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Identification and characterization of a novel heparin-binding peptide for promoting osteoblast adhesion and proliferation by screening an *Escherichia coli* cell surface display peptide library

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Heparin/heparan sulfate (HS) plays a key role in cellular adhesion. In this study, we utilized a 12-mer random *Escherichia coli* cell surface display library to identify the sequence, which binds to heparin. Isolated insert analysis revealed a novel heparin-binding peptide sequence, VRRSKHGARKDR, designated as HBP12. Our analysis of the sequence alignment of heparin-binding motifs known as the Cardin–Weintraub consensus (BBXB, where B is a basic residue) indicates that the HBP12 peptide sequence contains two consecutive heparin-binding motifs (i.e. RRSK and RKDR). SPR-based BIAcore technology demonstrated that the HBP12 peptide binds to heparin with high affinity (K_D = 191 nM). The HBP12 peptide is found to bind the cell surface HS expressed by osteoblastic MC3T3 cells and promote HS-dependent cell adhesion. Moreover, the surface-immobilized HBP12 peptide on titanium substrates shows significant increases in the osteoblastic MC3T3-E1 cell adhesion and proliferation.

Keywords: heparin; cell adhesion; titanium; BIAcore; osteoblast

Introduction

Heparin/HS and HSPGs are a group of macromolecules, which are characterized by long, unique carbohydrate chains attached to a protein core [1,2]. They exist as components of the ECM or as membrane-associated proteins on the surface of every eukaryotic cell and participate in diverse biochemical and physiological processes including cell adhesion and growth [3–5]. Heparin is a negatively charged polysaccharide consisting of a regular disaccharide repeat sequence (α-L-iduronic acid $\rightarrow$ D-glucosamine) with a high degree of sulfation. Thus, many proteins are expected to bind heparin via electrostatic interactions, as evidenced by the requirement of an increased salt concentration for the dissociation of specific heparin–protein complexes [6]. Indeed, substitution and chemical modification experiments demonstrated that positively charged residues were crucial for heparin binding [7,8]. However, electrostatic forces by themselves are probably not sufficient, as other negatively charged polymers cannot replace heparin [9,10], implying that a steric fit is required between the heparin and its binding site on the protein. Cardin and Weintraub identified a consensus sequence, BBXB (B represents a basic residue), that is present in many known heparin-binding proteins [11].

In recent years, titanium (Ti) has become a key material for biomedical applications, such as in orthopedic implant surgery, owing to its biocompatibility with human tissues and excellent mechanical properties [12]. Nevertheless, titanium-based surgical implantation is still associated with certain clinical challenges. One potential problem is the unpredictability of implant integration with the host bone and in clinical practice the osseo-integration of orthopedic implants is often incomplete, resulting in the risk of implant loosening over time. To enhance the biological acceptance of orthopedic implants and improve the post-implantation healing and integration responses, much effort has been made to promote the specific binding interactions between Ti and host cells by the introduction of biologically active factors, such as the surface immobilization of specific sequences of cell-adhesive peptide/proteins [13].

In this paper, we describe the identification and kinetic and functional studies of the synthetic peptide corresponding to the heparin/HS-binding sequence VRRSKHGARKDR, which is referred to as the HBP12 peptide. The present study demonstrates that the

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Abbreviations used: *HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; ECM, extracellular matrix, FC-1, first flow cell; RU, resonance units; FC-2, second flow cell; SPR, surface plasmon resonance; HGF, Human gingival fibroblasts; MEM, minimal essential medium; MTS, methyl terazol sulfate; SEM, scanning electron micrographs.*

HBP12 peptide is a highly selective heparin/HS-binding peptide, which recognizes heparin and cell surface HSPG and can support HS-dependent cell adhesion and proliferation.

Materials and Methods

Screening

The peptide display library, FliTrx (Invitrogen), was used to identify peptides with heparin-binding specificity. FliTrx is an *Escherichia coli* system that expresses a random 12-amino-acid peptide on the flagella tip of the bacteria. Each organism expresses a specific peptide, and the collection of all organisms expressing all possible amino acid combinations constitutes the displayed peptide library. Each peptide sequence is flanked by cysteine residues, which form a disulfide loop that constrains the expressed peptide. This conformational constraint has been shown to improve the binding of peptides to their target molecule. Our goal was to identify those peptides with binding specificity for heparin. Clones binding to heparin are then propagated, which should amplify those organisms displaying peptides with heparin-binding specificity.

One vial of the FliTrx Peptide Library was used to inoculate 50 ml of IMC medium (1 X M9 salts, 0.2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$) containing 100 µg/ml of ampicillin. Heparin (Sigma) was used as the target molecule in this study. Peptide screening was performed by biopanning, according to the manufacturer's instruction manual. After the seventh round of biopanning, several clones were isolated at random, and their sequences were analyzed.

Immobilization of Heparin

In these analyses, streptavidin was coupled to a CM5 sensor chip using the amine coupling kit (BIAcore, Uppsala, Sweden) with the procedures specified by the manufacturer. The streptavidin was immobilized in the FC-1 to 20 k RUs. BSA was immobilized in the FC-2 to 950 RUS as a negative control for background correction. BiHep was immobilized to the streptavidin-coupled CM5 sensor chip according to supplier's instructions.

BIAcore Binding Analysis

The evaluation of the kinetic parameters was performed using a SPR-based BIAcoreX biosensor (BIAcore). To determine the binding kinetics of the peptide, a series of samples with various concentrations were injected during the association phase for 5 min (40 µl/min). The dissociation phase was allowed to proceed for a period of 10 min. The flow cell could be regenerated with 3-min injection of 1.0 M NaCl, which removed the peptides while leaving the heparin intact. The kinetic analysis of the sensograms obtained from the interaction of various analytes with the immobilized BiHep was based on the following rate equation:

$$dR/dt = k_a C R_{max-}(k_a C + k_d)R_t \quad (1)$$

where dR/dt is the rate of change of the SPR signal (RU) because of the interaction of the analyte with the immobilized BiHep at time t seconds, K_a and K_d are the association and dissociation rate constants, respectively, C is the concentration of thr analyte, and R_{max} is the maximum analyte binding capacity to BiHep in RUs. Both the association- and dissociation-rate constants were determined from the analysis of the sensograms using BIAevaluation software, version 3.0. All binding curves were corrected for background and bulk refractive index by the subtraction of the values obtained from the reference flow cells. The models were fitted both globally across the data sets and for a single concentration.

Cell Adhesion Assay

HGFs were cultured in α-MEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The HGFs were serum starved for 24 h, harvested by the addition of 0.02% trypsin, 1-mM EDTA, resuspended in α-MEM, washed three times with α-MEM containing 100 µg/ml soybean trypsin inhibitor and 1% BSA, and plated at 5×10^4 cells/well in α-MEM. Twenty-four well plates were coated with recombinant proteins overnight at 4 °C and then blocked with 1% (w/v) BSA in PBS for 30 min, and then rinsed with PBS. Suspensions of control cells were maintained in BSA-coated plates for the indicated time. After 60 min at 37 °C, the adherent cells were washed twice with PBS, fixed with 3% paraformaldehyde (Sigma), and stained with 0.25% (w/v) Crystal violet (Sigma) in 2% (v/v) ethanol/water. After extensive washing with distilled water, the plates were allowed to dry. The absorbance was read at 570 nm and the nonspecific adhesion was determined in the wells coated with 1% BSA as a negative control.

MTS Assay

Cell growth was assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] tetrazolium assay (Celltiter96 AQ Non-radioactive Cell proliferation Assay, Promega, USA), which measures the number of viable cells as described by the manufacturer. This assay measures the conversion of MTS into a water-soluble formazan product that has an absorbance maximum at 490 nm. Cells were seeded onto Ti substrates at a density of 5000 cells/cm^2. After the incubation of the cells at 37 °C for 1, 3, and 7 days, MTS (40 µl) was added, followed by additional incubation at 37 °C for 4 h. The optical density was measured at 490 nm using an ELISA reader (Tecan).

Sequence Analysis

The sequence analysis of the random peptide region of the isolated clones was performed with an ABI PRISM 3700 DNA Analyzer (PE biosystems, CA), using the FliTrx forward-sequencing primer recommended in the manufacturer's instruction manual and the Bigdye Terminator Cycle Sequencing FS Ready Reaction kit for the sequence reaction.

Results

Oligopeptide Sequence of the Isolated Clones by Panning Experiment

In this work, we used an *in vitro E. coli* system (FliTrx *E. coli* peptide display library) for the identification of peptide sequences that bind to heparin. Following the panning of the FliTrx library against heparin, colonies were picked randomly for insert analysis. The resulting sequences were then analyzed for repeats. Among these sequences, one single peptide sequence (VRRSKHGARKDR) was found to be predominant. This putative heparin/HS-binding peptide was designated as the HBP12 peptide. The BB*X*B motif, where B represents a basic residue, has been shown to be a

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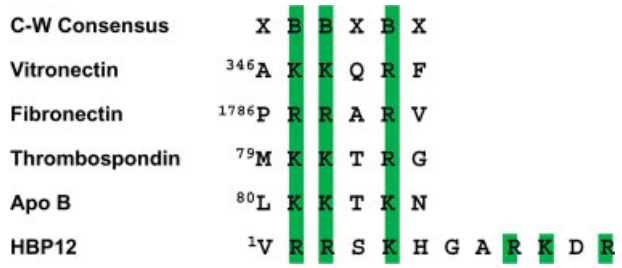

Figure 1. Sequence alignment of heparin-binding peptide/proteins. Cardin and Weintraub consensus sequence (C–W consensus), XBBXBX (B = basic residues, in green).

consensus heparin-binding motif for several proteins [11]. As shown in Figure 1, inspection of the HBP12 sequence showed that it had two consecutive heparin-binding motifs (i.e. RRSK and RKDR).

Binding of HBP12 Peptide to Heparin

The binding kinetics and affinity between the HBP12 peptide and heparin were assessed via SPR. A series of binding sensorgrams was obtained upon the injection of various concentrations of the HBP12 peptide to heparin immobilized on the streptavidin-coupled CM5 sensor chip. The direct binding studies using SPR-based BIAcore technology clearly demonstrated that the HBP12 peptide binds to heparin with high affinity. The equilibrium dissociation constants, K_D, were estimated via a nonlinear regression fitting to a Langmuir binding model. The HBP12 showed K_D values of 191 nM for heparin under physiological condition (Table 1). HBP12 exhibited a fast association rate with heparin (k_a, $1.04 \times 10^5 \times M^{-1}\ s^{-1}$) biosensor surfaces. The resultant complexes between HBP12 were stable as illustrated by slow dissociation rates (k_d, $1.99 \times 10^{-2} \times s^{-1}$).

Osteoblastic MC3T3-E1 Cell Adhesion to HBP12 Peptide

To investigate the biological significance of the HBP12 peptide, we explored whether its heparin-binding property was relevant to the process of cell adhesion. Osteoblastic MC3T3-E1 cells, an osteoblastic cell line derived from mouse calvaria, were used for the detailed examination of cell adhesion to the HBP12 peptide. Preliminary studies determined that 1 μM was the optimal coating concentration of the HBP12 peptide and this concentration was used for routine osteoblastic MC3T3-E1 cell adhesion assays (data not shown). The osteoblastic MC3T3-E1 cells were plated on dishes coated with HBP12 peptides. When the MC3T3-E1 cell adhesion to HBP12-coated dish was quantitated by the crystal violet staining of fixed cells, HBP12 exhibited significantly higher adhesive activity than the nontreated control. Notably, the osteoblastic MC3T3-E1 cells also failed to attach to a peptide containing a randomly scrambled sequence (SRHKVRARDRGK) of the same amino acid composition (Figure 2). Therefore, although positively charged residues were crucial for heparin binding, electrostatic forces by themselves are probably not sufficient, suggesting that a steric fit as well as electrostatic forces is required between the cell surface HSPGs and their binding site on the HBP12 peptide.

Table 1. BIAcore binding studies with immobilized heparin and HBP12 peptide

Analyte	k_d ($s^{-1} \times 10^{-2}$)	k_a ($M^{-1}\ s^{-1} \times 10^5$)	K_D (nM)
HBP12	1.99	1.04	191

Binding curves were best fit to a 1 : 1 Langmuir binding model using the BIAevaluation software version 3.0. The association rate (k_a), dissociation rate (k_d), and the dissociation constant ($K_D = k_d/k_a$) are shown. Goodness of fit was indicated by χ^2, values which were less than 5.

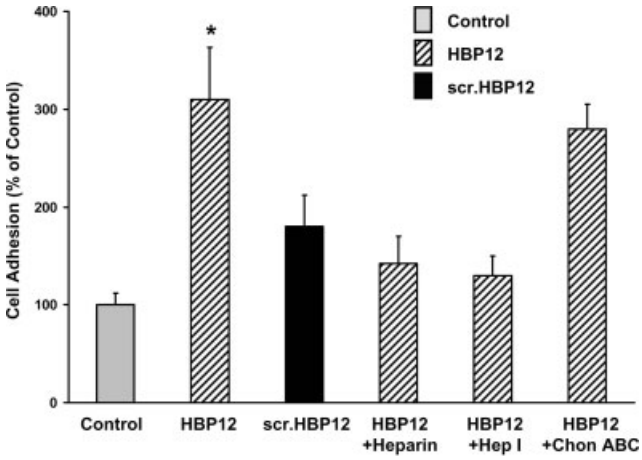

Figure 2. HBP12 peptide-mediated cell adhesion. Twenty four-wells culture plates were individually coated with HBP12 peptide (1 μM) or scrambled HBP12 peptide (scr.HBP12, 1 μM). MC3T3-E1 cells were pretreated with heparinase I (Hep I, 2 units/ml), or chondroitinase ABC (Chon. ABC, 2 units/ml) or co-incubated with heparin (5 μg/ml) prior to plating. The data represent the mean of three separate determinations ± SD and are representative of three experiments ($^*P < 0.05$).

Moreover, the osteoblastic MC3T3-E1 cell adhesion to the HBP12 peptide was inhibited in the presence of 5 μg/ml heparin. These results suggest that the prior occupancy of the heparin-binding sites in the HBP12 peptide by soluble heparin might interfere with its ability to bind to the cell surface HSPGs. To test this possibility, the cell surface HSPGs were removed from the MC3T3-E1 cells by treatment of the cells with heparinase I, an enzyme that acts on highly sulfated HSPGs [14]. The heparinase I digestion of the MC3T3-E1 cell surfaces reduced the number of HBP12-binding sites by 70%. In control experiments, the treatment of the MC3T3-E1 cells with chondroitinase ABC failed to inhibit the HBP12-mediated adhesion of MC3T3-E1, showing that the cell surface HSs, but not the chondroitin sulfates, contribute to the cell adhesive properties of HBP12 (Figure 2). Taken together, these results support that the HBP12 peptide contains binding sites for cell surface HSPGs and promotes osteoblastic MC3T3-E1 cell adhesion.

Effect of Surface-immobilized HBP12 on Osteoblastic MC3T3-E1 Cells

Next, we examined the effects of the surface treatment of Ti with the HBP12 peptide on the osteoblastic MC3T3-E1 cell functions. Representative SEM of the MC3T3-E1 cells attached to the HBP12 peptide- or noncoated Ti surfaces after 1 h of incubation are shown in the insets of Figure 3. The osteoblastic MC3T3-E1 cells plated on the HBP12 peptide-coated Ti surfaces attached and spread better compared with the noncoated Ti control. This result confirmed that the presence of HBP12 peptide sequences on the Ti surface supports osteoblastic MC3T3-E1 cell adhesion.

The effect of surface-immobilized RGD on the proliferation of osteoblastic MC3T3-E1 cells was examined by the MTS assay. Figure 3 shows that the absorbance of formazan produced by

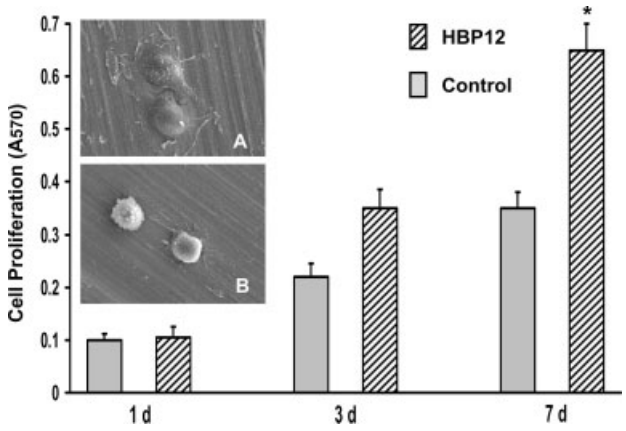

Figure 3. Cell proliferation on the HBP12-immobilized Ti surfaces after 1, 3, and 7 days determined by methyl terazol sulfate (MTS) assay. The results are given as O.D. values and represent the mean ± SD from three separate experiments (* $P < 0.05$). The inset SEM images are the HBP12-immobilized Ti surfaces (A) after exposure to an osteoblastic MC3T3-E1 cell suspension (10^4 cells/cm^2) for 1 h. Noncoated Ti substrates (B) were used as a control.

the cells on the HBP12 peptide-coated Ti is significantly higher compared with that of nontreated Ti control after 7 days of culture ($P < 0.05$). This result again suggests that the HBP12 peptide immobilized on the external surface of the Ti substrate is the main operator involved in enhancing osteoblastic MC3T3-E1 cellular activity.

Discussion

Heparin/HS exists ubiquitously as a component of proteoglycans on cell surfaces and plays important roles in various biological processes including the reorganization of the cytoskeleton and the assembly of focal adhesions [15]. We previously showed that the heparin-binding domain of fibronectin enhances the cell adhesion and proliferation of osteoblasts [16]. More recently, we reported that the heparin-binding domain of fibronectin enhances the cell adhesive and spreading activities through the cooperative mechanism of the RGD-containing central cell-binding domain of fibronectin in human MG63 osteoblastic cells [17]. In the present study, we have identified a cell adhesive synthetic peptide from a 12-mer random *E. coli* cell surface display library. This synthetic heparin-binding peptide sequence, VRRSKHGARKDR, binds to heparin with high affinity ($K_D = 191$ nM) and promotes HS-mediated cell adhesion and osteoblastic proliferation in osteoblastic MC3T3-E1 cells. Consistent with our results, it was recently reported that the synthetic heparin-binding sequences from human fibroblast growth factor-2 promoted osteoblast adhesion and differentiation through the interaction with cell surface HSPGs [18].

The immobilization of biologically active factors on the surface of biomedical devices has emerged as a promising strategy to enhance the host healing responses to implanted devices [13]. Specific sequences of cell-adhesive peptide/proteins immobilized on the surface constitute the most widely used candidate biologically active factors [19]. Among these, RGD peptide is by far the most effective and most often employed peptide sequence for stimulated cell adhesion [20]. Strategies to make surfaces more conductive to cell attaching and spreading by attaching RGD-containing peptides have been developed for numerous materials [21–24].

Herein, we demonstrated that the surface-immobilized HBP12 peptide on Ti substrates supports osteoblastic MC3T3-E1 cell adhesion and proliferation. To the best of our knowledge, this is the first report on a synthetic heparin-binding peptide derived from a 12-mer random peptide library promoting osteoblast adhesion and proliferation.

Acknowledgements

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