

Biological Effects of Fibronectin Type III 10 domain on Human Osteoblast-like cells

Chang-Seok Lee¹, Jun-Hyeog Jang², Tae-II Kim^{1,2}, Yong-Moo Lee¹, In-Chul Rhyu¹
Chong-Pyoung Chung^{1,2}, Soo-Boo Han¹, Young Ku^{1,2}

Department of periodontology, College of Dentistry, Seoul National University¹
Intellectual Biointerface Engineering Center, Seoul National University²

I. Introduction

Fibronectin(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 consists of disulfide-linked 235 kDa monomers, which are composed of three domains: i.e., domain types I, II, and III.⁽¹⁾ Integrins are cell surface heterodimeric receptors that link the ECM with the intracellular cytoskeleton. Through the interaction with integrin, FN plays an important role during cell adhesion and osteoblast differentiation.⁽²⁾ Therefore FN has been expected to be applied to the surface of implants, GTR membrane and bone graft materials to enhance their biological effects.^(3,4,5) 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.⁽⁶⁾

Extensive analyses revealed that within the lengthy FN molecule regions involved in cell adhesion there are several specific integrin recognition sequences. It has been reported that the Arg-Gly-Asp (RGD) sequence is located in FN III 10, and the Pro-His-Ser-Arg-Asn (PHSRN) sequence which serves as a synergistic site is located in FN III 9.⁽⁷⁾

Whole natural protein usage have problems such as reduced material availability, immunogenicity, relatively high cost, high molecular weight, enzymatic degradation and sterilization.⁽⁸⁾ Therefore, synthetic small peptides which contain the active binding sequences of the natural protein such as the Arg-Gly-Asp (RGD) sequence and the Pro-His-Ser-Arg-Asn (PHSRN) sequence can be an attractive alternatives.⁽⁹⁾

Recently, it was reported that FN III 7-10, FN III 9-10 synthetic peptides are effective in osteoblast adhesion.^(10,11) Their effects are similar to FN whole

*This work was supported by the Korean Science and Engineering Foundation(KOSEF) through the Intellectual Biointerface Engineering center at Seoul National University

corresponding author : Young Ku, Department of periodontology, College of Dentistry, Seoul National University, 28 Yongon-Dong, Chongno-Ku, Seoul, 110-749, Korea (E-mail : guy@snu.ac.kr)

protein. However, it was also reported that biological activity of RGD is significantly lower than that of the complete FN protein.^(12,13,14,15) Therefore, it is necessary to design optimal peptides which possess comparable biological effects to FN in an attempt to induce favorable cellular activity.

The purpose of this study is to evaluate the biological effect of FN III 10 in the aspects of osteoblastic cell adhesion, cell proliferation, alkaline phosphatase activity, and to compare its activity with FN.

II. Materials and Methods.

1. Preparation of FN Type III 10

Native plasma FN was used (American Peptide Company, Sunnyvale, CA) and FN III 10 was expressed by using recombinant DNA technology. FN cDNAs were amplified from adult human brain cDNAs library. Polymerase chain reaction (PCR) primers were designed to recognize FN III 10 as follows.

Forward : 5'-GCTGGTACCGGCCAACAAATCAACA-3'
Reverse : 5'-TCGGATCCTAAGTTCGGTAAT-TAAT-3'

The thermocycling parameters used in PCR were as follows: annealing, 1min at 55°C; extension, 2min at 72°C; denaturation, 1min at 94°C.

After 30 cycles, amplified cDNA products were digested with *Nde*I and *Bam*HI and separated using PCR purification kit (Qiagen, Chatsworth, CA).

The FN III 10 proteins containing poly-histidine tag were expressed and purified using a Ni²⁺ affinity column under denaturing conditions according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The cell lysates and purified fusion proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining.

2. Cell Culture

HOS (Human osteosarcoma) cell (KCLB, Korea) were maintained in RPMI solution supplemented with 10% fetal bovine serum and 1% gentamycin at 37°C in a 5% CO₂ atmosphere. Each medium was changed every 3-4 days.

3. Peptide coating

The concentration of FN groups used was 0.5 μM. For peptide coating 500 μl solution of FN groups were inserted into each wells, and maintained overnight at 4°C;

At 1 day, 24 well plates were washed with phosphate buffer solution and then 500 μl of cell suspension were seeded onto 24-well plates coated with FN, FN III 7-10, FN III 10. In the control group, RPMI solution was used.

4. Cell adhesion assay

The concentration of cells used for cell adhesion assay was 2.5 × 10⁵/ml. 500 μl of cells in a serum free media were seeded into 24 well plates.

After maintaining cells in a serum free media for 1 hour, washing with 1% phosphate buffer solution, fixation with 3.7% formaldehyde, staining with 1% Crystal Violet were followed.

And then maintaining in room temperature for 24 hours, we observed cells by light microscope and measured optical density at 570nm to determine cell concentration.

5. Cell proliferation assay

The concentration of cells used for cell proliferation assay was 6.5 × 10⁴/ml. 500 μl of cells were seeded into 24 well plates.

Cell proliferation was measured at 4 day. Methyl thiazole sulfate (MTS) which is provided in CellTiter 96^R AQueous One Solution (Promega, USA) was used. By metabolically active cells, MTS is converted into deeply colored formazan product. So the degree of proliferation can be measured by measuring the optical density.

Cell proliferation was measured by manufacturer's protocol. Media was suctioned and 24-well plates were washed with PBS solution. And then media was changed. 100 μ l solution of MTS was added. After 2 hours optical density was measured at 570nm.

6. Alkaline phosphatase activity

ALPase was measured at 6 day by using *p*-nitrophenyl phosphate (*p*NPP) as a substrate. *p*-nitrophenyl phosphate is converted into *p*-nitrophenol by ALPase and it is measured spectrometrically at 405nm. This test was done with the same cells used for the cell proliferation test.

Cell lysis buffer was added to destruct cell membrane. 100 μ l cell suspension was collected and mixed with 50 μ l solution of *p*NPP. After 30 min 2N-NaOH was added to stop the reaction. Optical den-

sity was measured to determine ALPase activity.

7. Statistical analysis

Differences in biological effects of FN groups were analyzed through ANOVA and Tukey's test. $p < 0.05$ was considered statistically significant. Statistical test was performed by SAS program (SAS Institute Inc., Cary, NC)

III. Results

1. Cell adhesion assay

Figure 1 showed the results of LM image of HOS cell attachment on the control surface after 1 hour incubation. Figure 2,3,4 showed the results of LM image of HOS cell attachment on the FN groups coated surface after 1 hour incubation. At 1 hour HOS cells cultured on the FN groups coated surfaces were demonstrated very well spread and more flattened stellate shapes compared to the cells on control surfaces.

FN III 10 promoted osteoblastic cell adhesion and its cell adhesive effect was significantly greater than that of control, while it was similar to FN groups.

Figure 1. LM image of the cell attachment of control after maintaining cells in a serum free media for 1 hour ($\times 100$)

Figure 2. LM image of the cell attachment of fibronectin after maintaining cells in a serum free media for 1 hour ($\times 100$)

Figure 3. LM image of the cell attachment of fibronectin III 7-10 after maintaining cells in a serum free media for 1 hour ($\times 100$)

Figure 4. LM image of the cell attachment of fibronectin III 10 after maintaining cells in a serum free media for 1 hour ($\times 100$)

Figure 5. Cell adhesion at 1 hour

Table 1. Cell adhesion at 1 hour

| | C | FN | FN III 7-10 | FN III 10 |
|------|-------|--------|-------------|-----------|
| Mean | 1,126 | 2,758* | 2,915* | 2,388* |
| SD | 0,015 | 0,085 | 0,008 | 0,095 |

The concentration of adhesive cells in FN groups was statistically greater than that of control after 1 hour incubation (* $p < 0.05$)

Optical density which is measured after maintaining cells in a serum free media for 1 hour is shown in Figure 5. The mean optical density of control group was 1,126. The mean optical density of FN, FN III 7-10, FN III 10 was 2,758, 2,915, 2,388 (Table 1).

Adherent cell concentration in FN groups was statistically greater than that of control after 1 hour incubation. There was no significance between FN

III 10 and FN in the aspect of cell adhesion.

2. Cell proliferation assay

Optical density which is measured at 4 day after treated with MTS solution is shown in figure 6. MTS is converted into visible formazan by metabolically active cells. Afterall, cell proliferation can be mea-

Figure 6. Cell proliferation at 4 days

Table 2. Cell proliferation at 4 days (optical density)

| | C | FN | FN III 7-10 | FN III 10 |
|------|-------|-------|-------------|-----------|
| Mean | 0,519 | 0,576 | 0,560 | 0,547 |
| SD | 0,009 | 0,023 | 0,021 | 0,018 |

Figure 7. ALPase activity at 6 days

Table 3. ALPase activity at 6 days (optical density)

| | C | FN | FN III 7-10 | FN III 10 |
|------|-------|-------|-------------|-----------|
| Mean | 0,105 | 0,110 | 0,110 | 0,109 |
| SD | 0,002 | 0,002 | 0,002 | 0,003 |

sured by measuring optical density. The mean optical density of control group was 0,519. The mean optical density of FN, FN III 7-10 and FN III 10 was 0,576, 0,560, and 0,547 (Table 2)

At 4 day, cells treated by FN groups proliferated better than control group, however the difference

was not statistically significant. Cell proliferation level of FN and FN 10 groups was similar.

3. ALPase activity assay

Optical density which is measured at 6 day after

treated with *p*-nitrophenyl phosphate is shown in figure 7. The mean optical density of control group was 0.105. The mean optical density of FN, FN III 7-10, and FN III 10 was 0.110, 0.110, and 0.109. (Table 3).

At 6 day ALPase activity in FN groups was greater than that of control. But that difference was not statistically significant. The level of ALPase activities of FN and FN 10 groups was similar.

IV. Discussion

Since FN was reported by Morrison *et al.* in 1948, extensive analyses were performed for its interesting cell adhesive properties.⁽¹⁶⁾ And its potential application to implants, bone graft materials were evaluated in lab test and animal studies.^(3,4,5) It has been reported that bioactive glass promoted osteoblast-like cell adhesion, while unreacted glass, amorphous calcium phosphate, and synthetic hydroxyapatite didn't. Improved adhesion to reacted bioactive glasses resulted from enhanced cell receptor-FN interactions.⁽³⁾ It has been reported that coating of titanium implants with RGD peptides may increase peri-implant bone formation.⁽⁴⁾ Also It has been reported that RGDC peptide coating may enhance titanium rod osseointegration in the rat femur.⁽⁵⁾

FN is involved in cellular adhesion, cellular spreading, cytoskeletal organization, hemostasis, cellular migration, oncogenic transformation, embryonic differentiation, phagocytosis.⁽¹²⁾ FN interact various molecules such as fibrin, heparin, collagen and etc.⁽¹³⁾ Therefore whole natural proteins may cause some biological side effects. Peptides of diminished size, but similar effects is preferred.

Many researchers have reported that biological activity of RGD is significantly lower than that of the complete protein.^(12,13) It has been supposed that FN can stimulate cell growth by RGD- independent

mechanisms.⁽¹⁸⁾ Peptides which are smaller than FN but bigger than RGD were needed. Therefore we choosed FN type III 10 because it includes RGD motifs and its size is smaller than that of FN type III 7-10 or FN type III 9-10 of which effect is proven to be similar to the complete protein.^(10,11)

HOS cells were chosen for this study, for they have shown to be similar to osteoblasts in many respect.⁽¹⁹⁾ In this study, we found that FN III 10 has a similar effect to that of other FN groups in cell adhesion, cell proliferation, ALPase activity. The effect of FN III 10 on cell adhesion was significantly better than that of control. And its effect on cell proliferation, ALPase activity was better than that of control, but that difference was not significant. The possible reason is that FN is involved in early healing. It is effective in cell adhesion, but its effect is decreased as cell proliferate. Delvin, reported that the function of FN may be important in granulation tissue formation, by providing a template matrix for fibroblasts migration.⁽²⁰⁾ Greiliny *et al.* reported that after injury, the wound space is filled with a fibrin/fibronectin clot containing growth factors released by platelets and monocytes. In response to these factors, fibroblasts migrate into the fibrin clot and contribute to the formation of granulation tissue.⁽²¹⁾

We think the result of this study is encouraging. FN type III 10 has a smaller size but similar effect to that of other FN groups. Further study is recommended to identify the effect of specific sequences in FN type III 10.

V. Conclusion

1. FN III 10 showed significant osteoblastic cell adhesion effect compared to control, however, it revealed no statistical significance compared to FN and FN III 7-10.
2. Regarding cell proliferation and ALPase activity,

FN III 10 showed similar extent to FN and FN III 7-10. However, there exists no statistical significance between control and FN groups.

VI. References

1. Petersen T, Thgeren HC, Skorstengaard K, VibePedersen K, Sahl P, SottrupJensen L, Magnusson S: Partial Primary structure of bovine plasma fibronectin : three types of internal homology. *Proc Natl Acad Sci*. 1983;80: 137-141
2. Hynes RO: *Fibronectins*, Springer-Verlog 1990:1-6
3. Garcia AJ, Ducheyne P, Boettiger D: Effect of surface reaction stage on fibronectin- mediated adhesion of osteoblast- like cells to bioactive glass. *J Biomed Mater Res* 1998;40:48-56
4. Schliephake H, Schamweber D, Dard M, Rosler S, Sewing A, Meyer J, Hoogestraat D: Effect of RGD peptide coating of titanium implants on periimplant bone formation in the alveolar crest. An experimental pilot study in dogs. *Clin Oral Implants Res* 2002; 13:312-319
5. Ferris DM, Moodie GD, Diamond PM, Gioranni CWD, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation in vivo. *Biomaterials* 1999;20:2323-2331
6. Albrektsson T: Direct bone anchorage of dental implants. *J prosthet Dent* 1983;50:255-261
7. Pankov R, Yamada KM, Fibronectin at a glance, *Journal of Cell Science* 2002, 115, 3861-3863
8. Grzesik WJ, Ivanov B, Robey FA, Southerland J, Yamauchi M: Synthetic Integrin-binding peptides promote adhesion and proliferation of human periodontal ligament cells in vitro. *J Dent Res* 1998; 77: 1606-1612
9. Kantlehner M, Schaffner P, Finsinger D, Meyer J, Jonczyk A, Diefenbach B, Nies B, Holzemann G, Goodman S, Kessler H: Surface coating with cyclic RGD peptides stimulates osteoblast adhesion and proliferation as well as bone formation. *Chembiochem* 2000;1: 107-114
10. Nishizaka T, Shi Q, Sheetz MP: Position-dependent linkages of fibronectin-integrin-cytoskeleton. *Proc Natl Acad Sci USA*. 2000; 18: 97(2) 692-697
11. Kim TI, Jang JH, Lee YM, Ryu IC, Chung JP, Han SB, Choi SM, Ku Y: Design and biological activity of synthetic oligopeptides with Pro-His-Ser-Arg-Asn (PHSRN) and Arg-Gly-Asp (RGD) motifs for human osteoblast-like cell (MG-63) adhesion. *Biotechnology Letters* 2002; 24:2029-2033
12. Pierschbacher MD, Hayman EG, Ruoslahti E. Synthetic peptide with cell attachment activity of fibronectin. *Proc Natl Acad Sci USA* 1983; 80:1224-7
13. Akiyama SK, Aota S, Yamada KM. Function and receptor specificity of a minimal 20 kilodalton cell adhesive fragment of fibronectin. *Cell Adhes Commun* 1995 ;3:13-25
14. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *J Cell Sci* 1997;110:2187-96
15. Globus RK, Doty SB, Lull JC, Holmuhamedov E., Humphries MJ, Damsky CH. Fibronectin is a survival factor for differentiated osteoblast. *J Cell Sci* 1998; 111: 1385-1393
16. Hynes RO: *Fibronectins*. Springer-Verlag 1990:1-6
17. Kenneth C. Ingham. [http://c.edu/biochem/ingham/fnp age.htm](http://c.edu/biochem/ingham/fnp%20age.htm)
18. Sottile J, Hocking DC, Langenbach KJ. Fibronectin polymerization stimulates cell growth by RGD-dependent and -independent mechanisms. *J Cell Sci*, 2000; 113: 4287-4299
19. Clover J, Gowen M: Are MG-63 and HOS TE85

- human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone 1994;15 :585-591
20. Delvin H. Early bone healing events following rat molar tooth extraction, Cells Tissues Organs, 2000;167:33-7
21. Greiliny D, Clark RA. Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix, J Cell Sci, 1997 ;110:861-70

Fibronectin type III 10 도메인이 조골양 세포에 미치는 생물학적 영향

이창석¹, 장준혁², 김태일^{1,2}, 이용무¹, 류인철¹, 정종평^{1,2}, 한수부¹, 구 영^{1,2}

서울대학교 치과대학 치주과학 교실¹
서울대학교 지능형 생체계면 공학 센터²

1. 연구 목적

Fibronectin은 세포외기질의 주요 성분인 거대 당단백질로서, 조골세포의 부착과 증식 및 이동능에 중요한 역할을 담당한다고 알려져 있다. 이러한 fibronectin의 조골세포에 대한 영향을 실제 임상에 적용하기 위해서, 전체 fibronectin 단백질질을 사용하는 것은 면역학적으로나 경제적으로 많은 단점을 안고 있어서, 유효한 반응단위만을 추출하여 활용하는 것이 바람직한 방법으로 알려져 있다. 이 연구의 목적은 세포부착에 주로 관여하는 fibronectin type III분절 중 10번 도메인이 조골양 세포에 미치는 영향을 전체 fibronectin 단백질과 fibronectin type III 7-10 도메인 분절과 비교, 관찰하는 것이다.

2. 연구 방법

사람의 fibronectin을 기초로 한 적절한 primer로서, 유전자 재조합법을 이용하여 fibronectin type III 10 도메인과 fibronectin type III 7-10 도메인 분절을 얻었으며, 전체 fibronectin분자는 상용으로 준비하여 24-well 세포배양 용기에 도포하였다. 배양된 조골양세포(HOS cell)를 1×10^5 cells/well의 농도로 각 well에 분주하여 37°C에서 1시간 배양을 하였다. Cell adhesion assay를 실시하기 위해 10% formaldehyde로 고정시키고 1% Crystal Violet으로 염색하여 광학현미경을 관찰 후 2% SDS를 처리하여 microplate reader기를 이용하여 570nm에서 혼탁정도를 측정하였다. 음성대조군으로는 RPMI 용액을 사용하였다. 동일한 방법을 이용하여 준비된 35mm²배양접시에 HOS cell을 37°C에서 4일간 배양 후, MTS assay를 이용하여 세포 증식도에 미치는 영향을 관찰하였다. 6일째 405nm에서 활성화된 세포에서 분비된 p-nitrophenol을 이용한 alkaline phosphatase activity를 측정하였다.

3. 결과 및 고찰

Fibronectin type III 10 도메인은 HOS cell에 대한 생물학적인 효과면에서, 전체 fibronectin 분자 및 fibronectin type III 7-10 분절과 통계적으로 유사한 세포부착도를 보여주었으며, 세포증식도와 alkaline phosphatase 활성도면에서도 큰 차이가 나타나지 않았다.

이상의 연구결과로 볼 때, fibronectin type III 10 도메인이 조골세포의 증식을 목적으로 사용하는 생체재료의 표면개질 부착물질로 응용할 수 있는 가능성이 있다고 하겠다.