Tissue Engineering and Regenerative Medicine, Vol. 5, No. 4~6, pp 811-820 (2008)

| Original Paper |

Anorganic Bone Mineral Coated with Tetra-Cell Adhesion Molecule Enhances the Activation of Osteoblast-Like Cells

Jin-Woo Park[1], Seung-Hoon Kang[1], Shin-il Yeo[1], In-San Kim[2], Yong-Chul Bae[3] and Jo-Young Suh[1,4*]

[1]*Dept of Periodontology, School of Dentistry,* [2]*Dept of Biochemistry, School of Medicine,*
[3]*Dept of Oral Anatomy & Neurobiology College of Dentistry,*
[4]*IHBR, Kyungpook Nat'l Univ, 188-1, Samdeok 2Ga, Jung-Gu, Daegu, 702-412, Korea,*

(Received: Nov. 3rd, 2008; Accepted: Nov. 8th, 2008)

Abstract : The construction of a biomimetic environment for tissue engineering, and for implants and grafts should take into account the channels through which chemical and biomechanical signals that contribute to differentiation and morphogenesis are conducted. The most commonly used peptide for surface modification is arginine-glycine-aspartic acid(RGD), the signaling domain derived from fibronectin(FN). The proline-histidine-serine-arginine-asparagine(PHSRN) sequence serves as a synergistic site that enhances the binding affinity of the RGD sequence. Also ßig-h3 is a cell adhesive protein whose expression is highly induced by TGF-ß in several cell types. And ßig-h3 has been considered to promote cell adhesion and spreading through EPDIM of the 4th fas-1 domain sand YH present in each of the fas-1 repeat domains. Herein, we synthesized Tetra-Cell Adhesion Molecule(T-CAM), which is the recombinant protein containing an RGD sequence in tenth type III domain and a PHSRN sequence in the ninth type III domain of fibronectin, and YH sequence and EPDIM sequence in fourth fas-1domain of ßig-h3. Therefore, the purpose of this study was to evaluate the cellular activity of osteoblast-like cell to anorganic bone mineral coated with T-CAM , while comparing the results with those to anorganic bone mineral. The number of viable cells significantly increased for ABM/T-CAM group at 4 and 7 days after culturing, as compared to control and ABM. The of ALP mRNA was also similar in all groups on the 10 and 20 days after culturing. The expression of osteopontin mRNA was highest on ABM/T-CAM on the 10 and 20 days. A greater expression was observed on the 30th day of culturing for ABM and ABM/T-CAM, as compared to control. Although the ABM group also had the staining of intercellular bridges among the particles, significant staining on intercellular bridges, in the osteoblast-like cell culture of the ABM/T-CAM group, was much more intense and frequent. Compared to the ABM-grafted group, the defects of ABM/T-CAM-grafted groups revealed active new bone formation in the middle area of the defects. we suggest that the presence of T-CAM remarkably effects the differentiation of osteoblast-like cell grown on HA. Then ABM/T-CAM templates may be effective as endosseous grafts, and may serve as tissue engineered substitutes for autologous bone grafts.

Key words: Tetra-cell adhesion molecule, osteoblast-like cell, bone mineral

1. Introduction

Recently, tissue engineering, which applies methods from engineering and life sciences to create artificial constructs to direct tissue regeneration,[1] has attracted many scientists and surgeons with a hope to treat patients. In order to achieve successful regeneration of damaged organs or tissues based on the tissue engineering concept, several critical elements should be considered including biomaterial scaffolds that serve as a mechanical support for cell growth, progenitor cells that can be differentiated into specific cell types, and inductive growth factors that can modulate cellular activities.[2,3] Biomolecular recognition of materials by cells can be achieved by incorporation of cell-binding peptides into biomaterials via chemical or physical modification. The cell-binding peptides include short peptide sequences derived from intact ECM proteins as well as a native long chain of ECM proteins that can induce specific interactions with cell receptors. Therefore materials which are composed of short peptide sequences derived from intact ECM proteins, potentially mimic many roles of ECM in tissues.

*Tel: +82-53-600-7521; Fax: +82-53-427-3263
e-mail: jysuh@knu.ac.kr (Jo-Young Suh)

Extensive studies have been performed to render materials biomimetic. The surface modification of biomaterials with bioactive moleculesis a simple way to make biomimetic materials. Short peptide fragments have been used for surface modification in numerous studies. The use of a short peptide for surface modification is advantageous over the use of the long chain of native ECM proteins. The native ECM protein tends to be randomly folded upon adsorption to the biomaterial surface such that the receptor binding domains are not always sterically available. However, the short peptide sequences are relatively more stable during the modification process than long chain proteins such that nearly all modified peptides are available for cell binding. In addition, short peptide sequences can be massively synthesized in laboratories more economically. The biomimetic material modified with these bioactive molecules can be used as a tissue engineering scaffold that potentially serves as an artificial ECM providing suitable biological cues to guide new tissue formation.

The most commonly used peptide for surface modification is arginine-glycine-aspartic acid(RGD), the signaling domain derived from fibronectin(FN). FN is composed of three general types of homologous repeating units, termed types I, II and III,[4] which are arranged into protease-resistant domains. The central cell binding domain is composed of the fibronectin type III repeats and includes an RGD site in tenth type III.[5] Synthetic peptides containing this sequence block the attachment of some cells to fibronectin.[6-8] Furthermore, deletion of the RGD sequence and a nearby proline-histidine-serine-arginine-asparagine(PHSRN) sequence in repeat III profoundly reduces cell attachment to FN.[9] The RGD sequence interacts with several cell surface integrins, the major one being $\alpha 5\beta 1$.[10,11] Therefore within the structure of FN, an RGD sequence in tenth type III domain and a PHSRN sequence in the ninth type III domain are key consensus sites for cell adhesion.[12]

βig-h3 is a cell adhesive protein whose expression is highly induced by TGF-β in several cell types.[13] It has four internal repeat domains named fas-1, which have a homology with a Drosophila neuroadhesion molecule, fasciclin-1.[14] βig-h3 transcript was detected in a variety of human and mouse tissues including uterine tissue, heart, breast, prostate, skeletal muscle, testes, thyroid, kidney, liver, and stomach.[15] Although the physiological function of βig-h3 in the above organ is not known, it has been generally accepted that it serves as a cell adhesive substrate and interconnects different matrix components. And βig-h3 has been considered to promote cell adhesion and spreading through EPDIM of the 4th fas-1 domain sand YH present in each of the fas-1 repeat domains.[16,17] Herein we synthesized tetra-cell adhesion molecule(T-CAM), which is a recombinant protein containing an RGD sequence in tenth type III domain and a PHSRN sequence in the ninth type III domain of fironectin, and YH sequence and EPDIM sequence in fourth fas-1 domain of βig-h3. Therefore, the purpose of this study was to evaluate the cellular activity of osteoblast-like cell to anorganic bone mineral coated with T-CAM, while comparing the results with those of anorganic bone mineral.

2. Material and Methods

2.1 Peptide Synthesis

T-CAM is a fusion protein consisting of the 9th and 10th type III domains of fibronectin and the 4th fas-1 domain of βig-h3. Fragment of fibronectin cDNA, encoding amino acids 1330 to 1513 was generated by PCR and restricted to the Nde I and Nsil I sites. The Nde I/Nsil I fragment of fibronectin cDNA was inserted into the EcoR V sites of 4th fas-1 domain of βig-h3.[16] Plasmid constructs encoding T-CAM were transformed into BL21(DE3) for expression. Overnight bacterial culture was induced by 1 mM IPTG for 3 h and T-CAM protein was purified using Ni-NTA resin(Qiagen) according to the manufacturer's recommendations.[16]

2.2 Anorganic Bone Mineral(ABM)

Anorganic bone mineral in a particulate form (OsteoGraf/N-300, 250-420 μm) was obtained from Dentsply Friadent CeraMed Co(Lakewood, CO, USA). In this form, most of ABM exists as smooth, porous particles(250-420 μm, with a mean diameter of 300 μm). We selected this preparation of ABM because of the manufacture's certification that deproteination was complete based on Kjeldahl and carbon analyses(ASTMD4129).

2.3 Preparation of ABM/T-CAM

For study of the effect of T-CAM on cellular activity of osteoblast-like cell, the peptide was absorbed on ABM by incubating ABM for 24 h in a solution of the peptide in phosphate-buffered saline(PBS) in a ratio of 1.0 g ABM to 2.0 mL solution containing 200 μg T-CAM. The incubation was carried out at room temperature with gentle shaking to ensure equilibration of the peptide with all exposed surfaces of the microporous ABM. Following incubation, ABM was washed three times by shaking with 5×volume of PBS over a 24 h

period to remove unabsorbed peptide. The ABM powder was collected and dried in a dry oven. ABM with T-CAM absorbed peptide was made using sterilized preparations.

2.4 Osteoblast Culture on ABM and ABM/T-CAM

We used the mouse calvaria-derived MC3T3-E1 osteoblast-like cell line for this study. MC3T3-E1 cells were cultured in alpha-Modified Eagle's Medium(a-MEM, Gibco) supplemented with 10% fetal bovine serum(FBS, Gibco) and 500 unit/mL penicillin(Keunhwa Pharm, Co,), 500 μg/mL streptomycin (DongA Pharm, Co) and cultured in an atmosphere of 100% humidity, 5% CO_2, and 37. Cells were grown in 10% FBS containing 10 mM β-glycero-phosphate(ß-GP, Sigma), 50 μg/mL of ascorbic acid and 100 nM dexamethasone in 100 mm dishes or 24 well plates(Corning, Corning, NY, USA) for the experiments. The medium was changed every 3 days. The experimental groups were divided as follows(Control: polystyrene petri dishes, ABM: OsteoGraf/N-300 particles in polystyrene petri dishes, ABM/T-CAM : OsteoGraf/N-300 particles absorbed T-CAM in polystyrene dishes).

2.5 MTT Assay for Viable Cell Numbers

MTT[3-(4,5-dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide, thiazol blue] assay is transformed by mitochondrial dehydrogenases into formazan, enabling mitochondrial activity and cell viability to be assessed. The cultured cells were seeded at an initial density of 2×10^4 cells/well on each well which contains each particle of the 24 well plate. Control and two kinds of particles(30 mg/well)-ABM and ABM/T-CAM-were used in each group. The cells were cultured in an atmosphere of 100% humidity, 5% CO_2, and 37°C for 1, 4 and 7days. And then, removed medium and washed with PBS two times. Then, 250 μL of MTT solution prewarmed to 37°C was added in each well and incubated for 3 h in the same condition and 750 μL of dimethyl sulfoxide(DMSO) and 250 μL of glycine buffer were added. The solution was transferred to other set of wells which does not contain the particles. The optical density was measured at a wavelength of 570 nm by ELISA reader(Precision Microplate Reader, Molecular Devices, USA). And 4-day cultures were stained by hematoxylin and eosin(H&E).

2.6 Staining for Alkaline Phosphatase

Cultures for examination of the presence of alkaline phosphatase(ALP) were prepared in each group. Ten-day cultures were fixed in neutral-buffered 10% formalin and stained to localize the presence of ALP with a-naphthyl acid phosphate substrate and Fast-Blue RR salt using a kit(86-R) available from Sigma.

2.7 Gene Expression for Bone Matrix Protein

The cultured cells were seeded at an initial density of 8×10^4 cells/well which contains 150 mg/well each particles of the 100 mm petridishes. The cells were cultured in an atmosphere of 100% humidity, 5% CO_2, and 37°C during 10, 20 days for ALP and 10, 20 and 30 days for osteopontin and collagen type I.

Total RNA was extracted from cultured cells by using a modified acid phenol method. Briefly, the growth medium was removed and the cells were lysed with Trizol(Invitrogen Co, USA). The lysate was cleared and extracted with 1/10 volume of 1-bromo-3-chloporopane. The aqueous layer was collected in a new tube and precipitated with isoporpanol. After 75% ethanol washing, the pellet was air-dried and resuspended in DEPC-treated water, and quantified by A260/A280 measurement by using UV spectrometer(DU530, Beckman, USA).

Ten microgram of total RNAs were heated to 65°C or 15min in 50% formamide, 0.02% formaldehyde, 40 nM MOPS[3-(-N-morpholino)propanesulfonic acid), 10 mM sodium acetate, 1 mM EDTA, and 0.1 mg/mL ethodium bromide prior to gel electrophoresis on 1% agarose, 55% formaldehyde, 40 mM MOPS, 10mM sodium acetate, and 1 mM EDTA. The RNA was blotted onto Hybond-N+Membranes(Amersham Biosci, UK) in 20X SSC. The RNA was air-dried and then cross-linked by exposure to ultraviolet light. The probes were labelled with [a-P^{32}]-dCTP by a Megaprime DNA labelling system kit(Amersham Biosciences, UK). Prehybridization and hybridization were performed by using the Express Hyb solution(BD Biosci Clontech, USA). After hybridization, the membrane was washed in 2X SSC-0.1% SDS at room temperature and then in 0.1X SSC/0.1% SDS at 55 and exposed to Agfa X-ray film at -70°C with intensifying screens.

2.8 Staining with Alizarin Red for Mineralization

Red staining with Alizarin Red is an indicator of mineralization.[18,19] MC3T3-E1 cell cultures on ABM and ABM/T-CAM were prepared for staining by removing the medium, washing the cells with PBS, and fixing in 2% paraformaldehyde for 30 minutes at 4°C following which the cells were gently washed with H_2O. The cells were stained in a solution of 1% Alizarin Red S, pH 4.2 for 10 minutes at room temperature, and then, washing the cells with H_2O two times. The samples were air dried, and were examine and

photographed in a light microscope.

2.9 Animals for Ability of New Bone Formation

Two 9 mm diameter, full-thickness calvarial defects were made in each rabbit parietal bone using trephine bur, and four adult male New Zealand White rabbits were used in this experiment. Each defects was filled with ABM and ABM/T-CAM graft particles. The animals were sacrificed at 4 weeks after surgery and the specimens were processed for histological evaluation. Block sections including the defect and surrounding bone tissue were removed and they were fixed in 10% formalin, decalcified with EDTA, paraffin-embedded, and sectioned at a final thickness of 5 μm. The slices were stained by hematoxylin and eosin(H&E) and Masson's trichrome.

2.10 Statistical Analysis

Analysis of variance(ANOVA) was used to evaluate differences of cell viability among all the groups.

3. Results

3.1 ABM/T-CAM Provides Better Environment for Osteoblast-like Cells Growth

The MTT assay was carried out to evaluate viable cell numbers on the control, ABM and ABM/T-CAM group at 1, 4 and 7 day(Fig 1). The MTT assay indicated the increases in viable cell numbers of control, ABM and ABM/T-CAM according to the time. Cell numbers were significantly increased on ABM/T-CAM at 4 and 7 day($p<0.01$), compared to control and ABM. In contrast, cell numbers attached on ABM were significantly lower than these of control and ABM-T-CAM at 1, 4 and 7 day culture($p< 0.01$). Also we observed these results on H&E stain at 4 day culture(Fig 4).

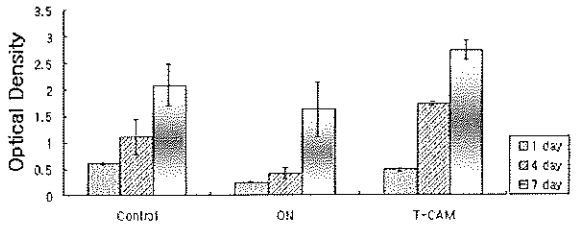

Figure 1. The optical density measured after culture for 1, 4 and 7 days at a wavelength of 579 nm by ELISA reader(n=3 in each groups)(Control: polystyrene petridishes, ABM: OsteoGraf/N-300 particles in polystyrene petridishes, ABM/T-CAM: OsteoGraf/N-300 particles absorbed T-CAM in polystyrene petridishes).

3.2 ALP Activity of ABM/T-CAM was Similar to Control and ABM

ALP is involved in bone formation and mineralization, and its expression considered to be evidence for osteogenic differentiation. In the present experiment, cells grew both on the surface of the culture dish and on the mineral particles with or without T-CAM. The cultures were stained for ALP 10 days after osteoblast-like cell were initially seeded. The control, ABM and ABM/T-CAM group were equally stained for ALP(Fig 5). Expression of ALP mRNA was also similar in all groups at 10 and 20 days(Fig 2).

3.3 The Expression of Osteopontin mRNA was increased in ABM/T-CAM

To prove that bone formation occurs in osteoblast-like cell

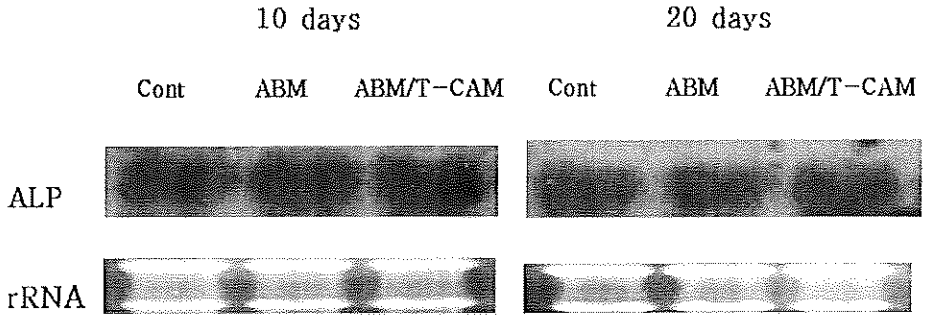

Figure 2. Gene expression of ALP of MC3T3-E1 cells cultured on control, ABM and ABM/T-CAM for 10 and 20 days(Control: polystyrene petridishes, ABM: OsteoGraf/N-300 particles in polystyrene petridishes, ABM/T-CAM: OsteoGraf/N-300 particles absorbed T-CAM in polystyrene petridishes).

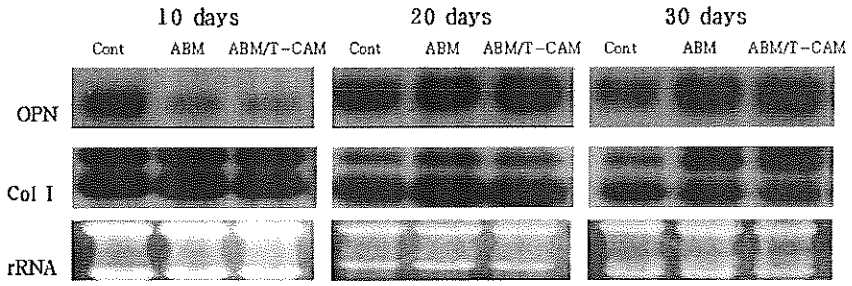

Figure 3. Gene expression of osteopontin(OPN) and α1(I) collagen(Col I) of MC3T3-E1 cells cultured on control, ABM and ABM/T-CAM for 10, 20 and 30 days(Control: polystyrene petridishes, ABM: OsteoGraf/N-300 particles in polystyrene petridishes, ABM/T-CAM: OsteoGraf/N-300 particles absorbed T-CAM in polystyrene petridishes).

cultures, we examined the expression of several genes implicated in osteoblastic differentiation. We therefore examined the expression of osteopontin mRNA and α1(I) collagen mRNA in osteoblast-like cells of control, ABM and ABM/T-CAM(Fig 3). Osteopontin is a major noncollagenous bone matrix protein and an early marker of differentiated osteoblast. The expression of osteopontin mRNA was the highest in ABM-T-CAM at 10 and 20 days, followed by ABM. It was lowest in control group. But the expression of osteopontin mRNA was highest in control group at 30 day and was very low in ABM and ABM/T-CAM.

Although little difference was seen in the level of expression

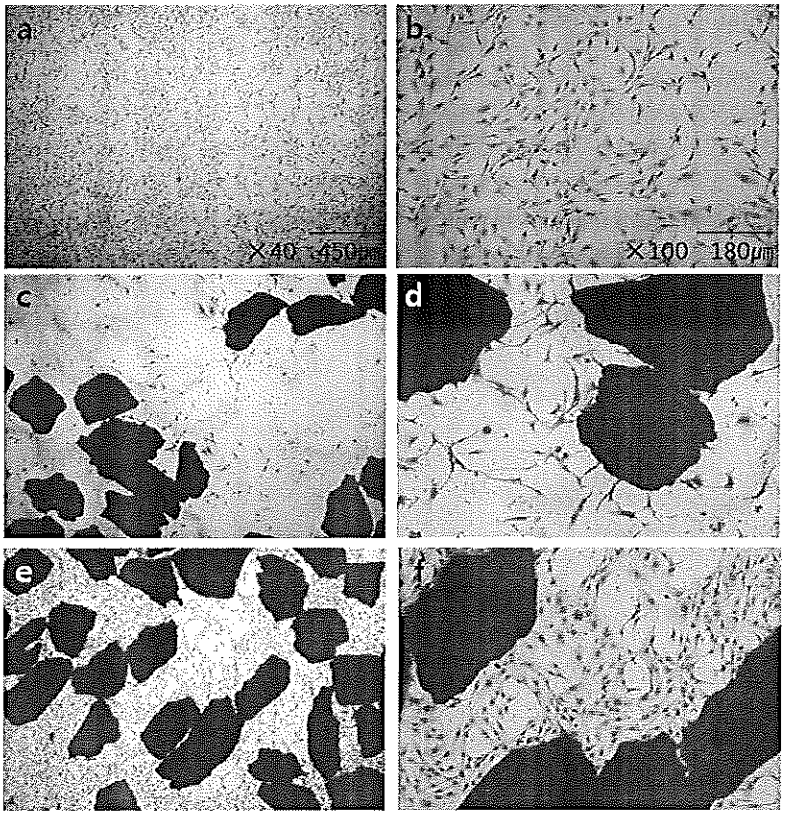

Figure 4. Cellular viability of control, ABM and ABM/T-CAM at 4 day culture stained by H&E; (a) Control(×40), (b) Control(×100), (c) ABM(×40), (d) ABM(×100), (e) ABM/T-CAM(×40), and (f) ABM/T-CAM(×100).

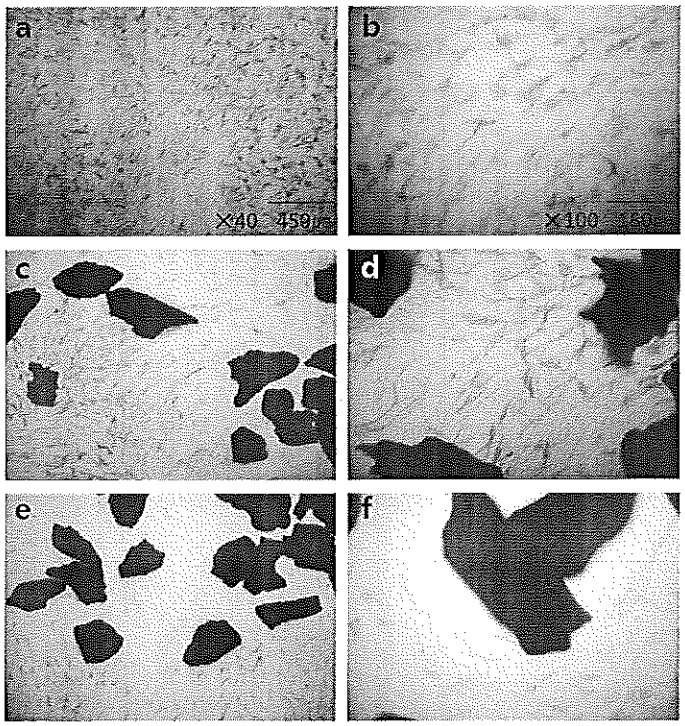

Figure 5. Staining for Alkaline phosphatase Activity on control, ABM and ABM/T-CAM at 10 day culture; (a) Control(×40), (b) Control(×100), (c) ABM(×40), (d) ABM(×100), (e) ABM/T-CAM(×40), and (f) ABM/T-CAM(×100).

of collagen RNA after 10 and 20 days in culture on control, ABM and ABM/T-CAM, greater expression was observed in 30 day culture in ABM and ABM/T-CAM compared to control.

3.4 ABM/T-CAM Enhances the Differentiation of Osteoblast-like cell

Staining of matrix with Alizarin Red dye is considered to be an indicator of mineralization[18,19] characterized by the deposition of Ca^{2+}.[20] Very strong staining with the dye was seen in osteoblast-like cell cultures on ABM as well as on ABM/T-CAM in 30 day cultures(Fig 6). Figs 6c and 6e show staining around isolated ABM and ABM/T-CAM particles. A halo of red stain consistent with Ca^{2+} deposition is seen in osteoblast-like cell matrix associated with ABM/T-CAM. Many of the cells in these cultures formed bridges across the gaps between adjacent particles. Tractional forces generated by these cells resulted in the clustering of particles. The staining of intercellular bridges was examined by focusing the microscope in planes above the culture dish surface(Figs 6d and 6f). Although ABM also had the staining of intercellular bridges among the particles, staining in intercellular bridges(arrows) in osteoblast-like cell culture on ABM/T-CAM was much more intense and frequent(Figs 6d and 6f). These observations are consistent with the induction of an osteogenic phenotype in osteoblast-like cell cultured on ABM/T-CAM and together with our observations on osteogenic gene induction, they strongly suggest that the presence of T-CAM remarkably effects the differentiation of osteoblast-like cell grown on HA.

3.5 ABM/T-CAM Enhances the New Bone Formation

The histologic findings at 4 weeks of healing are shown in Fig 7. A little amount of newly formed bone surrounding grafted particles was seen in the ABM-grafted defects(Fig 7a). Compared to ABM-grafted group, the defects of ABM/T-CAM-grafted groups revealed active new bone formation in the middle area of defects, almost complete bony bridges were observed in the ABM/T-CAM-grafted defects and amount of newly formed bone in ABM/T-CAM-grafted particles was observed higher than in ABM-grafted group(Fig 7b).

4. Discussion

The construction of a biomimetic environment for tissue engineering, and for implants and grafts should take into

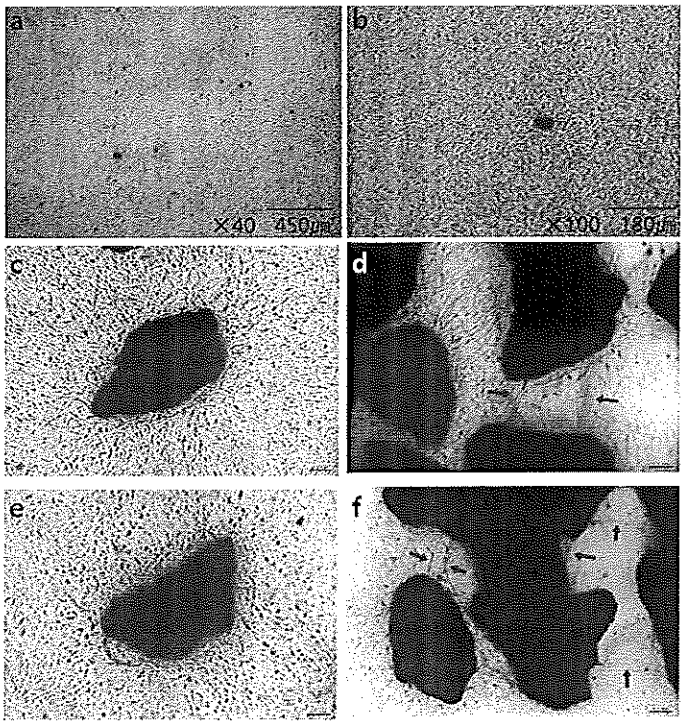

Figure 6. Staining with Alizarin red S on control, ABM and ABM/T-CAM at 30 day culture; (a) Control(×40), (b) Control(×100), (c) ABM(one particle×100), (d) ABM (particles×100), (e) ABM/T-CAM(one particle×100), and (f) ABM/T-CAM(particles×100).

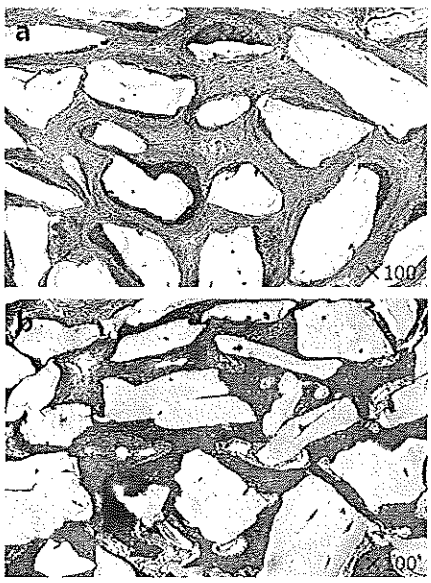

Figure 7. Histological examination of the calvarial defects of grafted with (a) ABM and (b) ABM/T-CAM at 4 weeks after surgery. Compared to ABM-grafted group, the defects of ABM/T-CAM-grafted groups revealed active new bone formation in the middle area of defects, and amount of newly formed bone in ABM/T-CAM-grafted particles was observed higher than in ABM-grafted group.

account the channels through which chemical and biomechanical signals that contribute to differentiation and morphogenesis are conducted. All anchored cells exist in 3-D compartments made up of an ECM. In this environment, cell-matrix interactions are mediated by highly specific receptors of cells and adhesion molecules of ECM. These interacitons facilitate the exchange of chemical and mechanical signals. Inert scaffolds lack mechanisms for providing flow of information to and from cells. We propose that incorporating T-CAM, which was considered to increase the cell-binding activity as composing of four cell adhesion molecules in a 3-D distribution, will facilitate the attachment, migration, and differentiation of cells in biologically inert graft materials. Such a graft material may be expected to serve as a hospitable biomimetic habitat for cells for tissue engineering and it may serve as a bioactive scaffold for bone formation.

One ECM protein that may provide information to osteoblasts during their differentiation is FN. FN is a heterodimeric ECM glycoprotein that has several cell- and matrix-binding domains.[21] The RGD sequence, present in FN type III repeat 10 of the central cell binding region, recognizes several members of the integrin family. These include the

specific integrin FN receptor α5β1 as well as several av-containing integrins, which also interact with other RGD-containing ECM components besides FN. A PHSRN sequence in FN type III repeat 9, referred to as the synergy sequence, has been found to selectively enhance binding of the FN RGD sequence to α5β1.[22,23] Also βig-h3 is an extracellular matrix protein that can be induced by transforming growth factor-ß in several cell types. Several studies suggest βig-h3 is involved in cell growth,[13] cell differentiation,[16,24] wound healing,[25] and cell adhesion,[26,27] although the underlying mechanisms for these effects are still unclear. The βig-h3 protein comprises 683 amino acids containing four homologous internal repeat domains. These domains are homologous to similar motifs in the *Drosophila* protein fasciclin-I and thus are denoted fas-1 domains. βig-h3 has been considered to promote cell adhesion and spreading through EPDIM and YH present in each of the fas-1 repeat domains.[16,17]

Then we synthesized T-CAM, which is the recombinant protein containing an RGD sequence in tenth type III domain and a PHSRN sequence in the ninth type III domain of fibronectin, and YH sequence and EPDIM sequence in fourth fas-1domain of ßig-h3, and was to evaluate the cellular activity of osteoblast-like cell to anorganic bone mineral coated with T-CAM , while comparing the results with those of anorganic bone mineral. We used the mouse calvaria-derived MC3T3-E1 osteoblast-like cell lines, which was known that the expression patterns of bone-related proteins were regulated in temporal manner during the successive developmental stages including proliferation(days 4-10), bone matrix formation/ maturation(days 10-16), and mineralization stages(days 16-30).[28] And we studied this experiment according to the above successive developmental stages.

To observe cellular viability or proliferation of osteoblast-like cells on ABM and ABM/T-CAM, we used the MTT assay. Cellular viability was increased significantly on ABM/T-CAM at 4 and 7 cultures, but cellular viability on ABM was significantly decreased according to time when compared to control and ABM/T-CAM. According to certification by the manufacturer, the ABM used in these studies is nearly pure hydroxyapatite and the preparations of AMB contained negligible amounts of endogenous organic material. But it was considered that ABM has a little cytotoxicity in this study and we could observe this results on the Fig 4 and it was supported by the report that small amount of protein are present in deproteinized cancellous bovine bone in close association with the mineral phase.[29] It was considered that the increase of cellular viability of ABM/T-CAM was due to four cellular adhesion molecules absorbed to hydroxyapatite, which was similar to other study.[30] They reported that FN fragment containing FN type III ninth-tenth domain fragment was no statistical difference compared to FN in the proliferation of periodontal ligament cells.

To prove that bone formation occurs in osteoblast-like cell cultures, we examined the expression of several genes implicated in osteoblastic differentiation. This study chose the mRNA expression level of α1(I) collagen, ALP and osteopontin, which are established markers of osteogenic differentiation.[28,31] The expression of α1(I) collagen was the highest at 20 days culture on all group and the expression of ALP was higher at 10 days culture than at 20 days culture on all group. This results means that MC3T3-E1 cells was well doing the successive developmental stages.[28] Osteopontin was expressed in a significantly higher amount for osteoblast-like cells cultured on ABM/T-CAM than on ABM at 20 days, the periods between bone matrix formation/maturation and mineralization stages. Osteopontin is a glycosylated phosphoprotein that is associated with the maturation and organization of the bone-like ECM. The regulation of osteopontin expression correlates with bone development where an increase in an osteopontin expression level reduces proliferation as well as induces differentiation of osteoblasts. And osteopontin expression is found only in the cells lining the parietal bones, which are known to contain fully differentiated osteoblats, while it was not found in the proliferating cells in osteogenic fronts.[32-34] Our results strongly support the idea that the ABM/T-CAM provide an appropriate environment for MC3T3-E1 cells to undergo differentiation into mature osteoblasts. The over-expressed osteopontin on ABM/T-CAM in our results implies that the biomimetic surface was involved not only in cell attachment but also in regulation of cellular processes that generate a specific signal transduction cascade that, in our case, enhanced the differentiation of the osteoblasts.

The main functions of osteoblast-like cells *in vivo* is to elaborate a mineralizing matrix. We examined this question histochemically using Alizarin Red S, a dye indicator of matrix mineralization characterized by the deposition of Ca^{2+}. We examined 30-day cultures on ABM and ABM/T-CAM for evidence of mineralization using Alizarin Red staining. although a halo red staining around isolated ABM and ABM/T-CAM was observed, there was marked staining associated with the 3-D cellular colony on the surface of ABM/T-CAM indicating that the cells in these colonies were involved in

mineralization. The interparticle bridges comprised of cells were seen in ABM and ABM/T-CAM cultures, but it was much more intense and frequent in ABM/T-CAM cultures. The cells in these bridges play a major role in the clustering of particles by generating tractional forces. The physiology of cells is profoundly affected by applied mechanical forces and displacements. Mechanical stimulation stimulates bone formation and inhibits bone resorption in cultured bone tissue and cells.[35,36] Intermittent compressive forces stimulated the release of an autocrine growth-factor from bone, suggesting that mechanical forces may modulate skeletal remodeling by affecting the production of local growth factors.[37] If ABM/T-CAM acts as a force-conducting ligand for osteoblast-like cells, this would explain the increased expression of osteopontin in MC3T3-E1 cells cultures on ABM/T-CAM. Several cell culture systems involving various osteogenic cell types suggested that the expression of osteopontin was increased in response to mechanical stimulation.[36,38-41]

In the present study, ABM particles showed biocompatible, osteoconductive in initial healing phase. However, ABM group did not reveal active bone formation. Compared with ABM group, ABM/T-CAM group showed more active bone formation in the margin of defect as well as in the middle areas of defect at 4 weeks. These findings indicated that peptide enhanced bone grafts probably evoked any effect to new bone formation of defect more than anorganic bone mineral, and also synthetic peptide related to cell adhesion contributed to these favorable new bone formation. The data presented here support our hypothesis that immobilizing T-CAM, composed of four cell adhesion molecules(RGD, PHSRN in fibronectin and EPDIM, YH in βig-h3) on ABM or hydroxyapatite, will enhance the ability of the ceramic matrix to serve as a host for osteoblast-like cells. In this environment, the osteoblast-like cells displayed cellular activity which is remarkable different from that of the same cells cultured on ABM in the absence of T-CAM. We believe that the improved attachment and proliferation of cells and changes in their ability are attributable to the composition of the composite which makes it, in effect, a reconstituted bone-like material. Our reason for this assertion is as follows. Although there are conflicting data on the distribution of integrins in osteoblasts, the FN receptors a3β1, a5β1, avβ3 and avβ5 were localized to normal bone tissue[42-44] and a5ß1 was identified in normal osteoblast cultures.[44,45] Both sequence of RGD and PHSRN in T-CAM are recognized by integrin a5β1 in osteoblasts-like cells.[46] EPDIM in T-CAM may be recognized by integrin a3ß1 in osteoblast-like cells because of mediating human corneal epithelial cell adhesion[16] and YH in T-CAM may be recognized by integrin avβ5 in osteoblast-like cells because of mediating human lung fibroblast cell adhesion.[17]

In conclusion, we suggest that the presence of T-CAM remarkably effects the differentiation of osteoblast-like cell grown on HA. Then ABM-T-CAM graft particles may be effective as endosseous grafts, and may serve as tissue engineered substitutes for autologous bone grafts.

Acknowledgements : It's work was supported by basic research grant of KMEST(M 10646020002-08N4602-00210).

References

1. JP Vacanti, R Langer, Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation, *Lancet,* **354**, 32 (1999).
2. G Khang, SH Kim, MS Kim, *et al.*, Recent and future directions of stem cells for the application of regenerative medicine, *Tissue Eng Regen Med,* **4(4)**, 441 (2007).
3. CA Heath, Cells for engineering, *TIBTECH,* **18**, 17 (2000).
4. KN Yamada, Fibronectin and other cell interactive glucoproteins. In: HAYED(ed.) *Cell Biology of Extracellular Matrix, New York: Plenum Press,* 111 (1991).
5. E Ruoslahti, Fibronectin and its receptors, *Annu Rev Biochem,* **57**, 375 (1988).
6. MD Pierschbacher, E Ruoslahti, Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule, *Nature,* **309**, 30 (1984).
7. KM Yamada, DW Kennedy, Dualistic nature of adhesive protein function: Fibronectin and its biologically active peptide fragments can auto inhibit fibronectin function, *J Cell Biol,* **99**, 29 (1984).
8. A Hautanen, J Gailit, DM Mann, *et al.*, Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor, *J Biol Chem,* **264**, 1437 (1989).
9. M Obara, MS Knag, KM Yamada, Site-directed mutagenesis of the cell-binding domain of fibronectin: Separable, synergistic sites mediate adhesive function, *Cell,* **53**, 649 (1988).
10. R Pytela, MD Pierschbacher, E Ruoslahti, Identification and isolation of a140 kD cell surface glycoprotein with properties expected of a fibronectin receptor, *Cell,* **40**, 191 (1985).
11. E Ruoslahti, MD Pierschacher, New perspectives in cell adhesion: RGD and integrins, *Science,* **238**, 291 (1987).
12. AM Mardon, KE Grant, The role of the ninth and tenth type III domains of human fibronectin in cell adhesion, *FEBS Lett,* **340**, 197 (1994).
13. J Skonier, K Bennett, V Rothwell, *et al.*, ßig-h3: a transforming growth factor beta-responsive gene encoding asecreted protein that inhibits cell attachment *in vitro* and suppresses the growth of CHO cells in nude mice, *DNA Cell Biol,* **13**, 571 (1994).
14. T Kawamoto, M Noshiro, M Shen, *et al.*, Structural and

phylogenetic analyses of RGD-CA P/ßig-h3, a fasciclin-like adhesion protein expressed in chick chondrocytes, *Biochim Biophys Acta,* **1395**, 288 (1998).
15. J Skonier, M Noshiro, L Madisen, *et al.,* cDNA cloning and sequence analysis of ßig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-ß, *DNA Cell Biol,* **7**, 511 (1992).
16. JE Kim, EH Kim, EH Han, *et al.,* A TGF-ß-inducible cell adhesion molecule, ßig-h3, is down regulated in melorheostosis and involved in osteogenesis, *J Cell Biochem,* **77**, 169 (2000).
17. JE Kim, HW Joeng, JO Nam, *et al.,* Identification of motifs in the fasciclin domains of the transforming growth factor-ß-induced matrix protein ßig-h3 that interact with the avß5integrin, *J Biol Chem,* **29**, 46159 (2002).
18. S Keila, S Pitaru, A Grosskopf, *et al.,* Bone marrow from mechanically unloaded rat bones expresses reduced osteogenic capacity *in vitro*, *J Bone Miner Res,* **9**, 321 (1994).
19. PV Bodine, J Green, HA Harris, *et al.,* Functional properties of a conditionally phenotypic, estrogen-responsive, human osteoblast cell line, *J Cell Biochem,* **65**, 368 (1997).
20. CM Stanford, PA Jacobson, ED Eanes, *et al.,* Rapidly forming apatitic mineral in an osteoblastic cell line(UMR 106-01 BSP), *J Biol Chem,* **270**, 9420 (1995).
21. RO Hynes, Fibronectins, *Springer-Verlag, New York*, 546 (1990).
22. HS Jang, JS Rim, ES Lee, *et al.,* Influence of fibronectin, type I collagen and vitronectin on migration of osteoblast, *Tissue Eng Regen Med,* **1(2)**, 190 (2004).
23. RD Bowditch, M Hariharan, EF Tominna, *et al.,* Identification of a novel integrin binding site in fibronectin. Differential utilization by beta 3 integrins, *J Biol Chem,* **269**, 10856 (1994).
24. SC Dieudonne, KM Kerr, T Xu, *et al.,* Differential display of human marrow stromal cells reveals unique mRNA expression patterns in response to dexamethasone, *J Cell Biochem,* **76**, 231 (1999).
25. IM Rawe, Q Zhan, R Burrows, *et al.,* Molecular cloning and in situ hybridization in corneal tissues, *Invest Ophthalmo & Visual Sci,* **38**, 893 (1997).
26. WW Lee, JS Park, HJ Do, *et al.,* Inducement of chondrogenic differentiation in injectable hydrogels embedded in mouse embryonic stem cells and growth factor for neocartilage formation, *Tissue Eng Regen Med,* **5(1)**, 103 (2008).
27. S Ohno, M Noshiro, S Makihira, *et al.,* YRGD-CAP((beta)ig-h3) enhances the spreading of chondrocytes and fibroblasts via integrinalpha(1) beta(1), *Biochim Biophys Acta,* **1451**, 196 (1999).
28. JY Choi, BH Lee, KB Song, *et al.,* Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells, *J Cell Biochem,* **61**, 609 (1996).
29. Z Schwartz, T Weesner, DS Van, *et al.,* Ability of deproteinized cancellous bovine bone to induce new bone formation, *J Periodontol,* **71**, 1258 (2000).
30. TI Kim, JH Jang, CP Chung, *et al.,* Fibronectin fragment promotes osteoblast-associated gene expression and biological activity of human osteoblast-like cell, *Biotech Lett,* **25**, 2007 (2003).
31. YI Yang, HI Kim, JY Seo, *et al.,* Adult stem cells as cell therapeutics for angiogenesis, *Tissue Eng Regen Med,* **4(4)**, 484 (2007).
32. S Iseki, AO Wilkie, JK Heath, *et al.,* Fgfr 2 and osteopontin domains in the developing skull vault are mutually exclusive applied FGF2, *Develoment,* **124**, 3375 (1997).
33. S Iseki, AO Wilkie, GM Morris-Kay, Fgfr 1 and Fgfr 2 have distinct differentiation and proliferation-related roles in the developing mouse skull vault, *Development,* **126**, 5611 (1999).
34. MH Park, HI Shin, JY Choi, *et al.,* Differential expression patterns of Runx2 isoforms in cranial suture morphogenesis, *J Bone Miner Res,* **16**, 885 (2001).
35. T Kubota, M Yamauchi, J Onozaki, *et al.,* Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin, *Arch Oral Biol,* **38**, 23 (1993).
36. J Klein-Nulend, J Roelofsen, CM Semeins, *et al.,* Mechanical stimulation of osteopontin mRNA expression and synthesis in bone cell cultures, *J Cell Physiol,* **170**, 174 (1997).
37. J Klein-Nulend, CM Semeins, JP Veldhuijzen, *et al.,* Effect of mechanical stimulation on the production of soluble bone factors in cultured fetal mouse calvariae, *Cell Tissue Res,* **271**, 513 (1993).
38. I Owan, D Burr, C Turner, *et al.,* Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanicalstrain, *Am J Physiol,* **273**, 810 (1997).
39. GN Bancroft, VI Sikavitsas, J van den Dolder, *et al.,* Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner, *Proc Natl Acad Sci USA,* **99**, 12600 (2002).
40. RS Carvalho, A Bumann, JL Schaer, *et al.,* Predominant integrin ligands expressed by osteoblasts show preferential regulation inresponse to both cell adhesion and mechanical perturbation, *J Cell Biochem,* **84**, 497 (2002).
41. KM Choi, YK Seo, HH Yoon, *et al.,* Differentiation of human bone marrow-derived mesenchymal stem cell into fibroblast-like cell *in vitro* by mechanical tension, *Tissue Eng Regen Med,* **3(4)**, 423 (2006).
42. J Clover, RA Dodds, M Gowen, Integrin subunit expression by human osteoblasts and osteoclasts *in situ* and in culture, *J Cell Sci,* **103**, 267 (1992).
43. K Hultenby, FP Reinholt, D Heinegard, Distribution of integrin subunits on rat metaphyseal osteoclasts and osteoblasts, *Eur J Cell Biol,* **62**, 86 (1993).
44. EK Lee, JS Lee, HS Park, *et al.,* Stretch-stimulated cellular rearrangement and alteration of fibronectin matrix assembly, a-smooth muscle actin, and CD44 expression in human mesenchymal stem cells, *Tissue Eng Regen Med,* **2(2)**, 94 (2005).
45. CT Brighton, SM Albelda, Identification of integrin cell substratum adhesion receptors on cultured rat bone cells, *J Orthop Res,* **10**, 766 (1992).
46. A Krammer, D Craig, WE Thomas, *et al.,* A structural model for force regulated integrin binding to fibronectin's RGD-synergysite, *Matrix Biol,* **21**, 139 (2002).