In vivo assessment of Fibroblast growth factor(FGF)-Fibronectin fusion protein coating on titanium : Histomorphometric analysis in rabbit tibia

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I. Introduction

Physical characteristics of dental implants such as surface chemistry, electric charge, surface texture and porosity can be used to influence bone response in vivo. Other approaches involve treating implants with biologically active substances such as growth factors.

Recently, it was supposed to immobilize biological factors onto an implant surface in order to induce a specific cellular response and promote long-term device integration into bone.

Fibronectin(FN) is defined as an adhesive cell-surface protein. FN is a major glycoprotein in the extracellular matrix(ECM), which can be a ligand for a dozen members of the integrin receptor family. It usually exists as a dimer composed of two nearly identical 250kDa subunits linked covalently near their C-termini by a pair of disulfide bonds, and each monomer composed of three types of repeating units.

Integrins are cell-surface heterodimeric receptors that link the ECM with the intracellular cytoskeleton. FN plays an important role during cell adhesion and osteoblast differentiation, through the interaction with integrin. Therefore FN has been expected to enhance the biological effects of implants, GTR membrane, and bone graft material. Because osseointegration of titanium implants is a biological process that occurs by formation of new periimplant bone in direct contact with the implant surface, cell adhesive property is required for successful implantation[1].

FN has a remarkably wide variety of functional activities besides binding to cell surfaces through integrins. It binds to a number of biologically important molecules that include heparin, collagen/gelatin, and fibrin.

Fibroblast growth factor(FGF) is important signal-

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ing molecule that plays a key role in the control of a variety of biological functions including proliferation, migration, and differentiation. The biological activities of the FGF are mediated through high affinity FGF receptors(FGFRs). Recent reports showing that mutations in FGFRs have been linked to a number of human genetic diseases with severe impairment of bone formation including dwarfism, provide evidence that FGF signaling is important for bone metabolism. By Jang et al(2002), fibroblast growth factor, especially FGF-2, enhances FN-mediated adhesion in human osteoblast-like MG63 cells[4].

Therefore, it is assumed that FN and FGF are the promoting factors for bone formation with synergic effects. The purpose of this study is to evaluate the biological effect of FN and FGF fusion protein coated titanium implant.

II. Materials and Methods

1. Preparation of FN type III 9-10 domains

FN cDNAs were ampilified from adult human cDNA library. PCR primers were designed to recognize type III 9-10 domains as follows. FN9F: 5' -GCTGGTACCGATACCATCATCCCAGCTG-3' FN10R: 5' -GCCAAGCTTATGGTTTGTCAATTTC-3' . The PCR products were cloned into pBAD/His A(Invitrogen, Carlsbad, USA) in-frame with the C-terminal 6X His tag. The FN III 9-10 fusion proteins containing poly-His tag were expressed and purified using a Ni^{2+} affinity column under denaturing conditions according to the manufacturer' s protocol (Invitrogen, Carlsbad, USA).

2. Preparation of FGF-contained FN peptides

Basic fibroblast growth factor(bFGF) were amplified from human cDNAs. PCR primers were designed to recognize bFGF: forward "bF1" primers, 5'- GAGCTCGAGGCAGCCGGGAGCATCACC-3' reverse "bF2" primers, 5'- TTCGAATTCTCAGCTCT-TAGCAGACAT-3'. PCR was performed with 1 min of pre-denaturation at 94℃, 1 min of annealing at 58℃ and 2 min of extension at 72℃. After 35 cycles, amplified cDNAs were digested by BglII and KpnI. After digestion, PCR product were in-frame ligated into the multiple cloning sites of pBAD-His-FN III 9-10 with the C-terminal 6X His tag. The bFGF-FN III 9-10 fusion proteins containing poly-His tag were expressed and purified using a Ni^{2+} affinity column under denaturing conditions according to the manufacturer's protocol (Invitrogen, Carlsbad, USA).

3. Implants

All implants have smooth surface and were custom fabricated for fit in a rabbit tibia model. The main body of the implant had a length of 6 mm and a diameter of 3.5 mm.

Implant fixtures were immersed in a 3.5㎍/ml solution(1:1 ethanol:water) of FN overnight at room temperature in closed containers with gentle mixing. All implants were sterilized in 70 % ethanol and stored for 1 day in sterile PBS prior to surgery.

The FGF-FN fusion protein coated implant were used to test group animals, while implants without surface coating were used to control group animals.

4. Experimental animals and surgical procedure

A total of 12 rabbits weighing 2.5kg were used. They are all physically healthy. They were bred and paired off in the each cage during experimental periods. And they had an adaptation period of one

week before the operation.

After elevating fascial and periosteal flap following skin incision of bilateral iliac bone, we performed fixture-installation on Brånemark protocol with exposure of 2-3 top screws. On each iliac bone, two fixtures were installed 1cm apart. Distal specimens were used to estimate removal torque and basal specimens were used to histometric analysis.

Study was designed to divided into 2 groups , composed of 6 rabbits, and each rabbit had got 4 implant fixtures. After installation, fascial and periosteal membrane were sutured with 4-0 chromic cat-gut(Ethicon Ltd England), and skin sutured with silk material.

5. Measurement of removal torque and specimen preparation

After 4 weeks of fixture installation, experimental rabbits were euthanized. Following flap elevation as stated above, removal torques were estimated from distal specimen of each iliac bone. As soon as cut the iliac bone, it was emerged into 10% neutral formalin solution for 3 weeks, and mesially installed fixtures were cut by 3 mm thickness parallel to fixture long axis of fixtures. Then they were dehydronized and resin-embedded bone embedding solution (Polyscience, Inc. Warrungton, U.S.A).

With a cutting and grinding machine (Exakt Apparatebau, Norderstedt, Germany), thin specimens were made of 15㎛ thickness and observed light microscope (Olympus Co. Tokyo, Japan) after toluidine blue staining.

6. Histomorphometric analysis

Prepared specimens were photographed with digital camera and two parameters, implant bone to implant contact(%), bone formation area(%), were measured with TDI scope program(×40 magnification, 640×480 pixels, Techsan digital imaging, Seoul, Korea).

The bone to implant contact was calculated by a percentage of one-hundredth parts after choosing the successive 3 peaks bone-contacted that is the superlative, measuring peak' s length and the length of the bone attached to the fixture surface. The bone surface was measured at the same area, and the bone formation area was measured by percentage.

7. Statistical analysis

Student t-test was performed to compare the differences between test and control groups were analyzed in the aspect of bone to implant contact(BIC, %), bone formation area(%), and removal torque.

III. Results

1. Bone to implant contact

The light microscope images of both groups show that test group has bone to implant contact of 55.67% and control group has 43.45%(Figure 1,2).

Because BIC means the magnitude of osseointegration, it is known that test group have more osseointegration. It shows significant difference between 2 groups($p<0.05$). The test group has better results(Table 1).

2.Bone formation area

Test group has bone formation area of 78.01%, when control group has 74.82%(Table 1). The bone formation area of test group are more than control group, and it shows statistical significant differences($p<0.05$, Figure 4).

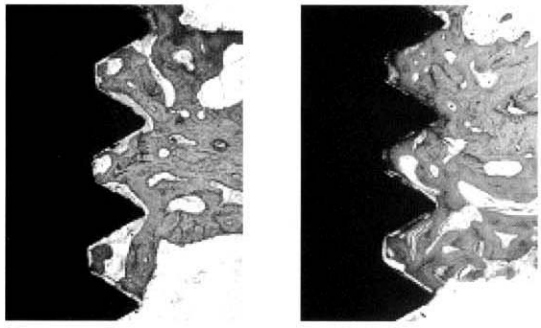

Figure 1. Control group

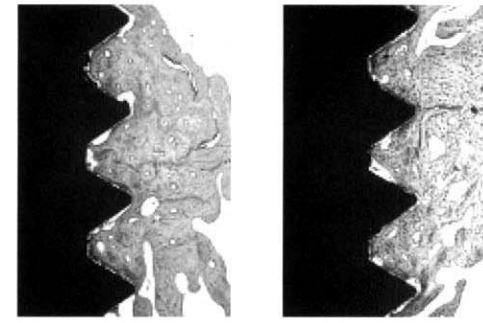

Figure 2. Test Group

Table 1. Statistical analysis (*p <0.05)

	Mean(SD)	
	Control	FGF-FN
Bone to implant contact	43.35(10.26)	55.67(12.77)*
Bone formation area	74.82(8.20)	78.01(10.52)*
Removal torque	4.28(1.72)	4.94(2.05)

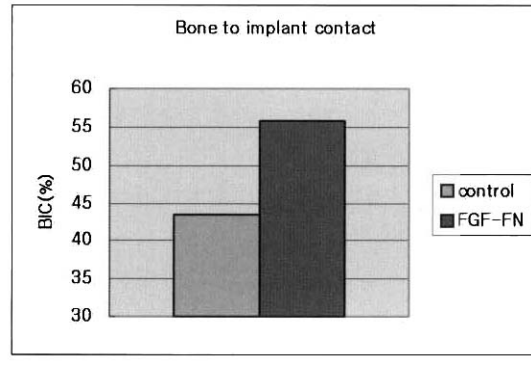

Figure 3. Bone to implant contact

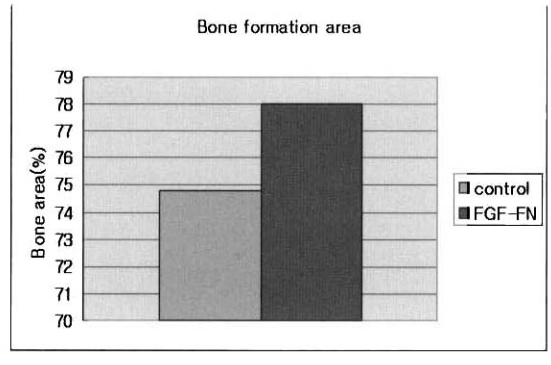

Figure 4. Bone formation area

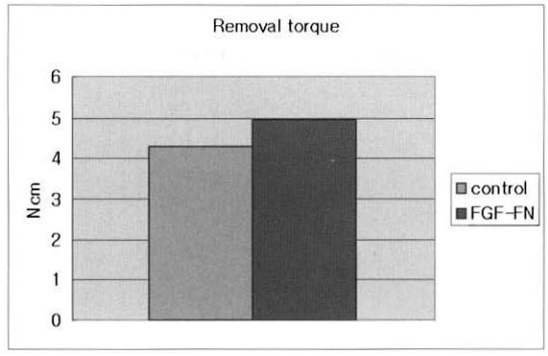

Figure 5. Removal torque

3. Removal torque

After 4 weeks of operation, test group has removal torque of 4.94Ncm, while control group, 4.28Ncm(Figure 5). Between 2 groups, there are no significant difference(p〉0.05, Table 1).

IV. Discussion

Many studies on the dental field have investigated that It is known that the success rate of implant is above about 90%. The clinical data on osseointegrated implants presented by Adell(1981) and Brånemark, et al.,(1981, 1983) regarding success rates have been well received and supported. Adell evaluated 734 osseointegrated implant cases and found a success rate in the maxilla of 88% after one year and 84% after five to twelve years. For the mandible, he found a success rate of 94% after one year and 93% after five to twelve years. Brånemark reported an overall success rate 96.5% for five years, and 81% in the maxilla and 91% in the mandible over 15 years, in 350 cases. At present, over fifty osseointegration implant centers are doing one to six-year longitudinal follow-up studies and are reporting success rates at 90-100% for mandibular cases[19),(20),(21)].

Success rate has been improved by several technologies such as implant morphology, implant type, and implant surface modification, etc. For example, there are impressed tissue-tac texture, diffusion-bonded microsphere interface, grit blasted/acid etched depth structuring, and coatings.

This study shows whether organic coating of implant surfaces with FN and FGF has influence on the early osseointegration and offers evidence that titanium implants coated with small, synthetic peptides stimulate bone formation in vivo. This work supports numerous earlier studies showing positive effects of Arginine-Glycine-Aspartate(RGD) peptides and related peptides and their ability to enhance osteoblasts gene/protein expression, migration and mineralization in culture.

In the previous decade, local application of bioactive polypeptides used to regenerate damaged periodontal tissues has been investigated, and the results have demonstrated that several cytokines are effective for periodontal regeneration. Among them, basic fibroblast growth factor(FGF-2; bFGF) is a potent mitogen for a wide variety of mesoderm- and neuroectoderm-derived cells, and has been shown to be produced in the brain, pituitary gland, kidney, corpus luteum, and adrenal gland. In addition, FGF-2 acts in angiogenesis, neurogenesis, and embryonic

development, indicating its broad range of biological significance. In vivo studies have demonstrated that a topical application of exogenous FGF-2 enhances the healing process of duodenal ulcers, chronic pressure sores, and bone fractures. Furthermore, we recently reported that local application of exogenous FGF-2 enhanced periodontal regeneration following experimental alveolar bone defects in beagle dogs and primates. In those experiments, it was noteworthy that no epithelial downgrowth, ankylosis, or root resorption were observed in the FGF-2 applied sites. This strongly suggests that FGF-2 plays an important role in the process of periodontal tissueregeneration, and that it may also influence or regulate the functions of gingival epithelial(GE) cells, which are important components of periodontal tissue. Although the biological effects of FGF-2 on human PDL cells have been extensively investigated, no reports regarding the effects of FGF-2 on GE cells are known.[11]

First described as fibroblast mitogens extracted from bovine brain and pituitary, basic and acidic FGF(βFGF, αFGF) represent a family of polypeptide factors that have many activities in addition to growth stimulation[22]. Basic FGF, in particular, has the ability to induce all the steps necessary for new blood vessel formation(angiogenesis), both in vivo and in vitro. FGFs have strong affinity for heparin(heparin-binding growth factors) and other anionic molecules and bind avidly to BMs. Basic FGFs is present in the extracts of many organs and is elaborated by activated macrophages, whereas acidic FGF is confined to neural tissues.

Nagai et al. studied low and high dose basic FGF on bone formation. It showed that among 23 FGFs, FGF-2 is an important regulator of osteoblast activity[18]. And FGF-2 enhances FN-mediated cell adhesion of human osteoblast-like MG63 cells[4].

A recent study provided only weak evidence that coating of titanium implants with RGD peptides in the present form and dosage may increase periimplant bone formation in the alveolar process[9]. RGDC peptide coating may enhance titanium rod osseointegration in the rat femur[2]. RGDS promotes minieralization between bone and implants in vitro[7]. And modified titanium surface with FN type III 7-10 domain fragment had favorable biologic effect on osteoblast cells[5].

FGF and FN are the components of extracelluar matrix which play important roles in cell migration, differentiation and proliferation. On coated by them, titanium can lead to improve activities of osteoblasts and osteocyte, and osseointegration. And experimental results support it.

At BIC or bone formation area, there are statistically significant difference between control and test groups. But the removal torques of 2 groups are not statistically different. In 3-4 weeks, no signs of proper osseointegration and after 3 months, high proportion of bone to implant direct contact were reported[23]. Because healing of bony sites may be dense connective tissue and osteoid starts of mineralization at 4th week, the removal torque between 2 groups may not be different.

The study shows that FGF-FN fusion proteins improve osseointegration through acceleration of bone formation following the promotion of amplification of osteoblasts. Therefore, FGF-FN fusion protein can be used as organic surface-coating materials for increasing the early success rate of implants, though clinical significant difference is observed. Further studies of long-term result may have to be verified.

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-국문초록-

섬유아세포 성장인자와 파이브로넥틴 복합 단백질로 처리한 타이태늄의 생물학적 효과 : 가토의 경골을 이용한 조직계측학적 분석

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파이브로넥틴은 세포외기질에 존재하는 당단백질로 세포의 부착, 이동, 성장 및 분화에 관여하며, 섬유아세포 성장인자는 세포의 증식, 이동 및 분화에 영향을 주는 중요한 성장인자로 알려져 있다. 최근 연구에 의하면, 파이브로넥틴은 조골세포의 타이태늄 임플란트 표면으로 이주와 증식 및 골생성을 촉진하며, 섬유아세포 성장인자는 파이브로넥틴에 상승작용을 한다고 보고된 바 있다. 이 실험의 목적은 파이브로넥틴 및 섬유아세포 성장인자의 복합 단백질을 이용하여 타이태늄 임플란트의 골 반응을 알아보는 것이다.

체중 2.5 kg 내외의 건강한 18 마리의 웅성가토를 준비하여 무균 사육하였고, 순수 타이태늄을 절삭가공하여 직경 3.5 mm, 길이 6 mm 의 machined surface를 지니는 screw type 의 임플란트를 준비하였다.

사람의 유전자를 기초로, 유전자 재조합법을 통해, 적절한 primer를 이용하여 얻은 섬유아세포 성장인자를 파이브로넥틴 III 형 분절의 9-10 번 도메인에 결합시켜 얻은 복합 단백질을 준비된 임플란트에 표면처리하여 실험군으로 하였고, 표면처리하지 않은 임플란트를 대조군으로 하여, 가토의 좌우 경골에 각각 2 개씩의 임플란트를 식립하였다.

4주 후, 가토를 희생시켜 각 경골 당 한 개의 임플란트에서 뒤틀림 제거력을 측정하였고 나머지 임플란트 식립 부위에서는 경골을 포함하는 조직표본을 제작하였다. 조직표본상에서 골접촉이 가장 좋은 3 개의 나사산의 길이를 측정하고, 나사와 접촉하는 골의 길이를 측정하여 골-임플란트 접촉도를 구하고, 같은 부위에서 나사산 사이의 면적과 골이 차지하는 면적을 비교하여 골생성률을 얻었다. 실험군과 대조군의 결과는 Student t-test 를 이용하여 신뢰도 95% 수준에서 통계학적 유의성을 검정하였다.

파이브로넥틴과 섬유아세포 성장인자의 복합 단백질로 표면처리된 임플란트와 표면처리를 하지 않은 임플란트는 뒤틀림 제거력에서는 통계적 유의성이 나타나지 않았으나, 골-임플란트 접촉도와 골생성률에서 복합 단백질로 처리된 임플란트가 통계적으로 유의하게 높은 결과를 보였다.

이상의 연구결과로, 섬유아세포 성장인자와 파이브로넥틴 복합 단백질로 처리한 타이태늄 임플란트가 주변 골 형성을 촉진시켜, 골유합을 증진시킴을 알 수 있었다. 따라서, 복합 단백질이 타이태늄 임플란트의 성공률을 높이기 위한 표면개질 물질로 이용될 가능성을 확인할 수 있었다.

주요어 : 섬유아세포 성장인자, 파이브로넥틴, 세포부착, 초기골유합