matrix composition rates[2]. In addition, IGF-I decreases collagen degradation in calvaria and increase recruitment of osteoclasts[3]. In vitro studies on direct effect of IGF-I on bone formation, it was found that IGF-I promotes bone collagen synthesis, and stimulates DNA and protein synthesis[4,5]. When endogenous IGF activities were inhibited with IGF antibodies, IGF receptor anti-bodies, or inhibitory IGF binding proteins, osteoblast proliferation and collagen synthesis were reduced[6]. In the bone healing process, IGF-I has been shown to promoting critical size of bone in bony defect of irradiated rats[7].

IGF expression is relatively high in cells of developing bone periosteum and growth plate, healing fracture callus tissues[8], and developing ectopic bone tissue induced by demineralized bone matrix[9]. There are differences in IGF expression among the various osteoblast cell models, human osteoblast cells produce primarily IGF-II, rodent osteoblast cells produce primarily IGF-I. Transformation of osteoblast cells may alter IGF gene expression, particularly IGF-I. In addition, differences in the state of osteoblast development during in vitro culture can impact results.

IGFs may also be important in mediating effects of systemic hormones on bone formation[10,11]. Such systemic agents, particularly PTH and steroid hormones, have been shown to regulate the synthesis of IGF-I in cells of osteoblastic lineage. Cyclic AMP induces insulin-like growth factor I synthesis in osteoblast enriched cultures[12], and PTH also has promotion effect by stimulating adenylate cyclase in osteoblast, estradiol enhances proliferation of osteobalst[13]. 1.25-Dihydroxy-vitamin D_3 also regulates IGF-I produc-tion[14], and PGE_2 stimulates IGF-I synthesis in rat osteoblast cultures[15].

On the other hand, IGF-I also has been reported to increase osteoclast formation from mouse osteoclast precursors[16,17], and has been suggested that IGF-I may act as an autcrine regulator of osteoclasts. In recent study, clinical IGF-I injection subcutaneously for 6 days increased serum markers for bone resorption as well as bone formation[18]. These studies indicate that in addition to the well known anabolic effects of IGFs on osteoblasts, IGFs may also stimulate cells of osteoblastic cell lineage and increase bone resorption. Furthermore, whether bone cells register a mitogenic or differentiated response to IGF stimulus may reflect receptor population and receptor cross reactivity and depend on cell type and osteoblast lineage. For many of the same reasons, differential functions for IGFs in bone have been difficult to define in vivo.

The effect of combinations of other growth factors with IGF-I on bone has been assessed in vitro and in vivo. In calvarial organ culture the combination of IGF-I plus TGF or, IGF-I plus PDGF increased bone matrix formation more than TGF, PDGF, IGF-I individually[19]. IGF-I has been shown to synergistically increase osteoblast mitogenesis in cultured bone cells when combined with other growth factors such as b FGF, PDGF, or TGF, and EGF[20]. Maxi-mal proliferation of cultured adult human osteoblasts was seen with a cocktail of IGF-I, PDGF, TGF- and EGF[21]. Thus, several studies suggest that IGF-I or II combined with other growth factors may augment the osseous turnover process.

Recently, there have been research achievements concerted in the fields of cell and molecular biology to understand the mechanisms of polypeptide growth factors on the repair and regeneration of periodontal tissues. Many growth factors are large polypeptides that have specific receptor-mediated interactions with their target cells, and such interactions are associated with cell proliferation in vitro. By contrast, the efficacy of growth factors in vivo is not so clearly established. This is in part the result of the difficulty of administering growth factors and measuring their cellular response in vivo.

Based on many understandings of IGF-I, this study investigated on the local regulatory effect of IGF-I to periodontium totally, both in an autocrine and paracrine manner, to examine the effect of IGF-I directly on osteoblast, and indirectly on osteoblast via periodonal fibroblast as a major cell of the periodontal ligament. For understanding of the humoral effect of adjacent tissues, the effect of periodoatal fibroblast and gingival fibroblast on osteoblast via cellular paracrine manner was examined indirectly by use of conditioned medium of each cell culture.

MATERIALS AND METHODS

Experimental design

GF (gingival fibroblast) and PDLF (periodontal ligament fibroblast) was obtained from male Sprague Dawley rats between six to eight weeks of ages, and OB was obtained from parietal and frontal calvarial bone of Sprague-Dawley 21d-old fetus. After each cell was incubated 24 hours, for collecting supernatants, different concentrations of IGF-I (1ng, 10 ng, 100ng/ml) was added in GF and PDLF cells, and supernatant from these cultures put into the primary OB culture with $1X10^4$cell/ml, 1ml/well. The experimental group was divided into six groups-groupI : control OB, groupII : IGF-I treated OB, groupIII : OB culture with conditioned medium from PDLF, groupIV : OB culture with conditioned medium from IGF-I treated PDLF, group V : OB culture with conditioned medium from GF, and group VI : OB culture with conditioned medium from IGF-I treated GF. Alkaline phosphatase assay, cell proliferation measuring using MTT assay, total protein measurement with BCA kit, BMP expression assay, collagen synthesis assay, and examination of bone nodule formation were performed in each groups of OB.

Primary culture of rat gingival fibroblast & PDL cells

For the primary culture of gingival fibroblasts and periodontal ligament (PDL) cells we used male Sprague-Dawley rats (DaeHan Animal Lab, Seoul, Korea) between six to eight weeks of ages. Animals were killed by ether inhalation anesthesia and overdose of KetamineHCl (Yuhan Co., Seoul, Korea) & Xylazine HCl. (Beyer Korea Co., Seoul, Korea) First, the mandible was separated in aseptic conditions, and then the mandible was disinfected with chlorhexidine and hydrogen per-oxide. Using a sharp scalpel, gingiva surrounding the molar teeth was dissected. The molar teeth were extracted with modified root pickers.

Primary gingival culture

Gingival tissue of the rat was washed in culture media (Dulbecco's Modified Eagle Medium (D-MEM), Gibco BRL.,Gaithersburg, USA) with 5X concentration of antibiotic-antimycotic mixture (Penicillin, Streptomycin, Fungizone) (Gibco BRL., Gaithersburg, USA) three times. Then it was minced with iris scissors into less than a square millimeter blocks. That minced tissue was incubated in trypsin-EDTA solution (Gibco BRL., Gaithersburg, USA) at 37? for 30 min. It was washed with media and incubated with D-MEM with 10% Fetal Bovine serum (FBS, Gibco BRL., Gaithersburg, USA)

Primary PDL cell culture

The teeth of the rat were washed in culture media (D-MEM) with 5X concentration of antibiotic-antimycotic mixture three times. They were incubated in the collagenase & Trypsin-EDTA solution for 20 min in a 37℃ water bath with continuous stirring. Then supernatant was collected, washed and plated in a 60mm culture dish (Corning Co., N.Y., USA). These procedures were repeated six times. Usually the third to fifth digest was good for yielding PDL cells. Cells were cultured in D-MEM (10% FBS) for one week. Then cells were trypsinized, counted and used for the experiments.

Calvarial osteoblast culture

21d-old fetuses of Sprague Dawley rats (12-15

fetuses) were used. The fetuses were killed in ether vapor and disinfected in 70% ethanol. For the removal of calvarial bone, a scalp flap was opened and the calvaria was cut from the frontal and parietal bones together. The collected calvarial bones were cut into smaller pieces (3-5mm) and put into the trypsin-EDTA solu-tion with 0.1% collagenase (Gibco BRL., Gaithersburg, USA). Tissues were incubated at 37℃ with continuous stirring for 10min, 10min, 20min, 20min, and 20 min, in sequence. For the experiment we used the third to fifth collections as primary osteoblast culture. Cells were put into a 60mm dish with 5ml D-MEM (10% FBS) for one week. Cells were trypsinized, counted and use directly for the experiment.

Basic experimental procedure

For preparing the primary osteoblasts required in this experiment, the OB were carried into 12 well culture plates and cultured 24hours in D-MEM with 10% FBS. Three different concentrations of IGF-I (Sigma Co., St. Louis, USA) (1ng/ml, 10ng/ml, 100ng/ml, 1ml/ well) were put into the culture plates of GF and PDLF, and these were incubated for an additional 12 hours. The supernatant of GF and PDLF culture media was collected and filtered with 0.2l sterilizing filter, and added to the primary OB culture plates (each well contained 1 $X10^4$ cells, 1ml/well). The OB culture was incubated for 24 hours, and the cells were lysed by three repeated freezing and thawing cycles.

Alkaline phosphatase assay

After 24 hours of incubation, the OB prepared from the basic experimental procedure were washed with HBSS (Gibco BRL., Gaithersburg, USA), and were lysed with two repeated freezing and thawing cycles. These cell lysates were homogenized and 20l aliquots were taken for assay. The aliquotes were incubated with 200l of p-nitrophenyl phosphate (p-NPP) (Sigma Co., St. Louis, USA) at 37℃ for 30 min. Spectrophotometric assay for alkaline phosphatase activity at 405nm was done using a Shimadzu UV-1601 Spectrophotometer (Shimadzu Co., Tokyo, Japan).

Measurement for BMP expression by Western blot hybridization

The prepared OB obtained from the basic experimental procedure. For western blot hybridization, antibody : anti-BMP2/4 polyclonal goat antiserum raised against peptides sequence 391-408 at C-terminal of BMP-4 (Santa Cruz Biotechnology, Santa Cruz, USA) and anti bovine BMP antibody (R&D System, Minneapolis, USA) were added to the cultured plates. Total protein of OB was extracted by addition of a protein lysis buffer. The protein concentration was measured using BCA reagent (Pierce Co., Rockford, USA). The extracts were electrophoresised in SDS-PAGE gel using the Laemmli method[22].

Measurement for cell proliferation

For measuring of OB proliferation, this experiment used MTT assay. The endpoint of MTT assay is an estimate of cell numbers. MTT reduction as a cell viability measurement is now widely chosen, because MTT (water soluble tetrazolium dye) is reduced by liver cells to a purple formazan product that is insoluble in aqueous solutions. The OB from the basic experimental procedure was used. After incubating OB for 24 hours, the media was changed; 200l of MTT (Sigma Co., St. Louis, USA) was added to the media. The plates were wrapped in aluminum foil and incubate for an additional 4 hours. The media was removed and 200l of DMSO (Sigma Co., St. Louis, USA) was added for resolving MTT-formazan crystal. The absorbance of final solution of each group was measured at 570nm with a Shimadzu spectrophotometer.

Measurement for total protein

After 24 hours of incubation the OB were washed with HBSS and lysed with two freezing and thawing cycles.

The cell lysate was homogenized and 100l aliquots were taken for assay. In this procedure, the BCA Protein Assay Kit (Pierce, Rockford, USA) was used.

Aliquotes were incubated with 1ml of BCA reagent

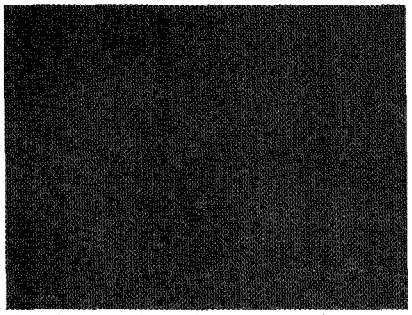

Fig. 1. Rat gingival fibroblast (Phase contrast microscopic view, X40)

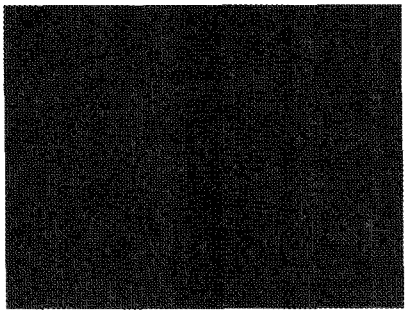

Fig. 2. Rat periodontal ligament fibroblast (Phase contrast microscopic view, X40)

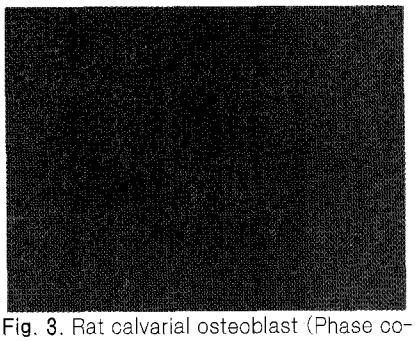

Fig. 3. Rat calvarial osteoblast (Phase contrast microscopic view, X40)

(Pierce, Rockford, USA) at 37℃ for 30 min. The purple colored active products were formed by chelation of two molecules of BCA with one cuprous ion, this water soluble complex exhibited a strong absorbance at 562nm.

Spectrophotometric assay was done for total protein at 562nm using a Shimadzu Spectrophotometer.

Measurement for collagen synthesis

The OB cell layer was rinsed with HEPES buffer. All cells in the culture plates were scrapped off and brought to 0.5mol/l acetic acid. In these combined media, the protein of the cell layer was digested with pepsin (0.1mg /ml) (Sigma Co., St. Louis, USA) at 4℃ for 4 hours. The pepsin-resistant collagen chain was then precipitated with 3mol/l NaCl overnight. After this step, the precipitate was collected by centrifugation at 8000rpm for 10 min.

The aliqoutes were dialyzed against 0.5mol/l NH_4HCO_3 overnight. The dialysates were freeze-dried and electrophoresised in SDS-PAGE gel using the Laemmli method. These gel plates were transferred to the PVDF membrane, and immunoblotting with anti-type I collagen antibody (clone I-8H5, Oncogene Research product, Cambridge MA, USA) was done.

ALP activity in situ

The OB were washed with HBSS, and the cells were fixed with 4% paraformaldehyde solution for 1 hour. Then the OB were washed again in Tris Buffer (pH 8.0). 1ml of BCIP-NBT solution was put into each well plate and they were incubated in a dark room at room temperature. The phase contrast microscopic view was recorded with a Spot digital camera (Diagnostic Instruments, Michigan, USA).

Bone formation in vitro

For examining the development of mineralized nodule in OB cultures, the OB was incubated in alpha MEM (Gibco BRL., Gaithersburg, USA) supplemented with ascorbic acid or organic phosphate ester (-glycerophosphate (Sigma Co., St. Louis, USA)) enriched medium. The culture plates were cultured for two weeks, and formation of bone nodules were checked with Von Kossa Staining in microscopic observation.

RESULTS

Cell culture

Gingival fibroblast showed spindle shaped cells, densely packed and growing in a parallel arrangement. Some area showed storiform growth patterns (Fig. 1). PDL cells were different from gingival fibroblast in individual cell morphology, showing more flattened appearance with stellate cytoplasmic processes and growing in a loosely arranged storiform or irregular pattern (Fig. 2). Primary rat calvarial osteoblast showed polygonal shaped cells with round nuclei. When confluent, cells were densely packed and inter-cellular space was uneven (Fig. 3). In some areas, cells were packed more densely and looked multi-layered.

Table 1. alkaline phosphatase activity of osteoblast

	1ng		10ng		100ng	
Control	0.357	0.059	0.425	0.095	0.380	0.064
IGF	0.402	0.137	0.599	0.139	0.583	0.116
PDLF	0.550	0.134	0.583	0.156	0.621	0.181
PDLF+IGF	0.528	0.131	0.602	0.108	0.597	0.111
GF	0.494	0.124	0.419	0.124	0.384	0.114
GF+IGF	0.471	0.129	0.489	0.132	0.400	0.129

Table 2. Adjusted result from absorbance data

	1ng		10ng		100ng	
Control	1.000	0.000	1.000	0.000	1.000	0.000
IGF	1.142	0.389	1.429	0.309	1.583	0.472
PDLF	1.591	0.351	1.427	0.436	1.678	0.608
PDLF+IGF	1.506	0.383	1.441	0.221*	1.610	0.413*
GF	1.408	0.371	1.009	0.262	1.025	0.297
GF+IGF	1.312	0.274	1.179	0.296	1.060	0.293

* P<0.05, t-test, significant difference compared to control group of each concentration

Table 3. MTT assay for cell proliferation activity

	1ng		10ng		100ng	
Control	0.936	0.279	0.958	0.156	1.016	0.372
IGF	1.164	0.364	1.357	0.246	1.492	0.269*
PDLF	1.093	0.268	1.236	0.336	1.355	0.435
PDLF+IGF	1.309	0.143	1.427	0.231	1.263	0.341
GF	0.864	0.268	1.151	0.345	0.950	0.352
GF+IGF	1.028	0.372	0.932	0.241	1.005	0.266

* P<0.05, t-test , significant difference compared to control group of each concentration

Table 4. Total protein measurement of osteoblast

	1ng		10ng		100ng	
Control	1.398	0.267	1.505	0.213	1.495	0.281
IGF	1.544	0.184	1.618	0.255	1.543	0.187
PDLF	1.578	0.147	1.635	0.187	1.611	0.199
PDLF+IGF	1.693	0.216	1.627	0.206	1.625	0.267
GF	1.571	0.205	1.334	0.278	1.509	0.041
GF+IGF	1.401	0.103	1.365	0.103	1.542	0.133

P<0.05, t-test

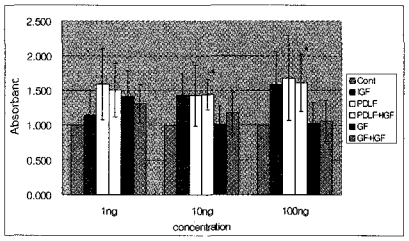

Fig. 4. Alkaline phosphatase activity of osteoblast

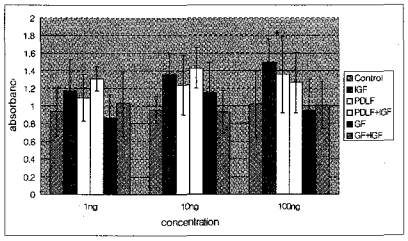

Fig. 5. MTT assay for cell proliferation activity

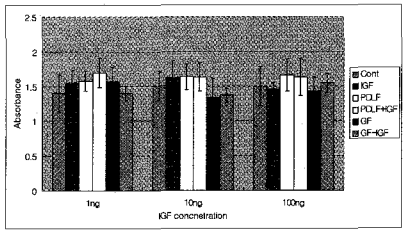

Fig. 6. Total protein measurement of osteoblast

The result of alkaline phosphatase assay

Alkaline phosphatase expressions were increased in the group with direct IGF treatment at concentration of 10 and 100ng/ml and all the group of PDL-IGF super natant treatment. The PDL cell culture supernatant only treated group also showed increased expression but was not significantly different from the untreated control statistically (Fig. 4, Table 1, 2). Gingival fibroblast culture supernatant-treated group showed no

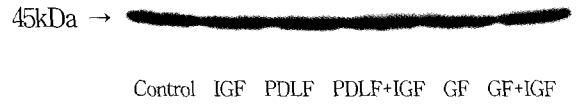

Fig. 7. BMP2,4 expression measurement (Western blot)
Six groups :
Control : control osteoblast
IGF : IGF-I treated osteoblast
PDLF : osteoblast culture with conditioned medium from periodontal ligament fibroblast
PDLF+IGF : osteoblast culture with conditioned medium from IGF-I treated periodontal ligament fibroblast
GF : osteoblast culture with conditioned medium from gingival fibroblast
GF+IGF : osteoblast culture with conditioned medium from IGF-I treated gingival fibro blast

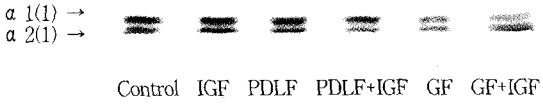

Fig. 8. Type I collagen measurement (Western blot)
Six groups :
Control : control osteoblast
IGF : IGF-I treated osteoblast
PDLF : osteoblast culture with conditioned medium from periodontal ligament fibroblast
PDLF+IGF : osteoblast culture with conditioned medium from IGF-I treated periodontal ligament fibroblast
GF : osteoblast culture with conditioned medium from gingival fibroblast
GF+IGF : osteoblast culture with conditioned medium from IGF-I treated gingival fibroblast

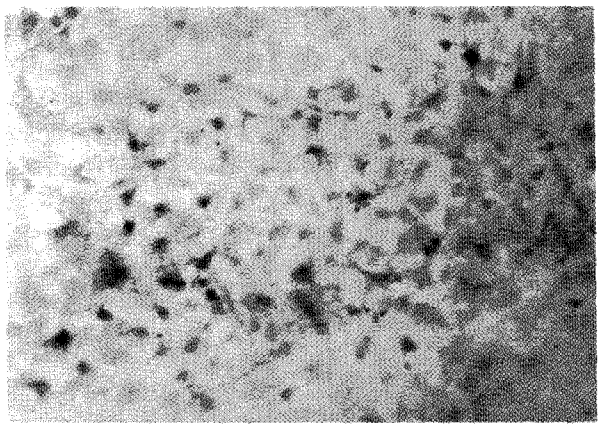

Fig. 9. In situ alkaline phosphatase staining (Microscopic view, X100) : Control

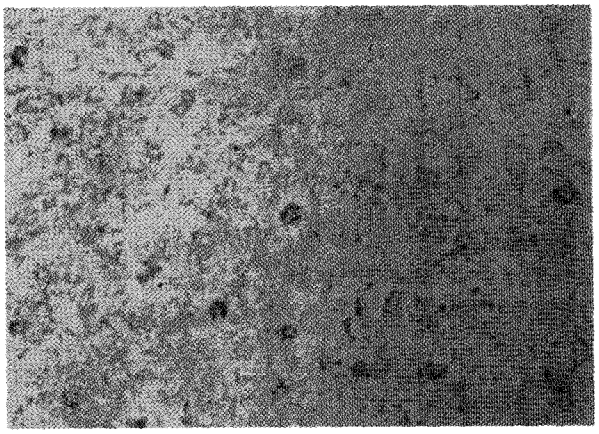

Fig. 10. Bone nodule formation (Von Kossa staining, X100) : Control

increase in alkaline phosphatase activity.

The result of MTT assay

Direct IGF treatment in 100ng/ml concentration showed increased in viable cell number measured by MTT assay. Other experimental groups showed little increase but statistically insignificant (Fig. 5, Table 3).

Result of total protein measurement

Total protein amount was not increased with IGF treatment in all experimental groups (Fig. 6, Table 4).

Result of BMP expression in western blot

The entire experimental group showed BMP 2, 4 expression in western blot. There were no differences between control and experimental groups at 24 hours of experiment (Fig. 7).

Type I collagen measurement

There was no significant difference between control and experimental groups in type I collagen measurement (Fig. 8).

In situ alkaline phosphatase staining

Experimental groups with 10-100ng IGF treatment or IGF treated PDL cell culture supernatant-treated groups showed increased alkaline phosphatase staining (Fig. 9).

Bone nodule formation

There was no significant difference in examining of mineralized bone nodule formation between control and experimental groups. No defining bone nodule was found in the control group or the experimental group after 2 weeks from the start of this experiment (Fig. 10).

DISCUSSION

The three peptide hormones, or growth factors, in IGF family-insulin, IGF-I, IGF-II, have approximately 50 percent of their amino acid in common. Insulin is synthesized in the beta cells of the pancreas as pro-insulin, which is cleaved to form insulin and C peptide. The IGFs, which are synthesized primarily by liver, retain the C peptide and have an extended carboxy terminus. Insulin circulates at picomolar concentrations and has a half-life of minutes. The IGFs, on the other hand, circulate at higher (nanomolar) concentrations and largely bound to one of six IGFBPs that modulate IGF activity[23]. These binding proteins, like the IGFs, are synthesized primarily in the liver. IGFs and their binding proteins are also produced locally by most tissues, where they act in an autocrine or paracrine manner.

Insulin acts primarily on the liver, muscle, and adipose tissues[24], whereas the IGFs are important in the function of almost every organ in the body[25]. Both IGFs are essential to embryonic development[26], and nanomolar concentrations of both are maintained in the circulation into adult life. After birth, however, IGF-I appears to have the predominant role in regulating growth, whereas the physiologic role of IGF-II is unknown.

Insulin, IGF-I, and IGF-II bind specifically to two high-affinity membrane-associated receptors that are tyrosine kinase. Insulin activates the insulin receptor, and both IGFs activate the IGF receptor (a third receptor, the IGF-II mannose-6-phosphate receptor, binds IGF-II but has no known intracellular signaling actions). Activation of either the insulin receptor or the IGF-I receptor evokes similar initial responses within the cell[27]. However, since insulin regulates metabolic functions, the final pathways these hormones activate within the cell must be separate and distinct.

The interactions of growth hormone with its hepatic receptor stimulate expression of the IGF-I gene and the release of the IGF-I peptide[28]. Serum concentrations of IGF-I usually parallel 24-hour mean serum concentrations of growth hormone by the pituitary. How the liver regulates the synthesis of IGF-II is unknown. Circulating IGFBPs limit the access of the IGFs to specific tissues and the receptor for IGF-I and insulin. Of six IGFBPs, $IGFBP_3$ binds more than 95% of the IGF-I in human serum. The IGF-IGFBP-acid labile protein subunit complex has a serum half-life of many hours. Once released from the complex, the IGFs leave the circulation and enter target tissues with the aid of other IGFBPs. Growth hormone increases the serum concentrations of both the acid labile subunit and $IGFBP_3$.

Some IGFBPs bind the growth factors with greater affinity than do the IGF receptors, thereby preventing the activation of intracellular signaling pathways. The affinity of these binding proteins for the IGFs can be reduced by protease cleavage, or increased phosphorylation of the binding protein, or by the binding of the protein to the surface of cells rather than the extracellular matrix. The reduced affinity enhances the biologic activity of the IGFs by increasing the amount of free growth factor available to IGF receptors. The actions of insulin and the IGFs are differentiated by the IGFBPs, which do not bind insulin but do direct the IGFs to their specific receptors. As a matter of fact, IGFBPs serve more than just transport protein, they can enhance or inhibit IGF activity. IGF activity at the receptor level depends on their concentration, their tissue source, the presence of IGFBP specific protease, and their binding affinity to extracellular matrices. To

make matters more complicated, systemic regulation of these IGFBPs is also quite varied.

Tissue IGFs (locally produced IGFs) are important in the activity of several organ systems. Growth hormone, parathyroid hormone and sex steroids regulate the production of IGF-I in bone, whereas sex steroids are the main regulators of local production if IGF-I in the reproductive system. The functions of circulating IGF-I are becoming clearer, but the actions of locally produced IGFs have yet to be identified.

IGFs' anabolic effects on wound healing process of soft and hard tissues have been recently focused on in the dental fields. In the normal wound healing process, multiple growth factors act in concert to form an intricate molecular arrangement regulating the activity of cells within and adjacent to a wound. At the wound margins, several growth factors are released within a few hours after injury such as PDGF, TGF-, EGF -like protein, and IGFs[29] in the plasma exudate. These growth factors may regulate the repair and/or regenerative process. In the periodotium, IGF-I has been shown to be chemotactic for cells derived from the periodontal ligament[30]. IGF-I also has strong effects on periodontal fibroblast mitogenesis and protein synthesis in vitro. IGF-I receptors have also been localized on the surface of periodontal fibroblasts[31]. The roles of IGF-II on parameters of periodontal fibroblast and gingival fibroblast metabolism have not been reported to date. Both IGF-I and IGF-II are found in large amount in bone, with IGF-II being the most abundant growth factor in bone matrix in human[32]. IGF-I produced by osteoblasts and stimulates bone formation by inducing cellular proliferation and type I collagen biosynthesis. In bone, it has been reported that high levels of IGF-I are synthesized and secreted by osteoblasts and therefore regulates bone formation in an autocrine manner[33]. IGF-I also increases the number of osteoclastic multinucleated cells. When IGF -I applied to natural periodontal lesions in dogs and monkeys, only slight increases in new cementum and bone formation were found[33]. But combinations of other growth factors with IGF-I, IGF-I plus TGF , or IGF-I plus PDGF increased bone matrix apposition more than TGF-, PDGF, IGF-I individually. IGF-I has been shown to synergistically increase osteoblast mitogenesis in cultured bone cells when combined with other growth factors[34].

In order to understand the relationship between the osteoblast and paracrine effects of periodontal fibroblast, our study has investigated the effects of IGF-I on osteoblast via periodontal fibroblast and gingival fibroblast by making the conditioned medium of each cell culture. Conditioned medium from periodontal fibroblast has the significant enhancing effect of proliferation, protein synthesis, and alkaline phosphatase activity of osteoblast. But conditioned medium from gingival fibroblast has no significant activating effect. These differences may have been due to different growth properties of the two cell types, different lengths of cell cycle, or cellular reactivity to specific growth factors.

Haase et al. have investigated the effect of PDGF -BB, IGF-I, and Growth Hormone on DNA and proteoglycan synthesis by cultured human gingival and periodontal ligament fibroblasts in vitro[35]. PDGF-BB and IGF-I, but not growth hormone, were mitogenic for both periodontal ligament fibroblasts and gingival fibroblasts, but the periodontal ligament cells responded more strongly. In this study, periodontal ligament fibroblasts were consistently more responsive to PDGF-BB, IGF-I than were the gingival fibroblasts, this may reflect the level of expression of specific growth factor receptors by these cells or differences in down stream intermediates and requires further investigation. TGF have been reported to have the similar effect on periodontal ligament fibroblast and gingival fibroblast of human, periodontal fibroblast showed a significant increase in protein synthesis to TGF over time while that by gingival fibroblast was not significant as compared to the control[36]. Periodontal fibroblasts consist of a majority of undifferentiated fibroblast, thus these cells retain osteoblastic properties and high alkaline phosphatase levels[37].

In this experiment, alkaline phosphatase activities in fetal rat calvarial osteoblast-like cells were increased in response to the cell culture supernatant of PDL cells regardless of IGF-I. IGF also increased alkaline phosphatase activities of osteoblast-like cells in concen-

tration higher than 10ng/ml. This result suggests that the PDL cell itself has its own growth promoting effects for the osteoblasts without stimulation by the IGF-I. In this experiment, 10% of the fetal bovine serum was added to the growth medium. It means enough amounts of serum growth factors may be supplied to the cells. And also PDL cells and gingival cells can utilize growth factors other than IGF-I. The osteoblast-like cells, however, may need enough amount of IGFs in addition to the serum supplement. The gingival fibroblast showed no effects on the osteoblasts activity regardless of IGF stimulation. The gingival cell phenotypes are much simpler than periodontal ligament cells, and PDL cells may include sizable populations of osteoblast precursor and vascular endothelial cells. These two cell types may be responsible for the result. The primary gingival fibroblast culture also contains these two cell types, but the osteoblast lineage cell population would be much less. Another reason is that the proliferation rate of gingival fibroblast is faster than PDL fibroblast, so the second or third passage of the primary culture, which is used in this experiment, contains much more fibroblast populations.

Protein synthesis measurements showed no remarkable differences between the experimental groups. In this experiment, the results were measured at 24 hours. This may be the earliest time point for the measurement of protein synthesis. Protein synthesis increased only in the 100ng concentration of IGF.

PDL fibroblast may produce several growth factors for bone induction, especially from the bone cell lineage in response to IGF stimulation, but IGF-I itself even in high concentration has no growth promoting effect for primary osteoblast. It is observed that an increase in bone cell proliferation and alkaline phosphatase activities occurred after treatment of PDL fibroblast supernatant. The results of this experiment showed that the PDL cell is different from GF in terms of growth promoting activities for osteoblast. A PDL cell has its own bone inducing effect without any stimulation from GFs. Some growth factors have different growth promoting and regulatory effects for the PDL cells. For PDL osteoblast, it can be induced by several serum growth factors, which are included in the media.

CONCLUSIONS

For investigation of osteoblast activity in different environmental conditions, this experiment was conducted with six experimental groups. These were control (untreated osteoblast), IGF treated osteoblast group, osteoblastic culture with conditioned medium from periodontal fibroblast, osteoblast culture with conditioned medium from IGF-I treated periodontal fibroblast, osteoblast culture with conditioned medium from gingival fibroblast, and osteoblast culture with conditioned medium from IGF-I treated gingival fibroblast. And the results after adding different concentrations of IGF-I (1ng/ml, 10ng/ml, 100 ng/ml/well) are as follows :

1. Alkaline phosphatase expressions increased in the group with direct IGF-I treatment at concentration 10 and 100 ng/ml, and in all the group of PDLF-IGF-I supernatant treatment.
2. Direct IGF-I treatment with the concentrations of 100ng/ml showed an increase in viable cell number measured by MTT assay.
3. IGF-I treatment did not increase total protein amount.
4. The entire experimental group showed BMP2, 4 expression in western blot. There were no differences between control groups and other experimental groups.

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국문초록

IGF-I으로 처리한 치은 및 치주인대 섬유모세포가 골모세포에 미치는 영향

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이 연구의 목적은 IGF-I의 골모세포에 대한 직접적 작용효과와 IGF-I이 섬유모세포에 작용된 후 섬유모세포에서 유리된 인자를 포함한 조절 배양액이 골모세포에 어떤 영향을 미치는지에 대해 각각 치주인대 섬유모세포와 치은 섬유모세포를 이용하여 알아본 후, 치조골의 반응을 외적자극과의 사이에서 매개하는 치주인대의 주세포군인 섬유모세포를 통한 IGF-I의 골모세포에 대한 영향을 알아보는 것이다. 이를 위해 치주인대 섬유모세포와 치은 섬유모세포를 6-8주된 백서(Sprague-Dawley rat)에서 채집하고, 골모세포는 태생 21일된 동종백서에서 채집하여 기본배양을 한 후, 각 군을 6군으로 분리하여 1 x 10^4/well, (1ml/well)세포수로 분주한 골모세포 배양접시에 IGF-I을 1,10,100ng/ml로 각각 농도를 달리하여 그 효과를 알아보았다. 각각의 군은 대조군, IGF-I을 직접 투여한 골모세포 배양군, 치주인대 섬유모세포의 조절배양액을 이용한 골모세포 배양군, IGF-I으로 처리한 치주인대 섬유모세포의 조절배양액을 이용한 골모세포 배양군, 치은 섬유모세포의 조절배양액을 이용한 골모세포 배양군, IGF-I으로 처리한 치은 섬유모세포의 조절배양액을 이용한 골모세포 배양군이다. 조절배양액은 배양 36시간후(IGF-I 처리후 12시간 배양 포함) 채집하였고, 마지막으로 IGF-I 및 조절배양액으로 처리한 후에 추가 24시간 배양한 후, Alkaline phoaphatase 활성도, Western blot을 이용한 BMP발현, MTT를 이용한 세포증식, BCA kit을 이용한 총단백질량 측정, Western blot을 이용한 교원질 합성 계측 및 골결절의 생성을 관찰하였다. 본 연구의 결과는 다음과 같다.

1. Alkaline phosphatase활성은 10, 100ng/ml의 IGF-I으로 처리한 군과 치주인대 섬유모세포의 조절배양액을 이용한 군, IGF-I으로 처리한 치주인대 섬유모세포의 조절배양액을 이용한 군에서 대조군보다 더 높게 나타났다. 10, 100 ng/ml 의 IGF-I으로 처리한 치주인대 섬유모세포의 조절배양액을 이용한 실험군에서 유의성 있게 높게 나타났다.
2. 100ng/ml농도의 IGF-I으로 직접 처리한 군에서 골모세포증식이 유의성 있게 증가하였다.
3. 총단백질량은 IGF-I투여와 상관없이 대조군, 실험군 모두 유사하였다.
4. 모든 실험군에서 BMP2,4가 발현되었고, 대조군과 유의한 차이는 없었다.

이상의 결과에서 IGF-I의 투여여부와는 상관없이 치주인대 섬유모세포가 유리하는 물질이 골모세포의 활성을 증가시키는 것으로 나타났으며, IGF-I은 고농도일때만 유의성있게 골모세포 활성을 촉진함을 알 수 있었다. 따라서 이 연구를 통하여 치주인대 섬유모세포가 골모세포활성을 촉진 시키는 작용을 가지고 있음이 확인되었다.

주요 단어 : IGF-I, 골모세포, 치주인대섬유모세포