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Injectable gel with synthetic collagen-binding peptide for enhanced osteogenesis *in vitro* and *in vivo*

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Abstract

A synthetic peptide denoted as collagen-binding motif (CBM) was identified from osteopontin (OPN), a multisubunit extracellular matrix (ECM) protein, by enzymatic digestion with chymotrypsin. The aim of this study was to examine the feasibility of identified CBM peptide as an active component of gel type scaffold material in osteogenesis. The binding of CBM peptide to collagen was specific and presented high affinity. Cell adhesion and growth on CBM peptide-immobilized gel were significantly increased as compared with those on gel with control peptide or without peptide. The CBM peptide-immobilized gel increased osteoblastic differentiation, followed by marked bone formation in the rabbit calvarial defect sites at 4 weeks. Taken together, the injectable gel with synthetic CBM peptide has a potential to induce osteogenesis *in vitro* and *in vivo*, suggesting its clinical application in bone regeneration procedure.

Keywords: Collagen-binding motif; Osteopontin; Alginate; Injectable gel; Immobilization; Osteoblastic differentiation; Osteogenesis

Phosphoproteins, such as osteopontin (OPN), bone sialoprotein (BSP), dentine sialoprotein, and osteonectin, play a pivotal role in the mineralization of collagen fibril, both in bone and dentine [1,2]. OPN and dentinal phosphophoryn have demonstrated apatite formation when complexed with collagen fibrils, suggesting the possibility that binding domains to collagen are initiators for mineralization in bone and dentine [3–6]. Together with other sequences containing glutamic acid-rich peptide domains known to induce hydroxyapatite nucleation, the collagen-binding domain of these proteins has also been considered as an essential component of mineralization in bone and teeth [7,8]. To this regard, the collagen-binding domain of OPN has been thought to play a role in mineral formation in bone and teeth matrices. Although a couple of reports have demonstrated the collagen binding site of BSP [1,8], the critical collagen binding from OPN as well as its biologic activity has not yet been established. In this report, we describe isolation of a conserved sequence responsible for collagen binding denoted as collagen-binding motif (CBM) from OPN and binding affinity of the CBM peptide and collagen.

The ability to deliver or present biologically active molecules such as peptide or growth factor over time through proper scaffold to the site of tissue regeneration offers tremendous therapeutic efficacy. Especially for local tissue regeneration for example, bone formation, the local concentration of the applied bioactive peptide should be securely maintained. To this regard, injectable polymeric gel was chosen to present and maintain enough concentration of CBM at the bone defect site. Alginate is a natural

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linear polysaccharide and has several favorable properties as biomaterials due to non-toxicity, biodegradability, and ease of processing into desired shape [9]. Covalently crosslinked alginate hydrogels have been used for wound healing [10], bridging peripheral nerve [11], and bone regeneration [12]. Combination of alginate with growth factor has been attempted to enhance healing capacity in bone regeneration area [13]. The carboxylic acid functional groups in alginate offer the chemical immobilization site for bioactive molecules. The RGD peptide and growth factor have been covalently immobilized to alginate and cell attachment and tissue regeneration were increased compared with non-immobilized alginate [9,12].

Herein, we demonstrate the identification of CBM peptide from OPN, binding affinity between collagen and CBM peptide, effect of immobilization of CBM peptide to alginate gel on *in vitro* and *in vivo* osteoblastic adhesion, growth, differentiation as well as *in vivo* bone formation in rabbit calvarial defect.

Materials and methods

Synthetic peptides. Peptides corresponding to residues 150–177 (CBM) and 53–80 (Control) of OPN were prepared in mass quantities using peptide synthesizer (APEX 396, AAPP TEC, Louisville, KY, USA).

Affinity of CBM peptide to collagen using the BIAcore binding assay. All experiments were performed at 25 °C on a BIAcoreX instrument (BIAcore AB, Uppsala, Sweden). Purified type I collagen (Chemicon) in acetate buffer (pH 4.0) was covalently immobilized on a dextran-covered sensorchip surface (CM5 chip) by using *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) as crosslinkers. To determine the binding kinetics of each prepared peptide, samples with various concentrations of peptide solutions were applied to collagen-immobilized surface during the association phase for 2 min at a rate of 30 μl/min.

Immobilization of peptide to alginate. Peptide was covalently attached to the alginate based on carbodiimide chemistry, resulting in the formation of an amide bond between the carboxylic acid groups of the alginate and the amine terminus of the peptide [14]. Alginate was dissolved in MES buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) to obtain a 1% (w/v) solution. EDC was added to activate the carboxylic acid groups (50 mg EDC/g alginate), followed by the addition of sulfo-NHS in the molar ratio 1:2 to EDC (28 mg sulfo-NHS/g alginate). The resulting conjugate solution of peptide-alginate was then lyophilized, stored in airtight tubes at −20 °C for future use.

Cell culture of bone marrow stromal cells on CBM peptide-alginate gel surface. Human bone marrow aspirates were obtained during pelvic osteotomy after obtaining informed consent and the approval of the Institutional Review Board of Seoul National University Dental Hospital. Bone marrow stromal cells (hBMSCs) were isolated as described previously [15]. Alginate gels with various amounts of CBM peptide used were prepared in the 96-well plates. The hBMSCs (1×10^4 cells/well) were allowed to adhere for 3 h at 37 °C in serum-free α-MEM containing 0.1% BSA. Attached cells were fixed with 4% paraformaldehyde and incubated in 0.2% crystal violet dye for 15 min. Wells were washed thoroughly with distilled water and the crystal violet dye was extracted with 2% SDS solution. Absorbance was measured at 540 nm using a microplate reader (BIO-TEK, Winooski, Vermont, USA). To measure inhibition of attachment by collagen, collagenase treatment, cells were harvested with trypsin-EDTA, and 1×10^4 cells/100 μl were treated with either collagen (5 μg/ml) or collagenase (0.2 U/ml), heparinase I (0.2 U/ml), and chondroitinase ABC (0.2 U/ml) for 1 h at 37 °C with mixing every 15 min. In addition, to determine the cell growth on the peptide-gel surface, the hBMSCs (1×10^5 cells/well) were allowed to adhere for 24 h at 37 °C and incubated for further 1, 7, 14, and 28 days on the gel with CBM peptides, followed by the measurement as described above. All assays were performed in triplicate, with each experiment repeated at least three times.

Confocal microscopic observation of cultured cells on gels with CBM peptide. Peptide-immobilized alginate gel (350 μg peptide in 0.5 mg gel) was placed in 4-well Lab-Tek II Chamber Slides (Nalgen, Nunc International, Naperville, IL, USA) and hBMSCs were applied onto the gel of peptide at a density of 1×10^5 cells/well and then incubated for 24 h at 37 °C. Adhered cells were fixed with 4% formaldehyde in PBS for 10 min, and then, permeabilized for 5 min with 0.1% Triton X-100 in PBS. Cells were incubated with anti-vinculin antibodies (Sigma–Aldrich) diluted 1:3000 in 0.1% BSA/PBS for 1 h and then incubated simultaneously with Alexa 546-conjugated goat anti-mouse secondary antibody (Ab) (4 μg/ml; Molecular Probes, Eugene, OR) and Alexa 488-conjugated phalloidin (0.67 U/ml; Molecular Probes) in 0.1% BSA/PBS for another 1 h. Nuclear staining was conducted with Hoechst 33342 (5 μg/ml, Molecular Probes, Eugene, OR) and then observed using an Olympus FV-300 Laser Scanning Microscope operated with FLUOVIEW software (Olympus, Tokyo, Japan).

Alkaline phosphatase (ALP) activity of cells on CBM peptide-gel. The hBMSCs (2×10^5 cells/ml) were seeded on peptide-immobilized alginate gels (350 μg peptide in 0.5 mg gel) and incubated with in α-MEM supplemented with 10% FBS, 1% antibiotic–antimycotic, 10 mM sodium β-glycerol phosphate, 50 μg/ml L-ascorbic acid, and 10^{-7} M dexamethasone. After 7, 14, and 28 days of culture, cells on peptide-immobilized gel were detached by using 2.5% trypsin in 4 mM EDTA , and centrifuged at 70*g* for 5 min, followed by sonification. Aliquots of cells were incubated with 0.1 M glycine–NaOH buffer, 15 mM *p*-nitrophenyl phosphate solution, and 0.1% Triton X-100/saline at 37 °C for up to 30 min. The production of *p*-nitrophenol in the presence of ALP was measured by monitoring the absorbance of the solution at a wavelength of 405 nm.

Matrix mineralization assay and Smad expression of cells on CBM peptide-gel. Calcein staining for detecting newly developed mineral by the cells was conducted as follows. The hBMSCs (2×10^5 cells/ml) were seeded on peptide-immobilized alginate gels (350 μg peptide in 0.5 mg gel) and incubated as the same method described above. The culture was supplemented with 1 μg of calcein during the observation period for ease of detecting mineralized ECM in the cells under a confocal laser microscope (FV-300, Olympus Co., Tokyo, Japan). In addition, intracellular Smad1/5/8 expression of hBMSC cell (1×10^6/well) grown CBM-gel was measured by Western blot analysis using antibody anti-Smad (1:2000) and anti-pSmad (1:1000).

In vivo bone formation by CBM peptide-gel. The rabbits were anesthetized with an intramuscular injection of xylazine (3.5 mg/kg body wt.). After dissecting the calvarial disc, the samples including CBM peptide-gel (4 mg) matrices were placed into the defect. The gel without the CBM peptide served as control. The animals were sacrificed 4 weeks after implantation. The retrieved specimens were fixed in a formalin solution and embedded in super low-viscosity Embedding Media (Polysciences Inc., Pittsburgh, PA, USA) without prior decalcification. The coronal sections (20 μm in thickness) were sliced and stained with Hematoxylin–Eosin (Sigma). Microscopic examination was conducted using an Olympus BH-2 optical microscope (Olympus Optical Co., Osaka, Japan). Histomorphometric measurements of newly formed bone were conducted using an automated image analysis system equipped with a CCD camera on a light microscope.

Statistical analysis. All values are presented as means ± SE for all control and experimental (N = total number of independent cultures). Data were analyzed by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) post hoc test (StatView; SAS Institute, Cary, NC, USA). p values of <0.05 were considered significant.

Results and discussion

Identification of CBM and binding affinity to collagen

Enzymatic digestion of OPN provided five different fractions of peptides depending on the affinity toward collagen.

Among the five fractions, the one with highest binding affinity to collagen was collected, purified, and synthesized in mass quantity for further experiments. The amino acid sequence of the peptide was corresponding to the residues 150–177 of human OPN (GLRSKSKKFRRPDIQYP DATDEDITSHM) and thus termed as collagen-binding motif (CBM) (Fig. 1A). The sequence consists of positively charged amino acids, such as Arg, Lys, His, as well as hydrophobic Met, Phe, Tyr, alternating with Asp. It has been known that amino acids, including Phe, Tyr, and Arg, are an essential component to create self-assembly, which derived from hydrophobic interactions [8,16]. Acidic amino acids, such as Asp and Glu, are responsible for calcium binding, which increases mineral formation [17]. Accordingly, four Asp and one Glu residues in CBM were expected to deposit calcium in assembly with collagen.

The other sequence from the fraction with lowest collagen affinity, corresponding to the 53–80 sequence of OPN, was prepared and served as control to the CBM (Fig. 1A). The direct binding kinetics of peptide to collagen was examined using surface plasmon resonance (SPR)-based BIAcore technology (Fig. 1B and C). Control peptide displayed negligible association even at highest concentration in comparison with that by CBM. In contrast, CBM exhibited a fast association rate with collagen biosensor surfaces which depends on the concentration of applied CBM. In addition, the binding of CBM to collagen was specific, as the CBM peptide did not provide similar peaks seen in Fig. 1B when applied to heparin-, fibronectin-, or chondroitin-bound chip (Fig. 1C). The mechanism of collagen binding of this CBM peptide has to be established, however, considering the amino acid sequence in CBM is enriched by seven basic amino acids (Arg, Lys, and His) than other collected fragments from OPN, and average of pK_a value for the carboxylic acid in collagen and amino of basic amino acid is 4.1 and 9.3, ionic interaction may participate in collagen binding of the CBM peptide. The existence of ionic interactions between type I collagen and other matrix proteins, including BSP, has been reported elsewhere [7,8].

Cell adhesion on peptide-immobilized alginate gel

Recent approach has involved the immobilization of bioactive molecules, including peptides, protein, enzyme, or growth factor [18–21], on the surface to achieve a stable cell proliferation as well as differentiation of the implanted biomaterials. In this study, peptide was initially applied to the alginate at ratios of 0.001, 0.01, 0.1, 0.5, 1.0, 5.0/100 mg alginate and the immobilization efficiency of each sample was about 70%, therefore the actual peptide immobilized in alginate was approximately 0.7, 7, 70, 350, 700, and 3500 μg, respectively.

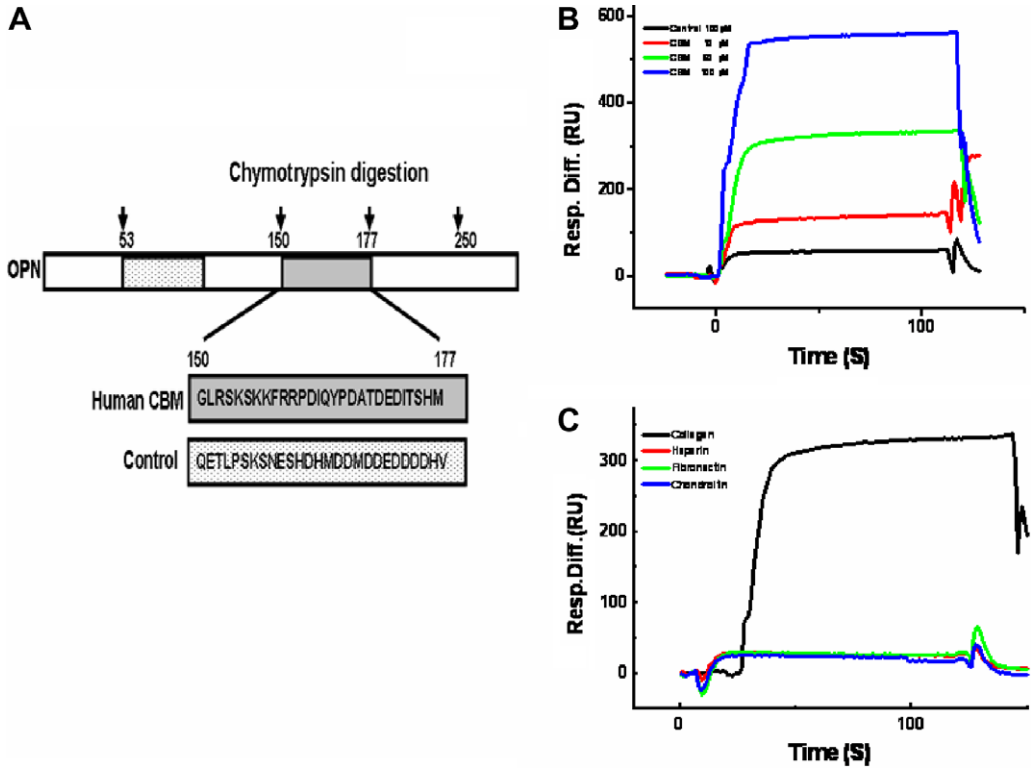

Fig. 1. The collagen-binding motif (CBM) peptide identified from osteopontin (OPN) by chymotrypsin digestion. (A) The CBM consists of amino acid sequence corresponding to that from 150 to 177 of OPN, and the peptide sequence corresponding to that from 53 to 80 of OPN served as control. (B) The binding of CBM peptide to type I collagen was measured by BIAcore analysis using various concentrations of CBM peptide (10, 50, and 100 μM). Control peptide was also measured by the affinity with collagen at 100 μM. (C) BIAcore analysis of CBM peptide to various extracellular matrix proteins including collagen, heparin, fibronectin, and chondroitin sulfate-immobilized chip. The concentration of CBM peptide was 50 μM.

Peptide-immobilized gel presented increased cell attachment in accordance with the amount of peptide during the immobilization process; however, it did not significantly increase further at the 100 μg of peptide amount (Fig. 2A), implying a saturation of cell attachment level could be achieved at peptide concentration of 100 μg/well. In addition, these results indicate that peptide quantitatively reacted with the alginate and was active in promoting cell adhesion. The control peptide did not increase cell attachment. Cell adhesion to CBM peptide was inhibited by the presence of 5 μg/ml collagen in the media (Fig. 2B). This result suggests that soluble collagen occupies the collagen-binding sites of peptide, interfering with ability of peptide to bind to cell surface collagen. Collagenase treatment decreased cell attachment to CBM by about 20%, compared to untreated cells. Treatment using heparinase and chondroitinase ABC had either less or no effect on cell attachment to CBM peptide. These results suggest that cell interaction with CBM can be mediated by cell surface collagen.

As seen in Fig. 2C, the CBM peptide-immobilized gel induced typical shape of actin stress (yellow) fiber of actively spread adherent cells as well as enhanced focal adhesion (red). Cells cultured on gel with either control peptide (Fig. 2D) or gel without peptide (Fig. 2E) showed few focal adhesions (red) at the cell membrane and actin stress fiber (green) in the cytosol. In addition, the cells pretreated with collagenase demonstrated few attachments even onto the CBM peptide-gel surface (Fig. 2F), indicating that the CBM peptide induced cell adhesion through binding with the cell surface collagen.

Cell growth and osteoblastic differentiation by CBM peptide-gel

The CBM peptide-immobilized gel exhibited higher cell proliferation in comparison with either gel with control peptide or gel without peptide and the level of growth was increased for 28 days (Fig. 3A). This result demonstrated that the immobilized peptide was maintained on the surface of gel without the leakage to the culture medium and allowed cell growth for up to 28 days, indicating that the CBM peptide-gel matrix can be employed as a scaffold for cell growth.

Fig. 3B shows the ALP activity in the cells grown on peptide-immobilized gel. The ALP is often used as a marker for increased osteoblastic metabolic activity and

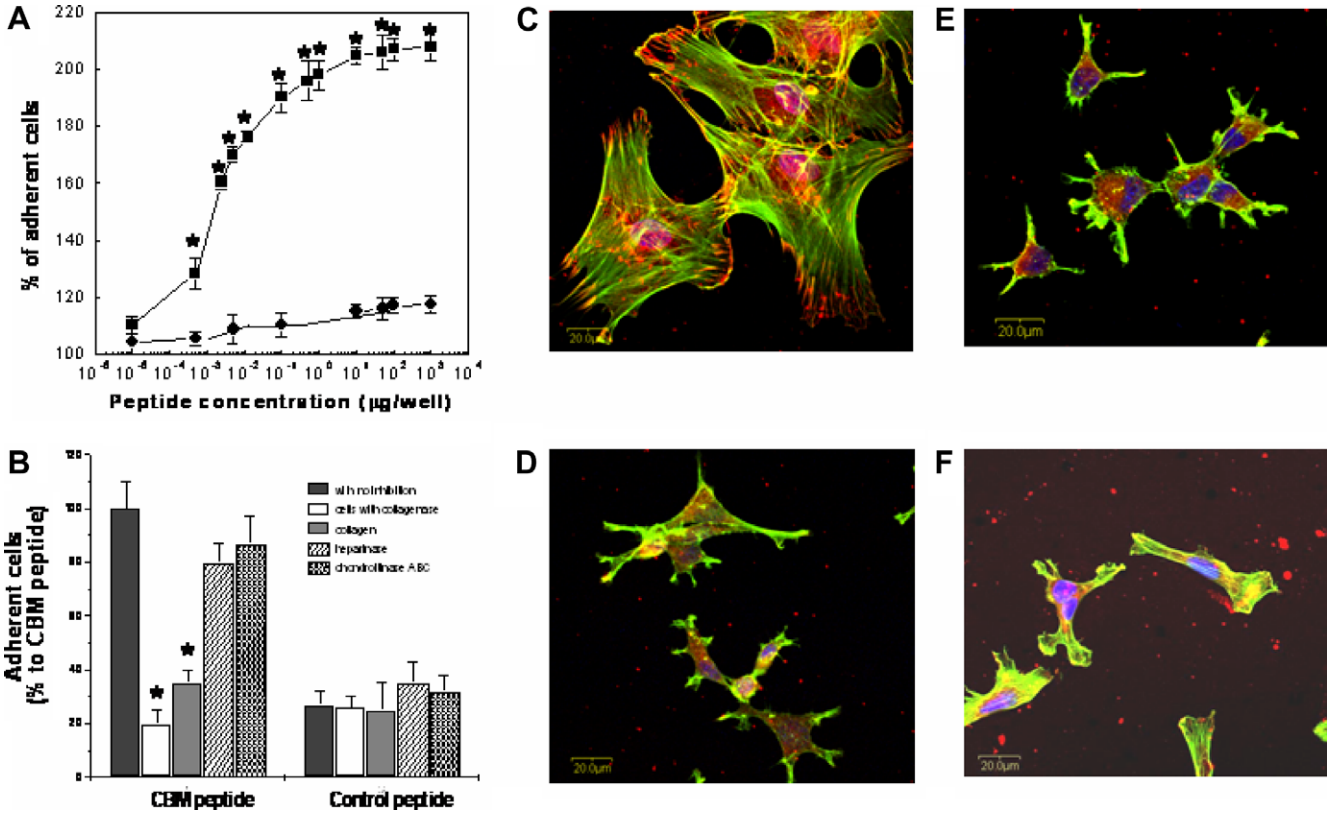

Fig. 2. (A) Effect of synthetic CBM peptide on hBMSC adhesion. Cell adhesion on (■) CBM peptide, (●) control peptide. Data obtained from triplicate experiments with at least four samples ($N = 4$) are presented as means ± SE for each peptide concentration and for control wells. $^{*}p < 0.05$, significantly different from control peptide. (B) Inhibition of cell attachment to CBM peptide by either collagen or enzyme treatment. Data are expressed as means ± SE. $^{*}p < 0.05$, significantly different from untreated cells. Morphology of attached hBMSCs on CBM-immobilized gel surface. The hBMSCs were applied to CBM-immobilized gel surface (C), control peptide immobilized surface (D), pretreated with collagenase then applied to CBM-immobilized gel surface (E), or applied BSA-coated well plate (F). Cells were incubated for 3 h, then fixed and stained for actin (green), focal adhesion (red), and nucleus (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

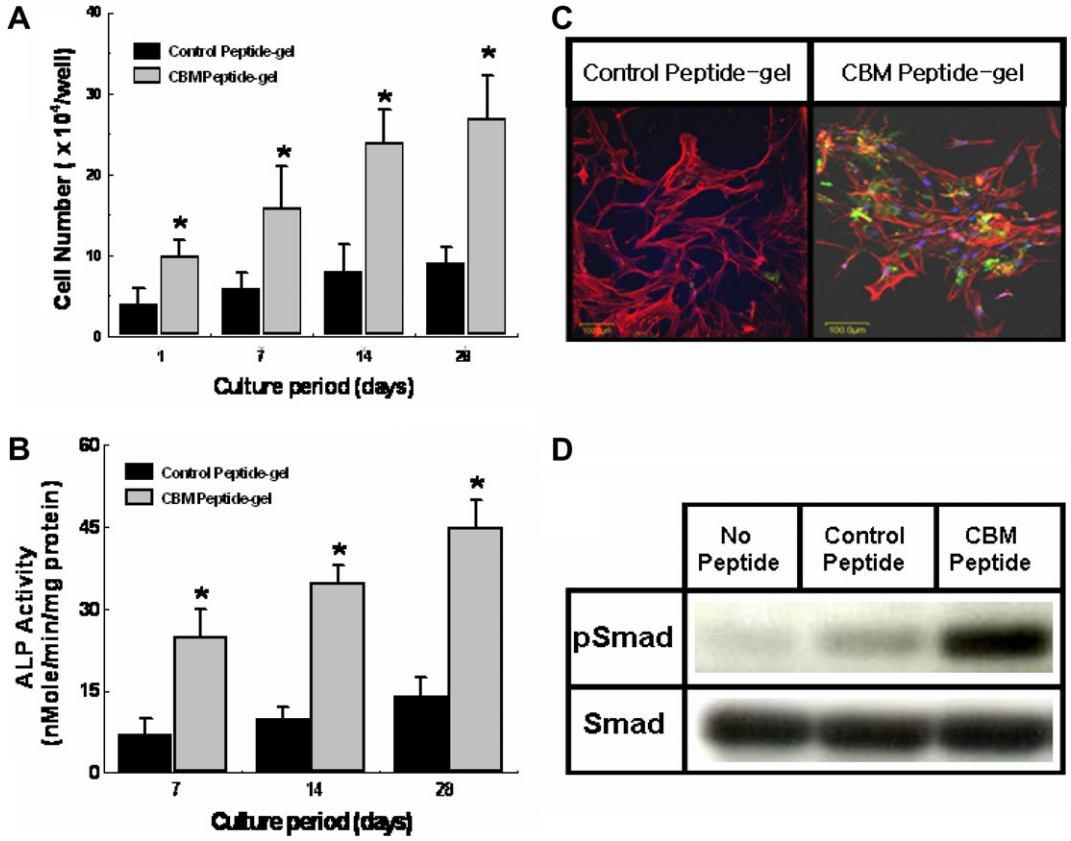

Fig. 3. (A) Effect of peptide-immobilized alginate gel on cell growth on CBM peptide-immobilized gel. Data represent means ± SE from triplicate experiments ($N = 4$). $^{*}p < 0.05$, as compared to no peptide-immobilized alginate at the same time point. (B) Effect of CBM peptide-immobilized alginate gel on the ALP activity of hBMSCS cultured for 7, 14, and 28 days. The data are expressed as nmol/min/mg protein and shown here as means ± SE from triplicate experiments ($N = 4$) $^{*}p < 0.05$, as compared to no peptide-immobilized alginate at the same time point. (C) Confocal micrographs of mineralization marker calcein incorporated into hBMSCs cultured on CBM peptide-immobilized gel surface (left) cells grown on control peptide-gel; (right) cells grown on CBM peptide-immobilized gel (original magnification: 80×). (D) Western blot analysis of the phospho-Smad (pSmad) expression of cells cultured on the well plate surface coated with CBM peptide-immobilized alginate gel.

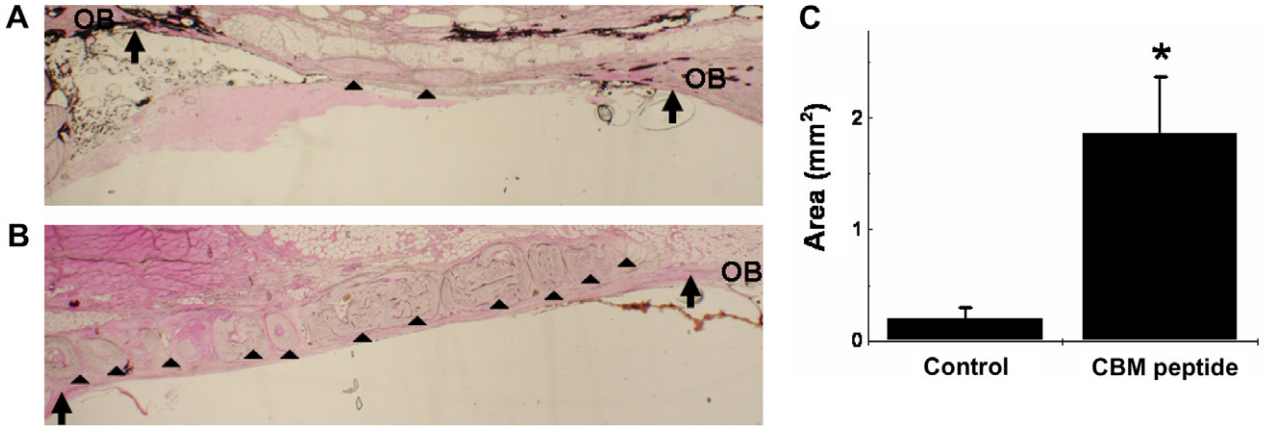

Fig. 4. Histological evaluation of CBM peptide-immobilized alginate gel in rabbit calvarial defects 4 weeks after implantation. (A) Negative control treated with gel without CBM peptide (original magnification 10×). (B) Bone defect treated with CBM peptide-immobilized alginate gel (original magnification 10×). Bone margin is indicated by an arrow (↑). New bone formation and bone marrow are indicated as arrowhead. The abbreviated terms, OB, NB, depict as old bone and new bone, respectively. (C) Histomorphometric result at 4 weeks. Total new bone is expressed as area (mm^2). $^{*}p < 0.05$, as compared to gel without peptide at the same time point.

an early indicator of osteoblastic differentiation [22]. Cells grown on gel conjugated with CBM peptide presented marked ALP activity up to 28 days, indicating enhanced osteogenesis by the peptide. Matrix mineralization was further examined in osteoblastic differentiation of hBMSCs cultures. Fluorescent yellow spots, indicative

of calcein uptake into newly mineralized extracellular matrix, were numerous when cells were grown on the gel with CBM peptide, but were barely detected in those cultured on either control peptide-immobilized gel or gel without peptide (Fig. 3C). When the cell undergoes osteoblastic differentiation inducing mineral creation, the level of Smad 1/5/8 is known to be increased. As seen in Fig. 3D, the expression of phospho-form of Smad (pSmad), activated form of Smad, was evident when cells were cultured on the surface of CBM peptide-gel while negligible expression was shown by gels with control peptide or without peptide, indicating that the CBM peptide of gel interacted with cell surface collagen and triggered intracellular Smad expression to direct osteoblastic differentiation.

Induction of bone formation in vivo

Fig. 4 shows histological sections of the rabbit calvarial defects with CBM-alginate gel at 4 weeks after surgery. No specimen revealed any evidence of infection or foreign body reaction, and all wounds showed a good healing response. In the negative control group where empty gel matrix without the peptide was applied, there was dense, fibrous connective tissue at the defect site with little amount of bone formation observed (Fig. 4A). In contrast, when CBM peptide was applied as a form of immobilized gel, the new bone formation originated from the bony borders directed toward the center of the defect (Fig. 4B). The magnified figure clearly demonstrates that newly formed bone already matured from the specimen treated with CBM-gel (Supplementary figure). The quantity of new bone was greater than that observed in the specimen treated with empty gel. Prominent new bone island was observed in the central region of the calvarial defects treated with CBM-gel, indicating active biomineralization *in vivo* condition. The quantification of the new bone area further proves that the CBM peptide-gel actively induced bone formation (Fig. 4C). Taken together, the applied gel with CBM peptide was able to induce significant new bone formation *in vivo*.

Conclusions

The synthetic collagen-binding peptide, termed CBM, was newly identified from osteopontin and chemically synthesized, immobilized to an alginate gel. The peptide presented specific collagen-binding affinity and was able to be immobilized onto the gel without any alteration of bioactivity. The gel with the CBM peptide induced increased cell adhesion, growth as well as osteoblastic differentiation of bone marrow stromal cells. *In vivo* study further confirmed the CBM peptide was active in bone formation when applied to the injectable gels. Taken together, CBM peptide-immobilized gel can be used as a bioactive scaffold for bone tissue regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.106.

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