

Chitosan/Calcium

Metaphosphate

1 . 1,2 . 1 . 3 . 3 . 4
1 . 1 . 1 . 1 . 1

1 , 2
3 , 4

I. 14.

가 .

가 ,

가

15.

가 , 가 , 가 가

. 가 , 가 , 가

가

가

가

2-4.

가

가

5-13,

가 , clonal osteogenic cell line MC3T3 - E1

3 1 matrix

56

16, 3 poly (lactide/gly -
colide) /hydroxy apatite(PLAGA/HA) poly -

*

(HMP - 99 - E - 10 - 0003)

mer matrix

PLAGA/HA matrix

가

Klokkevold 40 chitosan

39.

17. 3 poly (DL - lactide - co - glycolic acid) foam

Chitosan

가

18.

가

41 - 43 ,

44 ,

46 - 49

45

chitosan

. Chitosan

19 - 24 .

. Boyko 25

minocycline

chitosan

50 ,

Chitosan

41 .

26 ,

27,28 .

3

metaphosphate (CMP) phosphate

calcium monocalcium

chitosan



. Chitosan

가

가가

CMP

29

30,31

32가

Chitosan

51,52 . 가

CMP

33 ,

34,35 .

chitosan

CMP

53 ,

. Chitosan

hydroxyapatite beta - tricalcium

가

phosphate가

54 .

Baksh

55

36 -

CMP block

38 , chitosan

hydroxyapatite

가
 ,
 .
 2
 sulfide bond
 28 - 35KDa . 2
 PDGF polypeptide가
 56%
 ,
 3가
 PDGF - AA, PDGF - BB
 PDGF - AB가 56 - 58.
 PDGF - BB 59 PDGF
 IGF - 1 가 60.
 PDGF , 61,
 , 가
 62,
 가 63.
 chitosan .
 3
 가 chitosan CMP
 PDGF - BB loaded chitosan/CMP
 3
 가 가 .

Ca(H₂PO₄)₂ condensation
 Ca(PO₃)₂
 CMP . Chitosan
 (Showa Chemicals, Tokyo, Japan) 5g
 100ml 1% acetic acid chitosan
 . 3g CMP 100ml
 chitosan
 tripolyphosphate
 3 가
 chitosan/CMP
 PDGF - BB(Genzyme Co. Cambridge,
 USA)
 5 x 5 x 2mm 200ng
 PDGF - BB가 chitosan/CMP
 .
 (Jeol, USA)
 2. PDGF - BB
 Chitosan/CMP PDGF - BB
¹²⁵I - labeled
 PDGF - BB . 5 μ Ci ¹²⁵I -
 labeled PDGF - BB non - radioactive
 PDGF - BB radioactivity가 1
 μCi가 200ng
 . pH 7.4 phosphate
 buffer가 glass vial 15rpm, 37°C
 shaking water bath 4
¹²⁵I - labeled PDGF - BB
 gamma counter(Cobra II, Packard
 Instrument Company, CT, USA)

II.

1. Chitosan/CMP PDGF - BB loaded
 chitosan/CMP

1)

Bellows 64 ,

21

가

0.1% collagenase(Sigma, St Louis, MO, USA) 4mM EDTA 0.25% trypsin(Gibco, Grand Island, NY, USA) 1.5ml 37°C

가 Reacti - vial(Pierce, Rockord, IL, USA) 10, 20, 30, 50 70

ice - cold fetal bovine serum(FBS; Gibco) 50 70 10

Hank's balanced salt solution(HBSS; Gibco) 4 x 10⁴ cells/cm² 35mm

10% FBS 1% antibiotic - antimycotic solution (Gibco) - minimum essential medium(- MEM; Gibco) 37°C 100% 95%

5% CO₂

2) 가 가

4mM EDTA 0.25% trypsin

10 15%

FBS, 1% antibiotic - antimycotic solution, 10mM Na - glycerol phosphate(Sigma), 50µg/ml I - ascorbic acid(Sigma) 10⁻⁷M dexamethasone(Sigma) - MEM 5 x 10⁷ cells/ml 30

chi -

tosan/CMP PDGF - BB loaded chitosan/CMP 24 - well plates(Nunc, Rochester, NY, USA) , 20µl

10⁶ cells 가 가 3

well 가 1ml 2 - 3

3) 1, 7, 14, 28 well

HBSS 가 4mM EDTA 1ml 0.25% trypsin 5

HBSS 2

trypsin/HBSS hemo cytometer

4) (alkaline phosphatase, ALPase) 1, 7, 14, 28

0.5ml 가 1 0.1ml 0.1ml

0.1M glycin - NaOH buffer, 0.1ml 15mM para - nitrophenol phosphate(PNPP), 0.1% Triton X - 100 0.1ml 30 37°C 2.5ml 0.1N NaOH

가 ELISA reader 405nm

nitrophenol(PNP) para - PDGF - BB loaded chitosan/CMP
 1 x 1 x 1 mm
 5) - 4 - 0 5 - 0 silk(Ethicon, Somerville, NJ, USA)
 2 4 6
 glutaraldehyde 2)
 5 μm hema - 10%
 toxylin - eosin 5% trichloroacetic acid
 (Olympus BH - 2, Olympus Optical Co., Osaka, Japan) 5 μm
 Masson trichrome
 3
 4. system(Image Access Application, Bildanalys System AB, Stockholm, Sweden)
 video camera
 1) 250g Sprague - Dawley 5.
 36) 30mg/kg ketamine(Ketarar 6
 0.5% chlorhexidine ±
 U.S.A.) SPSS 8.0(SPSS,
 1% ANOVA 5%
 Tukey
 8mm trephination drill(3i III.
 Implant Innovation, Palm Beach Gardens, FL, USA) 1.
 65.
 chitosan/CMP chitosan/CMP PDGF - BB loaded

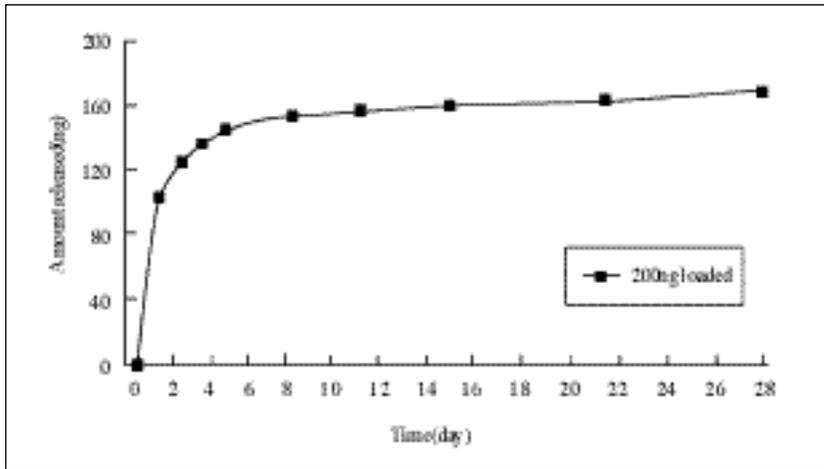


Figure 2. Release kinetics of ¹²⁵I - labeled PDGF - BB from chitosan/CMP sponge

Table 1. Cell proliferation in chitosan/CMP and PDGF - BB loaded chitosan/CMP sponge

Day	Number of cells (×10 ⁴ /sponge)	
	chitosan/CMP sponge	PDGF - BB loaded chitosan/CMP sponge
1	2.96 ± 0.73	4.22 ± 0.91#
7	7.36 ± 2.30**	9.84 ± 0.96***
14	9.98 ± 1.38**	13.59 ± 1.88***#
28	10.64 ± 1.96**	15.11 ± 0.95***#

** : P<0.01, as compared with 1 day in each group

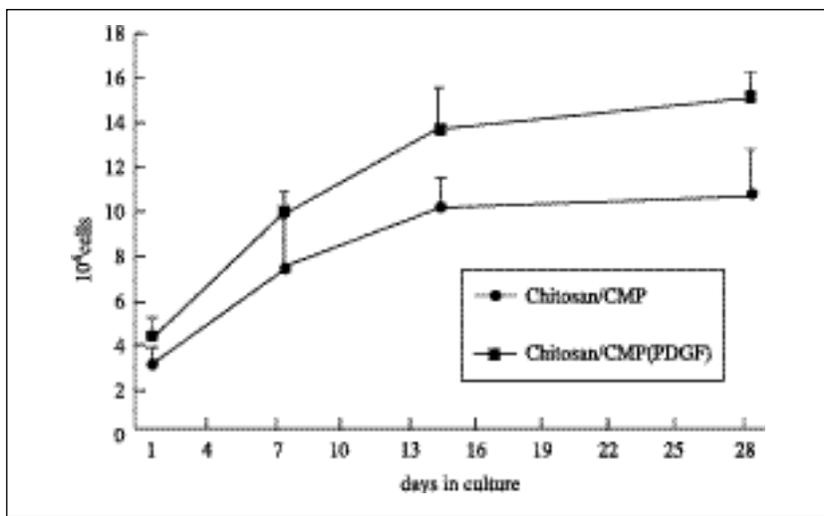


Figure 3. Cell proliferation in chitosan/CMP and PDGF - BB loaded chitosan/CMP sponge

chitosan/CMP (figure 1). 3 (Figure 2). 200ng PDGF - BB 100ng 5ng

가 100 - 200 μ m . 1 가 2 1 -

2ng PDGF - BB가 3. -

2. PDGF - BB

가 PDGF - BB 1 1) Chitosan/CMP PDGF - BB loaded chi - tosan/CMP

Table 2. ALPase activities of cells in chitosan/CMP and PDGF - BB loaded chitosan/CMP sponge

Day	ALPase activity (nM of PNP/30min/10 ⁴ cells)	
	chitosan/CMP sponge	PDGF - BB loaded chitosan/CMP sponge
1	3.71 ± 0.87	4.92 ± 0.52 [#]
7	5.61 ± 1.39 [*]	6.95 ± 1.22 ^{**}
14	7.19 ± 1.33 ^{**}	8.01 ± 2.17 ^{**}
28	7.84 ± 0.98 ^{**}	7.90 ± 1.77 ^{**}

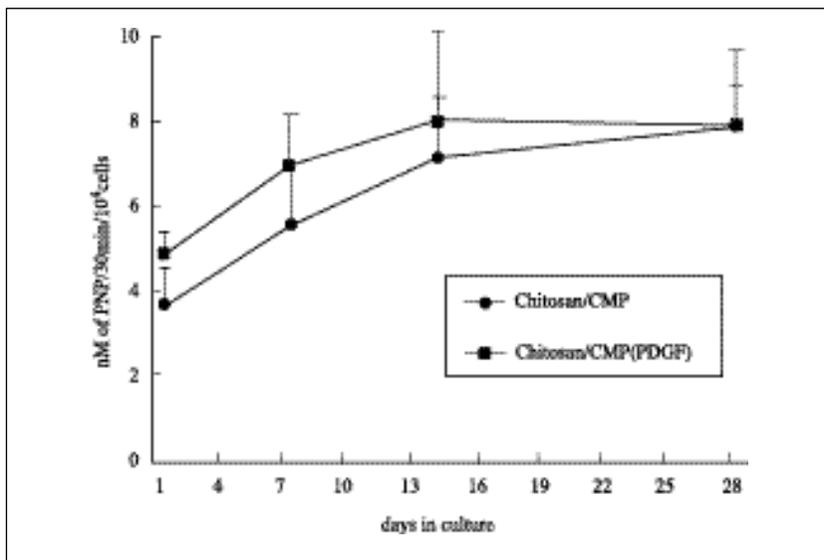


Figure 4. ALPase activities of cells in chitosan/CMP and PDGF - BB loaded chitosan/CMP sponge

Table 3. Area of new bone in rat calvarial defect

weeks	area of new bone ($\times 10^{-3}/\text{mm}^2$)		
	control	chitosan/CMP sponge	PDGF - BB loaded chitosan/CMP
2	139.33 \pm 41.06	269.83 \pm 78.43**	456.00 \pm 65.89***
4	251.67 \pm 92.03	518.17 \pm 100.30**	706.33 \pm 146.94***

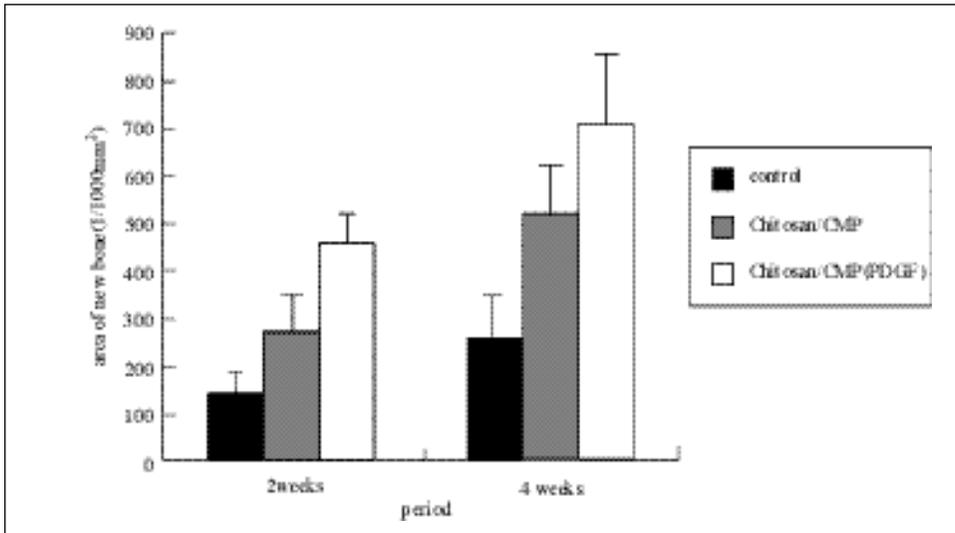


Figure 11. Area of new bone in rat calvarial defect

hemocytometer

28
(Table 1, Figure 3).

chitosan/CMP	1	Chitosan/CMP	PDGF - BB loaded chi -
chitosan/CMP	2.96 $\times 10^4$	14	tosan/CMP
cells/sponge	4.22 $\times 10^4$	가	가
		가	
	28	10.64	PDGF - BB loaded chitosan/CMP
$\times 10^4$	15.11 $\times 10^4$ cells/sponge	가	14
			가
가	14		(Table 2, Figure 4).
	가	가	
28	가	1	chitosan/CMP
	PDGF - BB loaded chi -	chitosan/CMP	PDGF - BB loaded
tosan/CMP	tosan/CMP	4.92nM / 30min / 10^4 cell	3.71

14 cell 7.19 8.01nM / 30min / 10⁴ PDGF - BB loaded chi - tosan/CMP 가 chi - tosan/CMP 1

가 loaded chitosan/CMP PDGF - BB (Figure 10), 4

3)

chitosan/CMP

(Figure 5, 6). 14 가 7

가 PDGF - BB loaded chitosan/CMP

(Figure 7), PDGF - BB loaded chitosan/CMP 가 가 4 가

(Table 3, Figure 11), 2 4 , PDGF - BB loaded chitosan/CMP PDGF - BB unloaded chitosan/CMP

(Figure 8).

4. Chitosan/CMP PDGF - BB loaded chitosan/CMP 가

IV.

2 4 2 (Figure 9),

3 가 가 chitosan/CMP PDGF - BB loaded chitosan/CMP . Chitosan 29 30,31 32가

3 66.

Chitosan/CMP

chitosan

, chitosan

hydroxyapatite phosphate가 beta - tricalcium phosphate가 17,66

36 - 39

chitosan

55 CMP

가

ascorbic acid, - glycerophosphate dex - amethasone 가

Bellows 64 가

63. 가 가

가 4.2 96% PDGF - BB 가 96

acid가 ascorbic

PDGF - BB 가 - glycerophosphate Dexamethasone

chitosan 67 PDGF

200ng PDGF - 가

BB loaded chitosan/CMP 68. dexamethasone PDGF

24 50% 69

PDGF - BB가 , 2 가 가 dexamethasone

1 - 2ng PDGF

PDGF - BB가

osteogenic cell line 가 clonal 가

가 3

3 가 ⁶⁴ spinner flask

3 가

가 ⁷⁰ 가

tosan/CMP PDGF - BB loaded chi - chitosan/CMP static

가 , PDGF - BB dynamic 가 static, dynamic bioreactor 가

가 , PDGF - BB ⁷² dynamic bioreactor 가

가 ^{66,71} 가 10^6 cells/cm^3

가 ⁷³ static 10^6

가 spinner flask

가 , - 가 가가 ,

가 , - 가 PDGF - BB loaded chi - tosan/CMP chitosan/CMP

가 가

spinner flask

가
 PDGF - BB loaded chitosan/CMP
 chitosan/CMP
 PDGF - BB unloaded
 chitosan/CMP

2
 PDGF - BB loaded
 가 PDGF - BB unloaded
 chitosan/CMP
 가
 PDGF - BB
 loaded chitosan/CMP
 PDGF - BB loaded
 chitosan/CMP

25 - 28

, PDGF - BB loaded chi -
 tosan/CMP 가

3

가

VI.

가

가

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V.

3

chitosan/CMP

PDGF - BB loaded chitosan/CMP

. PDGF - BB loaded

chitosan/CMP

PDGF - BB

1

가

, 2

1 - 2ng PDGF - BB가

가

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(1)

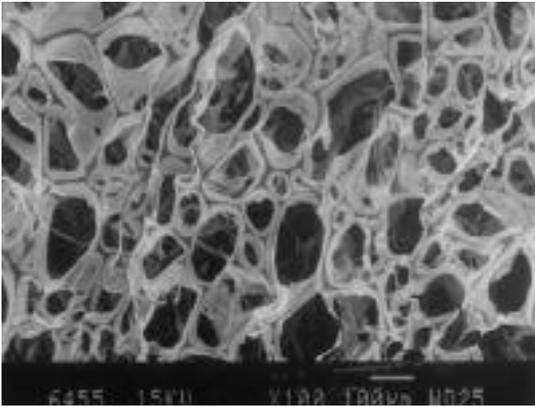


Figure 1

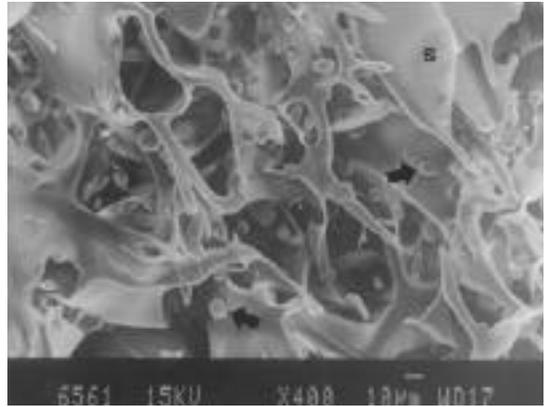


Figure 5A

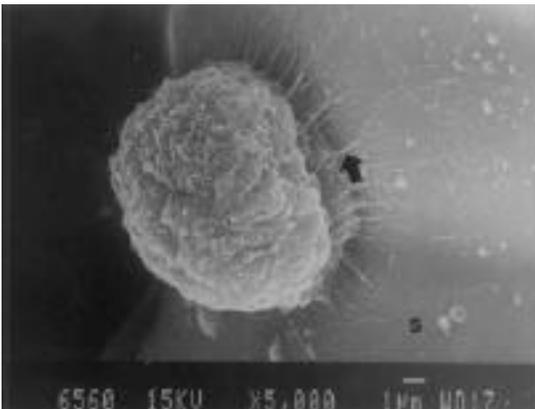


Figure 5B

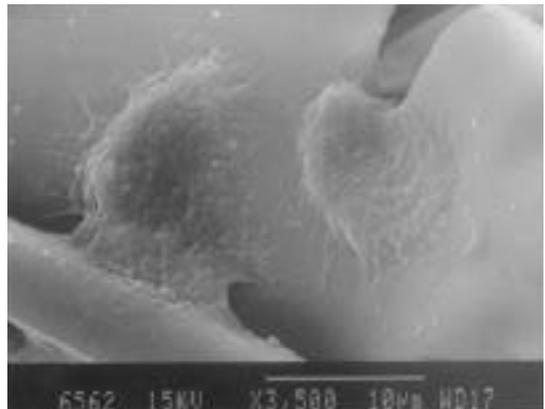


Figure 5C



Figure 6A

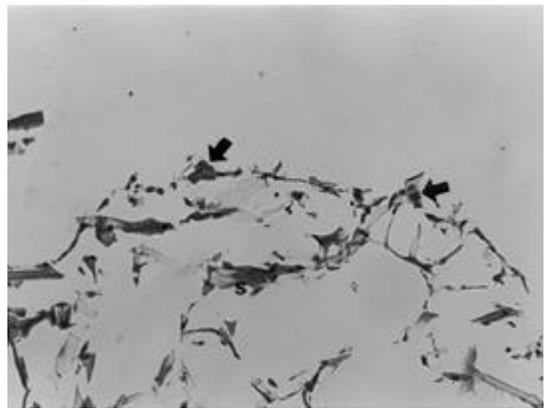


Figure 6B

(II)



Figure 7A

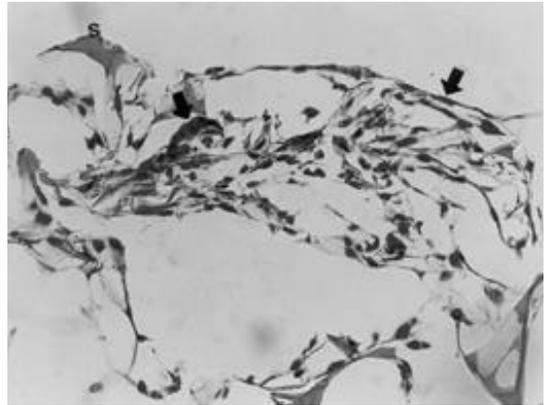


Figure 7B



Figure 8A

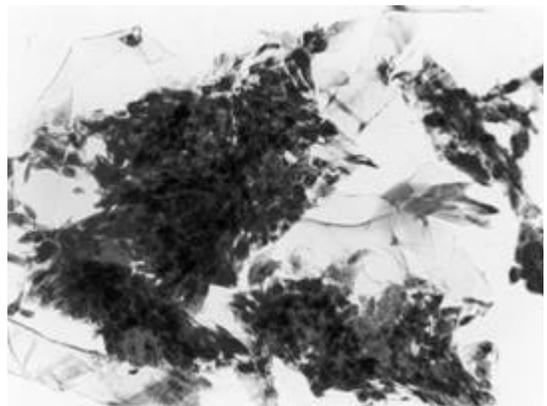


Figure 8B

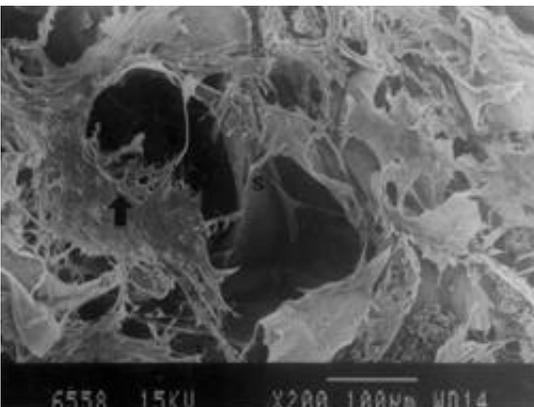


Figure 8C

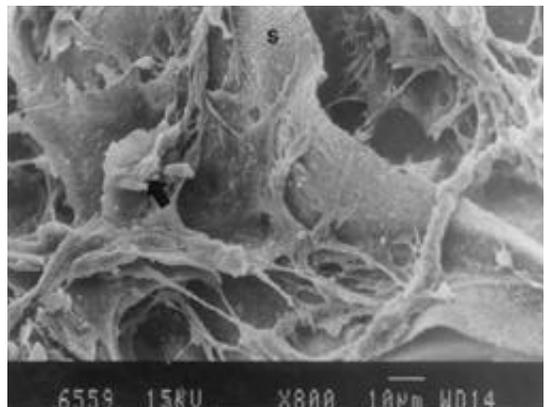


Figure 8D

(III)

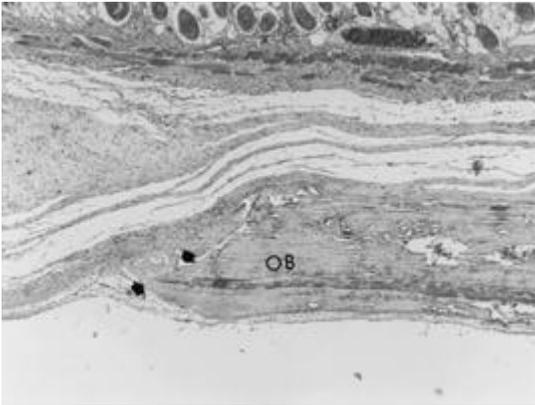


Figure 9A



Figure 9B

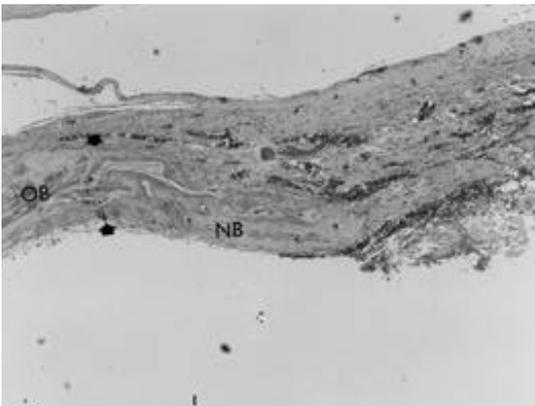


Figure 9C

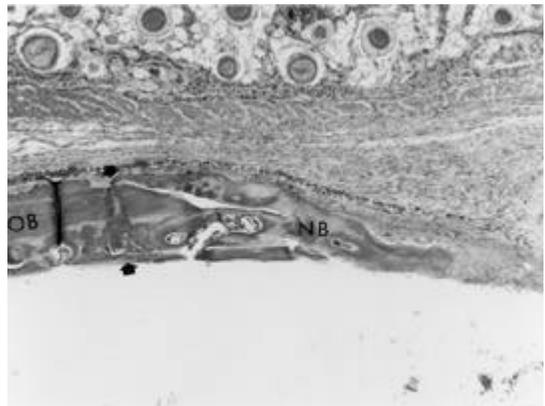


Figure 10A

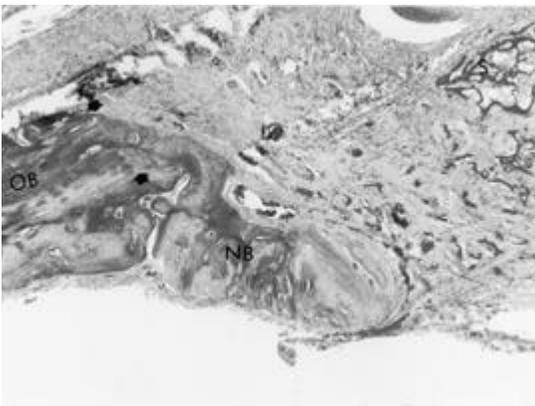


Figure 10B

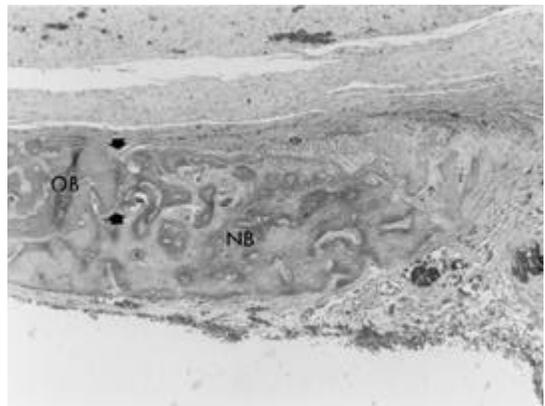


Figure 10C

- odontal regeneration. *J Clin Periodontol* 1989;16:545 - 548.
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Figure 1. Scanning electron micrograph of PDGF - BB loaded chitosan/CMP sponge x100. The sponge exhibits a 3 - dimensional porous structure and the pore size is 100 - 200 μm .

Figure 5. Scanning electron micrograph of osteoblastic cells cultured for 1 day. A: Seeded cells (arrow) attach to the PDGF - BB loaded chitosan/CMP sponge (S). x400, B: early stage of cell attachment x5000 (Arrow indicates cell process, S sponge), C: late stage of cell attachment x3500.

Figure 6. Cross sectional views through three - dimensional cultures of osteoblastic cells cultured for 7 days. Seeded cells (arrow) well attach to the sponges (S). A: chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100, B: PDGF - BB loaded chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100.

Figure 7. Cross sectional views through three - dimensional cultures of osteoblastic cells cultured for 14 days. Seeded cells (arrow) firmly attach and proliferate to form multilayer of cells on the sponges (S). A: chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100, B: PDGF - BB loaded chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100.

Figure 8. Cross sectional views through

three - dimensional cultures of osteoblastic cells cultured for 28 days. Seeded osteoblastic cells form nodules of bone - like matrix on the sponges. A: chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100, B: PDGF - BB loaded chitosan/CMP sponge, hematoxylin and eosin stain, original magnification x100, C: Scanning electron micrograph of mineralized matrix on the PDGF - BB loaded chitosan/CMP sponge x200, D: Scanning electron micrograph of mineralized matrix on the PDGF - BB loaded chitosan/CMP sponge x800. (Arrow indicates mineralized matrix, S sponge)

Figure 9. Histologic findings of calvarial defect sites at 2 weeks. A: Ungrafted control, figure illustrates one side of defect. Defect is filled with loose fibrous connective tissue, and there is no significant sign of new bone formation. Masson trichrome stain, original magnification x10. B: Defect grafted with chitosan/CMP sponge, figure illustrates one side of defect. Osseous tissues are growing from the edges of the defect. Masson trichrome stain, original magnification x10. C: Defect grafted with PDGF - BB loaded chitosan/CMP sponge, figure illustrates one side of defect. Significant regenerated new bone infiltrates from the edges of the

defect.. Masson trichrome stain, original magnification x5. (Arrow indicates original border of defect, NB new bone, OB calvarial bone, S sponge)

Figure 10. Histologic findings of calvarial defect sites at 4 weeks. A: Ungrafted control, figure illustrates one side of defect. Limited bone repair localized to the defect border. Masson trichrome stain, original magnification x10. B: Defect grafted with chitosan/CMP sponge, figure illustrates one side of defect. The bone regeneration is increasing from the defect border. Masson trichrome stain, original magnification x10. C: Defect grafted with PDGF - BB loaded chitosan/CMP, figure illustrates one side of defect. The bone regeneration is increasing from the defect border, the amount of regenerated bone is larger than that of chitosan/CMP sponge. Masson trichrome stain, original magnification x10. (Arrow indicates original border of defect, NB new bone, OB cal-

varial bone, S sponge)

- Abstract -

The Effect of Platelet Derived Growth Factor - BB Loaded Chitosan/Calcium Metaphosphate on Bone Regeneration

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Chitosan is a biodegradable natural polymer that has been demonstrated its ability to improve wound healing, and calcium metaphosphate(CMP) is a unique class of phosphate minerals having a polymeric structure. In this study, chitosan/CMP and platelet derived growth factor(PDGF - BB) loaded chitosan/CMP sponges were developed, and the effect of the sponges on bone regeneration and their possibility as scaf-

folds for bone formation by three - dimensional osteoblast culture were examined.

PDGF - BB loaded chitosan/CMP sponges were prepared by freeze - drying of a mixture of chitosan solution and CMP powder, and soaking in a PDGF - BB solution. Fabricated sponge retained its 3 - dimensional porous structure with 100 - 200 μm pores. The release kinetics of PDGF - BB loaded onto the sponge were measured in vitro with ^{125}I - labeled PDGF - BB. In order to examine their possibility as scaffolds for bone formation, fetal rat calvarial osteoblastic cells were isolated, cultured, and seeded into the sponges. The cell - sponge constructs were cultured for 28 days. Cell proliferation, alkaline phosphatase activity were measured at 1, 7, 14 and 28 days, and histologic examination was performed. In order to examine the effect on the healing of bone defect, the sponges were implanted into rat calvarial defects. Rats were sacrificed 2 and 4 weeks after implantation and histologic and histomorphometrical examination were performed.

An effective therapeutic concentration of PDGF - BB following a high initial burst release was maintained throughout the examination period. PDGF - BB loaded chitosan/CMP sponges supported the proliferation of seeded osteoblastic cells as well as their differentiation as indicated by high alkaline phosphatase activities. Histologic findings indicated that seeded osteoblastic cells well attached to sponge matrices and proliferated in a multi - layer fashion. In the experiments of implantation in rat calvarial defects, histologic and histomorphometric

examination revealed that chitosan/CMP sponge promoted osseous healing as compared to controls. PDGF - BB loaded chitosan/CMP sponge further enhanced bone regeneration.

These results suggested that PDGF - BB loaded chitosan/CMP sponge was a feasible scaffolding material to grow osteoblast in a three - dimensional structure for transplantation into a site for bone regeneration.

Key Words : c h i t o s a n / c a l c i u m metaphosphate sponge, platelet derived growth factor, three dimensional scaffold, bone regeneration