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Effect of immobilized cell-binding peptides on chitosan membranes for osteoblastic differentiation of mesenchymal stem cells

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Two cell-binding domains from FGF-2 (fibroblast growth factor-2) were shown to increase cell attachment and osteoblastic differentiation. Two synthetic peptides derived from FGF-2, namely residues 36–41 (F36; PDGRVD) and 77–83 (F77; KEDGRLL), were prepared and their N-termini further modified for ease of surface immobilization. Chitosan membranes were used in the present study as mechanical supportive biomaterials for peptide immobilization. Peptides could be stably immobilized on to the surface of chitosan membranes. The adhesion of mesenchymal stem cells to the peptide (F36 and F77)-immobilized chitosan membrane was increased in a dose-dependent manner and completely inhibited by soluble RGD (Arg-Gly-Asp) and anti-integrin antibody, indicating the existence of an interaction between F36/F77 and integrin. Peptide-immobilized chitosan supported human bone-marrow-derived mesenchymal-stem-cell differentiation into osteoblastic cells, as demonstrated by alkaline phosphate expression and mineralization. Taken together, the identified peptide-immobilized chitosan membranes were able to support cell adhesion and osteoblastic differentiation; thus these peptides might be useful as bioactive agents for osteoblastic differentiation and surface-modification tools in bone regenerative therapy.

Introduction

The application of growth factor to enhance tissue regeneration has resulted in good achievements in the field of tissue engineering and regeneration therapy [1–5]. In particular, the basic FGF-2 (fibroblast growth factor-2) is of interest as an important cytokine that induces the early healing process in bone and cartilage repair [6–8]. rhFGF-2 (recombinant human FGF-2) has been applied as a bioactive agent for bone repair in combination with a matrix such as collagen or demineralized bone [9,10]. The mechanism of FGF-2 involves its binding with an FGFR (FGF receptor), integrin or proteoglycan on the cell surface [11–16]. Despite its great potential as a tool for enhancing tissue-healing efficacy *in vivo*, clinical applications of controlled growth-factor release are hindered by two major shortcomings. One is that many of the growth factors have short biological half-lives. For example, when PDGF-BB (platelet-derived growth factor-BB) or TGF-β1 (transforming growth factor-β1) is intravenously injected, the compound's half-life ($t_{1/2}$) is less than 10 min *in vivo* [4,5]. The use of the peptides in regenerative therapy might have advantages over using the whole growth factor in terms of overcoming possible immunogenicity, short $t_{1/2}$ and tumour-related side effects. Peptides can be synthesized on a large scale, and the N-terminus of these peptides can be modified for ease of immobilization to present a high density of peptides. Therefore it was anticipated that the peptide in combination with biomaterials would provide effective regeneration capacity while reducing the shortcomings of whole growth factors. Mechanical supports of these peptides are required for enhancing cell adhesion, thereby inducing an early bone-repairing effect. Chitosan is an attractive biopolymer that has been widely utilized for tissue-repair

Key words: bone regenerative therapy, cell-binding domain, chitosan membrane, fibroblast growth factor-2, mesenchymal stem cell, osteoblastic differentiation.

Abbreviations used: ALP, alkaline phosphate; ALPase, alkaline phosphatase; α-MEM, α-minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; Fmoc, fluoren-9-ylmethoxycarbonyl; hBMSC, human bone-marrow-derived mesenchymal stem cell; hMSC, human mesenchymal stem cell; IRB, Institutional Review Board; KNIH, Korean National Institute of Health; OCPC, *o*-cresolphthalein complexone; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

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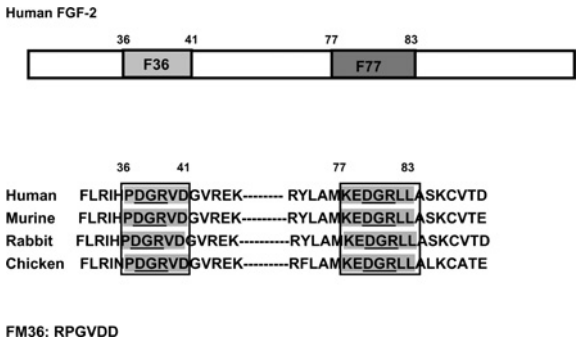

Figure 1 The amino acid sequences of mature FGF-2 across several species and the amino acid sequences of synthetic peptides

The synthetic peptide sequences in human, murine, rabbit and chicken FGF-2 are highly conserved and are highlighted in grey. F36 consists of six amino acids corresponding to the sequence between 36 and 41. F77 consists of seven amino acids corresponding to the sequence between 77 and 83. The two peptides include an RGD (underlined), and a GGC motif was introduced to the N-terminus of the peptides to improve the ease of conjugation on the surface of chitosan membranes. FM36 is a mismatched peptide of F36.

purposes, including bone and cartilage regeneration, and can be used as a supporting scaffold for cells [17,18]. Chitosan membrane, in the present study, was modified with the peptides, and we sought to examine whether the peptide enhanced the bioactivity of cells on the membrane.

We designed synthetic peptides derived from the cell-binding domain of FGF-2 that can support cell attachment and differentiation of mesenchymal stem cells into osteoblastic cells. Two different sequences of binding motif from FGF-2, F36 (PDGRVD) and F77 (KEDGRLL) were identified and chemically synthesized. FM36 is a mismatched peptide of F36 and was used as a control of F36 in the experiment to demonstrate the importance of RGD sequence for cell attachment and osteoblastic differentiation (Figure 1). In the present study, F36 and F77 peptides were immobilized on to the surface of chitosan membranes using a heterobifunctional cross-linker. Using hMSCs (human mesenchymal stem cells), we examined the effect of soluble RGD (Arg-Gly-Asp) and anti-integrin antibody on cell attachment to the peptide-immobilized chitosan membrane. Osteoblastic differentiation by immobilized peptide was also demonstrated by ALPase (alkaline phosphatase) activity and calcium deposition.

Materials and methods

Materials

α-MEM (α-minimal essential medium), DMEM (Dulbecco's modified Eagle's medium), HBSS (Hanks balanced salt solution), trypsin/EDTA, FBS (fetal bovine serum) and antibiotic/antimycotic solution were purchased from Gibco BRL (Grand Island, NY, U.S.A.). BSA was purchased from Sigma (St. Louis, MO, U.S.A.). The antibodies that block α1–α6 and β1 integrin function (β1 Integrin Partners Investigator kit) were purchased from Chemicon (Temecula, CA, U.S.A.). Other chemicals were obtained from Sigma.

Peptide synthesis

All peptides (Figure 1) were manually synthesized by the Fmoc (fluoren-9-ylmethoxycarbonyl) strategy using a peptide synthesizer (APEX 396; AAPP TEC, Louisville, KY, U.S.A.) and were prepared in the C-terminal amide form as described previously [19]. Amino acid derivatives and resins were purchased from AAPP TEC. The respective amino acids were condensed manually in a stepwise manner using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. *N*-dimethylformamide was used during the synthesis as a solvent. The resulting crude peptides were precipitated and washed with diethyl ether and were then purified by reverse-phase HPLC (using a Vydac C_8 column and a gradient of water/acetonitrile containing 0.1 % trifluoroacetic acid). The purity of each peptide was above 90 %.

Immobilization of peptides

The amount of immobilized peptide on the well plates was investigated by the method of Park et al. [20] with some modifications. The peptides were labelled with FITC at their N-termini [21]. Chitosan (98% deacetylated; molecular mass 300 kDa; Sigma) membranes were prepared by casting 2% (w/v) solution of chitosan in 2% acetic acid into non-tissue-culture plates (500 μl per well for 24 wells and 100 μl per well for 96 wells). Chitosan membranes were air-dried for 2 days, then neutralized with 0.1 M NaOH for 1 h, then washed with doubly distilled water until the pH was neutral. The chitosan membrane was allowed to react with SMCC [succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; 50 mM; Pierce] for 4 h to activate the amine groups in the chitosan. Various concentrations of FITC–peptide in PBS (pH 7.4) were added to the SMCC-linked chitosan and the reaction was allowed to take place at 4 °C overnight and the chitosan then washed three times with 8 M urea and PBS to remove unchanged peptides. The absorbance of FITC–peptide was detected at 494 nm using a microplate reader (BioTek, Winooski, VT, U.S.A.) [22]. The standard curve was then used to immobilize peptide with input concentration of peptide solutions for the 96-well plates. The plates were coated with 100 μl of 2% (w/v) BSA in PBS for 1 h, and then washed with PBS. These plates were then employed for the cell-adhesion and inhibition studies described below.

Cell culture

Human bone-marrow aspirates were obtained during pelvic osteotomy after obtaining informed consent and the approval of the IRB (Institutional Review Board) of KNIH (Korean National Institute of Health) and Seoul National

University. hMSCs were isolated from three patients (aged 23–40 years) as described previously [23] and plated in T75 flasks for continuous passaging in DMEM supplemented with 20% (v/v) FBS (Gibco BRL) and 1% antibiotic/antimycotic solution (Gibco BRL). The medium was changed twice weekly and cells were detached using trypsin/EDTA and passaged into fresh culture flasks at a ratio of 1:4 on reaching confluence. Cultures were incubated at 37°C under a humidified atmosphere containing 95% air and 5% CO_2. Overall procedure for obtaining cells from patients was approved by both the IRB of KNIH and the IRB of Seoul National University Dental Hospital.

Cell adhesion assay

The cell-adhesion assay was performed by the method of Mochizuki et al. [19]. Cells (1×10^4 cells per 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% (w/v) 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% (w/v) SDS solution. Then, the A_{540} of each well was measured using a microplate reader (BioTek). The absorbance of the Crystal Violet solution from the BSA-coated chitosan membrane without peptide treatment served as the control, and the A_{540} of the BSA-coated well was set to 0%. The percentage of adherent cells was determined by using the following equation:

$$\text{Percentage of adherent cells} = \left(\frac{\begin{array}{c}\text{absorbance of Crystal Violet solution} \\ \text{from peptide-immobilized chitosan}\end{array}}{\begin{array}{c}\text{absorbance of Crystal Violet} \\ \text{solution from the control}\end{array}} \times 100 \right) - 100$$

To examine the change in attachment due to the addition of RGD or heparin, 1 mM RGD or 20 μg/ml heparin was added to the cell suspensions before the cells were seeded on to the well, and the absorbance of the cultured wells was measured as described above. In addition, the effect of an integrin-binding antibody on cellular attachment was examined as described above, except for pretreatment with a functional integrin-blocking antibody (10 μg/ml) for 30 min at 37°C, with vortex-mixing every 5 min before plating. After 3 h of incubation, the attached cells were quantified as described above. All assays were performed in triplicate, with each experiment repeated at least three times.

Actin cytoskeleton of cultured cells

The 100-μM-peptide-immobilized chitosan in four-well Lab-Tek II Chamber Slides (Nalgen Nunc International, Naperville, IL, U.S.A.) were prepared by the same method as described above. Then, hMSCs (1 ml, 1×10^4 cells) were added and incubated at 37°C for 3 h. Cells were fixed with 4% formaldehyde in PBS for 10 min and permeabilized for 5 min with 0.1% Triton X-100 in PBS. Cells were blocked for 1 h with 1% BSA in PBS and incubated with anti-vinculin antibodies (Sigma–Aldrich) diluted 1:400 in 1% BSA/PBS for 1 h, followed by incubation for another 1 h with the mixture of Alexa Fluor® 546-conjugated goat anti-mouse secondary antibody (4 μg/ml; Molecular Probes, Eugene, OR, U.S.A.) and Alexa Fluor® 488-conjugated phalloidin (0.67 unit/ml; Molecular Probes) in 0.1% BSA/PBS according to the manufacturer's protocol. Nuclear staining was conducted with Hoechst 33342 (5 μg/ml, Molecular Probes) according to the manufacturer's protocol. Coverslips were mounted on to slips using ProLong Gold (Molecular Probes). The images of cells in each peptide-treated well were acquired using an Olympus FV-300 laser scanning microscope operated with FLUOVIEW software (Olympus, Tokyo, Japan).

Osteogenic differentiation

The 100-μM-peptide-immobilized chitosan membranes in 24-well plates (Nalgen Nunc International) were prepared by the same method as described above. To examine the osteogenic differentiation of cells, the hMSCs (1×10^4 per well) were cultured under an osteogenic medium (α-MEM containing 15% FBS, 1% antibiotic/antimycotic solution, 10 mM sodium β-glycerol phosphate, 50 μg/ml L-ascorbic acid and 10^{-7} M dexamethasone) for 14 days. The medium was changed twice a week. The ALPase activity was measured by fixing cultured cells with 4% (w/v) paraformaldehyde and staining them with a mixed solution of naphthol and Fast Red Violet according to the manufacturer's instructions for the ALP (alkaline phosphate) staining kit (Chemicon). The level of ALPase activity was also measured spectroscopically. After 14 days of culture, cells were washed with PBS, treated with 2.5% trypsin in 4 mM EDTA to detach cells, and centrifuged at 70 ***g*** for 5 min. The cells were sonicated on ice (Sonic dismembrator 550; Fisher, Pittsburgh, PA, U.S.A.) for 1 min at 110 W (50/60 Hz). Aliquots of the cells were incubated with 0.1 M of glycine/NaOH buffer, 15 mM of *p*-nitrophenyl phosphate solution and 0.1% Triton X-100/saline at 37°C for up to 30 min. The reaction was quenched by adding 0.1 M NaOH and placing the mixture on ice. The production of *p*-nitrophenol in the presence of ALPase was measured by monitoring the absorbance of the solution at a wavelength of 405 nm. Protein concentrations of the cell lysates were determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). ALPase activity is expressed as nmol of *p*-nitrophenol released/min per mg of protein.

The calcium phosphate precipitates from the cell cultures were stained using Alizarin Red S, as described elsewhere [19]. In brief, the cultured cells were fixed with 95% ethanol (4°C, 15 min), washed with deionized water, and then treated with 2% Alizarin Red S (Sigma) for 10 min.

The wells were washed several times with deionized water to remove the unbound stain and photographed using an Olympus inverted microscope. The calcium content in the cell lysates were measured according to a previously published method using Sigma diagnostic kit no. 587-A [24], which measures the amount of purple-coloured calcium–OCPC (*o*-cresolphthalein complexone) complex formed as a result of binding between OCPC and free calcium generated by cells. A 0.5 ml portion of the lysates was incubated at room temperature (25 °C) overnight with 0.5 ml of acetic acid (1.0 M) in order to extract calcium from mineralized constructs. A 10 μl portion of the samples was taken and placed in a 96-well plate. Equal amounts (150 μl) of calcium-binding reagent (OCPC, 0.24% 8-hydroxyquinoline and surfactant) and calcium buffer reagent (500 mM 2-amino-2-methylpropane-1,3-diol) supplied with the kit were added to each well, and the plate was incubated at room temperature for 10 min. The standards in concentrations ranging from 5 to 100 μg/ml were prepared using $CaCl_2$. The attenuance of each well was measured using a microplate reader at 575 nm. The calcium content from each sample was normalized to the total protein, determined by the Bradford protein assay (Bio-Rad Laboratories), at each time point.

Statistical analysis

All values are presented as the means ± S.E.M. for all control and experimental data (n = total number of independent cultures). Data were analysed by one-way ANOVA followed by Fisher's PLSD (protected least-squares difference) *post hoc* test (StatView; SAS Institute, Cary, NC, U.S.A.). A P value of < 0.05 was considered significant.

Results and discussion

Immobilization of peptide on to the chitosan membrane

Synthetic and natural biomaterials have been utilized clinically in tissue-regenerative procedures. Chitosan, which is a co-polymer of *N*-acetylglucosamine and glucosamine and is obtained from chitin through its deacetylation, is now well known for its numerous and interesting biological properties as a biocompatible, bioresorbable and bioactive polymer [17,25]. However, these materials alone are less bioactive towards cells *in vivo*; thus the surface immobilization approach using growth factors has been of interest to the medical and pharmaceutical community to increase the biological activity of the materials [20]. However, the application of growth factors itself has been beset by limitations, including a short $t_{1/2}$ *in vivo* as well as side effects caused by overdoses to compensate for the short $t_{1/2}$ [26–30]. Therefore it would be useful to have stable peptides with long-lasting activity that would have a direct effect on cells at the applied site of injury. By modifying the surface with FGF-2-derived synthetic peptides, these workers attempted to generate a biomimetic surface for tissue regeneration. Peptide-modified surfaces promoted cell attachment and osteoblastic differentiation with sequence-specific activity *in vitro*, implying the peptides' usefulness in preparing surface-active biomaterials for tissue engineering.

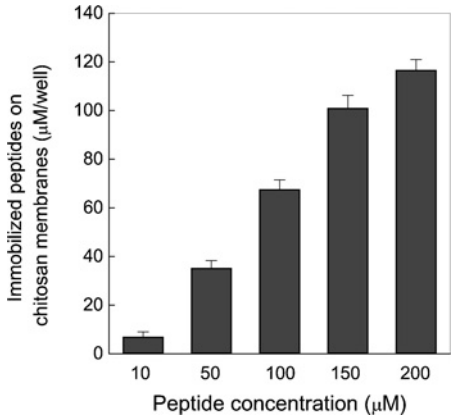

Figure 2 Immobilization of FITC-labelled peptide on the surface of chitosan membranes in accordance with the concentration of the initial amount of applied peptide (10, 50, 100, 150 and 200 μM)

Surface-immobilized peptide was measured as described in the Materials and methods section. Results were obtained from three sets of separate experiment with at least four samples ($n = 4$) and are expressed as means ± S.E.M.

Various RGD peptides were coated on titanium, silk, hydroxyapatite and polymer to promote cell functions such as attachment and differentiation [31–34]. Most RGD peptides were identified from extracellular-matrix components such as fibronectin and bone sialoprotein [35,36]. Two inverse sequences of the RGD cell-adhesive domains in FGF-2 were identified in a previous study [14]. However, the present study demonstrated that whole FGF-2 interacts with $\alpha v \beta 3$ integrin, and this interaction mediates the capacity of the angiogenic growth factor to induce adhesion and mitogenesis of endothelial cells. The RGD sequence is present in FGF-2, but the effects of those sequences on bone regeneration have not yet been demonstrated. Therefore we synthesized peptides F36 and F77, which contain the RGD sequence, and we investigated the biological activity on the osteoblastic differentiation of hMSC by F36 and F77.

Two bioactive peptides (F36 and F77) were synthesized using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry with a C-terminal amide, whereas the N-terminus was further modified with a spacer dipeptide (Gly-Gly) and cysteine for ease of further modification. The thiol functional group of cysteine was chemically immobilized on the amine group of chitosan by forming S–S bond through SMCC. The immobilization efficiency of peptide on plates was approx. 70% of initial peptide concentration (10–100 μM) and decreased to 60% at 200 μM (Figure 2). The immobilized peptide

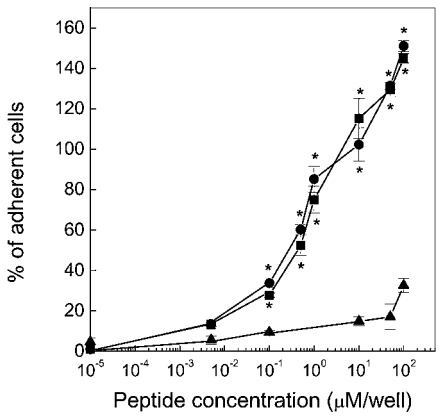

Figure 3 Effects of peptides on hBMSC adhesion

The cells were allowed to attach to F36-, F77- and FM36-immobilized chitosan membranes for 3 h and then were stained with Crystal Violet and detected as described in the Materials and methods section. The extent of adherent cells on the peptide-immobilized chitosan membranes was determined by measuring A_{540} and was expressed as a percentage of the absorbance measured in BSA-coated chitosan membrane. Results are presented as means ± S.E.M. for the control and for each concentration of peptide, and triplicate experiments were performed (n is at least 4). Symbols: ■, F36; ●, F77; ▲, FM36. *Significantly different from the control (cells on the FM36-immobilized chitosan membranes; $P<0.05$).

on chitosan membrane was not increased in proportion to input of peptide above 100 μM; therefore we used 100-μM-peptide-immobilized chitosan membranes in the *in vitro* test. To compensate for the decreased efficiency, excess concentration of peptide solution was applied to the well plates to reach the 100 μM peptide concentration, which was confirmed by quantification, as described in the Materials and methods section, of immobilization of peptide. The peptide concentration used in the cell-culture experiment was that of the immobilized peptide.

Cell adhesion on peptide-immobilized surface

The mismatching sequence of F36, termed as FM36, was used as a control for these peptides, whereas 1% BSA-coated chitosan membrane served as a negative control. The hMSCs were used to evaluate the cell-adhesion activity of the peptide. As seen in Figure 3, the peptides induced higher cell attachment in sharp contrast with the mismatched peptide (FM36), which showed poor cell attachment. F36 and F77 presented increased cell attachment in accordance with the concentrations of peptide, showing a saturation of the cell attachment level at

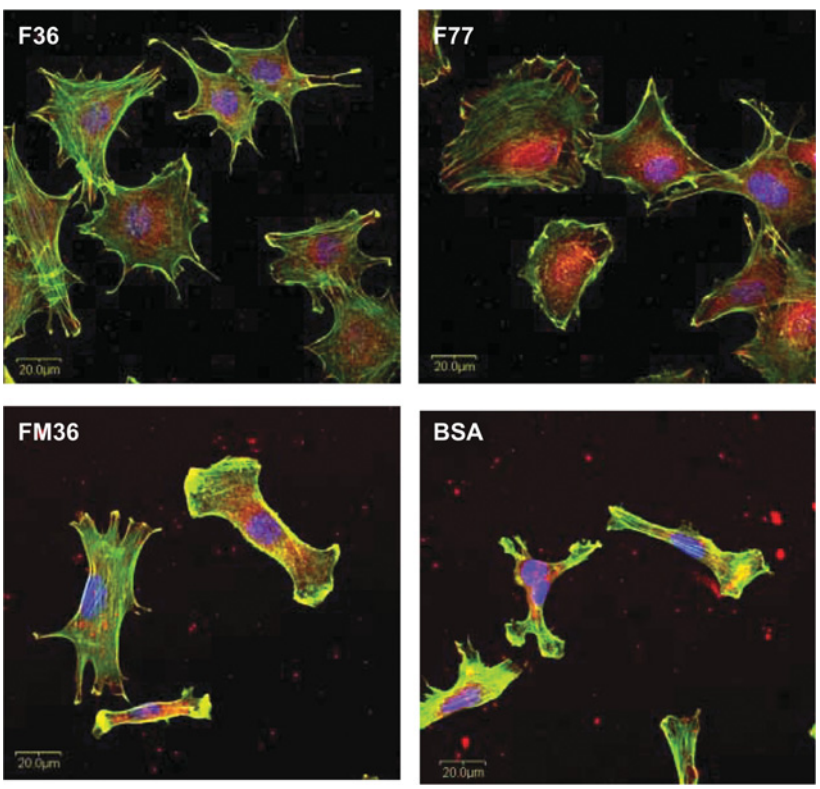

Figure 4 Morphology of attached hMSCs on a peptide-immobilized chitosan membranes

F36, F77 and FM36 (100 μM) were immobilized as described in the Materials and methods section. Cells were incubated for 3 h on each peptide and then were fixed and stained for actin (green), focal adhesion (red) and nucleus (blue). Actin stress fibres were visualized with phalloidin, focal adhesion was visualized with vinculin, and the nucleus was visualized with Hoechst 33342.

concentrations above 100 μM. The mismatching sequence, however, attenuated the adhesive activity of the F36 peptide towards cells, indicating that the sequences of F36 and F77 were specific cell-binding regions of FGF-2. The number of cells adherent to the peptide-immobilized surface was specific to the sequence and also depended on the amount of applied peptide. FM36 significantly reduced cell-adhesive activity when compared with F36, indicating that the cell surface specifically recognizes peptide sequences of F36.

Cell morphology

We compared the organization of the cytoskeleton of hBMSCs (human bone-marrow-derived mesenchymal stem cells) attached on the peptide-immobilized chitosan membrane by staining with vinculin and phalloidin to visualize focal adhesion and actin stress fibres respectively (Figure 4). Cells cultured on the BSA-coated chitosan served as controls and showed unspread shapes with poor microfilament bundles. The FM36 showed little actin stress fibre and no focal-adhesion complex in the cell membrane although it seemed to be having a more spread morphology than BSA-coated wells. The difference in morphology between BSA and FM36 was not so significant; thus FM36 could not support the cell attachment. However, in contrast with the control, F36 and F77 induced the shapes typical of actin stress fibre as well as vinculin-containing focal adhesion at the end of actin stress fibres (Figure 4). In the cell attachment test, we use the cell culture medium without FBS, which promotes cell attachment, to demonstrate the activity of peptide for cell attachment. The presence of RGD sequence in F36 and F77 enhanced cell attachment in short incubation times. The focal-adhesion and actin-stress-fibre formation in the cells also confirms that integrin may be involved in the cell adhesion to the peptides.

Interaction of peptide with cell surface molecules

We next evaluated the effect of RGD and heparin on the hMSCs' attachment to the peptide-immobilized chitosan membranes. Cell adhesion to the peptides F36 and F77 was inhibited by soluble RGD, but not by heparin (Figure 5A). RGD-dependent cell adhesion of hMSCs was studied through inhibition of cell adhesion assays using soluble RGD peptides as inhibitors [37]. If the cell adhesion interfered with heparin, this means the peptides bind to cell-surface heparan sulfate proteoglycans [38,39]. This result indicates that the cells attached to F36 and F77 in an RGD-dependent manner, suggesting integrin-mediated cell adhesion.

Antibody-blocking experiments were conducted to verify the type of integrin subunit that the peptides recognize (Figure 5B). The hMSCs express several integrin subunits including α1, α2, α3, α5, α6 and β1 [40]. As peptides F36 and F77 contain an RGD sequence, an antibody specific to integrin partially blocked adhesion to these peptides. Among antibodies against α1–α6 and β1, antibodies against α3, α5 and β1 showed inhibition effect

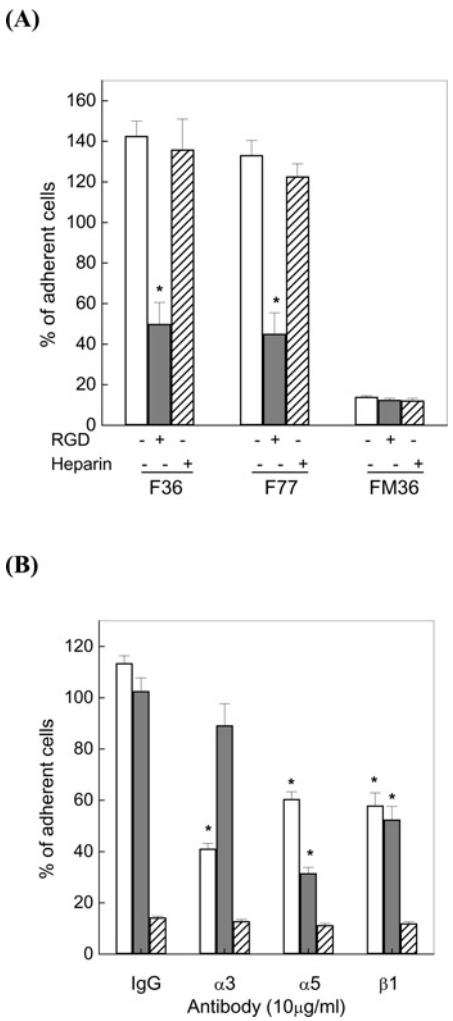

Figure 5 Effects of RGD and heparin (A) and integrin-blocking antibodies (B) on cell attachment to peptides

(**A**) The effect of RGD and heparin on cell attachment to peptides. F36, F77 and FM36 (100 μM) were immobilized on chitosan membranes as described in the Materials and methods section. The hMSCs were allowed to attach to the peptides in the absence or presence of 1 mM RGD or 20 μg/ml heparin added to the cell suspensions, and then the cells were plated. After 3 h of incubation, the attached cells were assayed by Crystal Violet staining. The results were obtained from at least three similar experiments, and means $\pm$ S.E.M. are indicated (*n* is at least 4). *Significantly different from control (cells that were not treated with RGD or heparin; $P<0.05$). (**B**) Effect of integrin-blocking antibodies on cell attachment to peptides. F36, F77 and FM36 (100 μM) were immobilized on chitosan membranes as described in the Materials and methods section. The hMSCs were allowed to attach to the peptides in the absence or presence of integrin-blocking antibodies added to the cell suspensions, and then the cells were plated. After 3 h of incubation, the attached cells were assayed by Crystal Violet staining. The results were obtained from at least three similar experiments, and means $\pm$ S.E.M. are indicated (*n* is at least 4). *Significantly different from control (IgG-treated cells; $P < 0.05$). Symbols: □, F36; ■, F77; ▨, FM36.

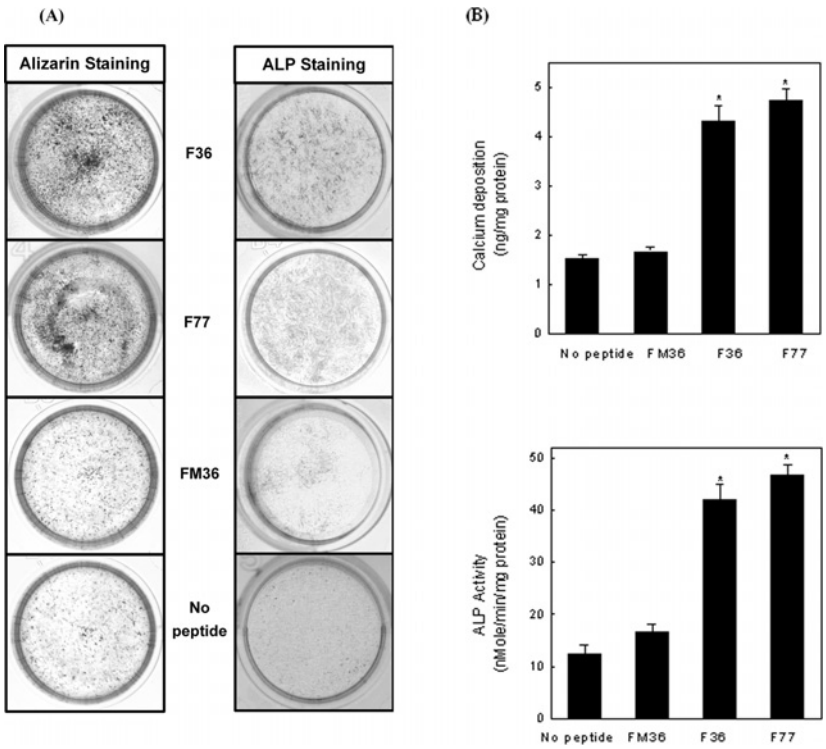

Figure 6 Effects of peptides on osteoblastic differentiation and matrix mineralization

(**A**) F36, F77 and FM36 (100 μM) were immobilized on chitosan membranes as described in the Materials and methods section, and hMSCs were cultured for 14 days. The ALP expression and mineralized matrix were visualized by ALP staining and Alizarin Red S staining respectively. (**B**) Measurement of calcium deposition and the ALP activity. The experiment was performed three times. *Significantly different from control (cells on the no-peptide-immobilized well; $P<0.05$).

on cell attachment to F36 and F77. The blocking antibodies against α3, α5 and β1 integrin subunits inhibited cell adhesion to the F36 peptide by 60, 50 and 50% respectively. Antibodies against α5 and β1 reduced adhesion to F77 by 70 and 50%. FM36 was not affected by the presence of antibodies against α3, α5 and β1. The inhibition of adhesion to peptides F36 and F77 by the addition of RGD and integrin antibodies demonstrated that integrin on the cell surfaces may be recognized by the peptides F36 and F77.

Osteogenic differentiation and mineralization

Figure 6(A) shows the expression of ALPase and mineralization in the cells on the F36- and F77-immobilized chitosan membranes. The ALPase enzyme is often used as a marker for increased osteoblastic metabolic activity and an early indicator of osteoblastic differentiation [41]. The expression was apparent in the cells cultured on F36- and F77-immobilized chitosan membranes. Cells treated with the mismatched peptide (FM36) did not display any significant ALPase expression, indicating the sequence-specific activity of the peptides. *In vitro* matrix mineralization was examined as an end-point indicator of the osteoblastic phenotype and the ultimate demonstration of the activity of F36 and F77 (Figure 6A). Enhanced mineralization was seen for cells cultured on the F36- and F77-immobilized chitosan membranes, in sharp contrast with either those cultured on the mismatched-peptide- or no-peptide-immobilized chitosan membranes. These results also demonstrate that the presence and the activity of immobilized peptides were maintained for 14 days in culture medium under cell culture conditions. When osteoblastic cells are cultured *in vitro*, mineralization, an indicator of late osteoblastic differentiation, occurs after 28 days [2,42]. Therefore, by using peptides, the time for differentiation could be shortened to 14 days.

The levels of ALPase activity and calcium were quantified and are shown in Figure 6(B), indicating that the peptide immobilized to the cells raises ALPase and mineralization induction, as compared with the cells on the no-peptide-immobilized chitosan, by 3–3.5-fold. The two peptides are sufficient to drive hMSCs into osteogenic differentiation, and they can be developed as therapeutic tools for bone regeneration. The number of glycine residues in the N-terminus of F36 and F77 can be modified to change the bioactivity of peptides. The N-terminus of the peptides can be further modified by introducing another sequence, and this makes it possible to immobilize the peptides on to biomaterials. It is beneficial to maintain the activity of the peptides for

a longer time by prolonging their presence on the surface of biomaterials. A major finding of the present study is that the peptides derived from the RGD domain of FGF-2 had good cell-attachment activity and osteogenic differentiation capacity. These peptides can be applied to biomaterials for tissue engineering to provide a bioactive/bioadhesive surface.

Conclusions

Two synthetic cell-binding peptides, F36 and F77, were immobilized on the chitosan membranes and tested for biological activity on osteoblast differentiation. They increased cell attachment in a dose-dependent manner by interaction with cell-surface integrin. Furthermore, the peptides supported the differentiation of hBMSCs into osteoblastic cells, as demonstrated by ALPase activity and mineralization. Taken together, the cell-binding peptides from FGF-2 could be applied on the surface of biomaterials for enhanced osteoblastic differentiations and bone regeneration.

Funding

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