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ScienceDirect
Biomaterials 29 (2008) 1005–1016
Biomaterials
www.elsevier.com/locate/biomaterials

Short-term effects of adhesion peptides on the responses of preosteoblasts to pBMP-9

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Received 30 July 2007; accepted 30 October 2007
Available online 26 November 2007

Abstract

Adhesion peptides are currently used to enhance the interactions of osteoblasts with biomaterials. However, little is known about the effects of adhesion peptides on cell responses to growth factors, especially the bone morphogenetic proteins (BMPs). We used adhesion peptides Ac-CGGNGERPRGDTYRAY-NH_2 (pRGD), derived from bone sialoprotein, and Ac-CGGDGEA-NH_2 (pDGEA), derived from collagen, which interact with $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins, respectively. We analyzed the effects of pRGD- and pDGEA-coated polystyrene (PS) on the responses of murine MC3T3-E1 preosteoblasts to a peptide derived from human BMP-9 (pBMP-9) in serum-free medium. After 1 h, pRGD favoured interactions with α_v while pDGEA bound β_1 integrin subunits. Adding pBMP-9 (400 ng/mL) increased the amount of α_v integrin subunits in cell membranes on pRGD-coated PS, but had no effect on β_1 integrin subunits. Only on this substratum, collagen type I mRNA was enhanced and the addition of pBMP-9 promoted the early cell differentiation, increasing their alkaline phosphatase (ALP) activity within 24 h. These cells also organized β_1 integrin subunits at their focal adhesion points. Inhibiting $\alpha_2\beta_1$ integrins by pDGEA pre-treatment decreased this ALP activity. It is therefore important to understand the impact of adhesion peptides on the early cell responses to growth factors in order to improve biomimetic materials.

Keywords: Adsorption; Alkaline phosphatase; BMP; Integrin; Osteoblast; Peptide

1. Introduction

Bone tissue engineering can be greatly improved by increasing cell attachment to biomaterials. Cell adhesive proteins like fibronectin, denatured collagen/gelatin, osteopontin, vitronectin and laminin have all been used to improve the function of biomaterials [1,2]. However, the purification of these proteins from other species enhances the risk of undesirable immune responses and infections [3]. Therefore, new strategies using specific adhesion peptides that mimic the proteins of the extracellular matrix (ECM) have been recently developed to control the interactions of cells and biomaterials [4–6]. The most commonly used tripeptide sequence is Arg-Gly-Asp (RGD), which is present in proteins such as osteopontin, bone sialoprotein and thrombospondin [7,8]. RGD peptides are recognized by specific heterodimeric $\alpha\beta$ transmembrane receptors, the integrins [9]. The integrin subunits α_1, α_2, α_3, α_4, α_5, α_6, α_v, β_1, β_3 and β_5 have all be found on osteoblasts [10,11]. However, the production of these integrins depends on the development stage of the osteoblasts [12]. The interactions between integrins and their specific ligands regulate cell functions like adhesion, motility, growth and differentiation [4,10]. The binding of RGD to integrins increases the attachment, growth and differentiation of osteogenic precursor cells and osteoblasts [13,14]. Other peptide sequences that are present in collagen type I, such as Asp-Gly-Glu-Ala (DGEA), can also modulate the behaviour of osteoblasts [15].

Growth factors like bone morphogenetic proteins (BMPs) may also influence the behaviour of osteoblasts [16]. BMPs are 30–38 kDa homodimers that are members of the transforming growth factor β superfamily [17]. Over 20 BMPs have been identified to date [18]. They are

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doi:10.1016/j.biomaterials.2007.10.047

synthesized as prepropeptides of about 400–500 amino acids [19]. The mature C terminus domain obtained after cleavage at an Arg-X-X-Arg sequence in the propeptide forms a dimer. BMPs are divided into classes (e.g. BMP-2/BMP-4 and BMP-9/BMP-10) based on their sequence homology [20]. Alignment of the amino acid sequences in the cysteine domains of BMPs also reveals difference among them [21]. For example, the sequence of BMP-7 is similar to that of BMP-6—87% identical in the cysteine-rich C terminus domain, but it is more distantly related to that of BMP-2—60% identical [21]. Recombinant human BMP-2 is the BMP most frequently used in clinical bone healing studies [22]. It also regulates osteogenic differentiation *in vitro* [23]. BMP-9 was recently shown to induce greater differentiation of osteoblasts than BMP-2 [24]. We have previously demonstrated the effect of a peptide derived from human BMP-9 (pBMP-9) to induce early preosteoblast differentiation similar to that generated by BMP-2 [25].

Several studies have demonstrated that growth factors and integrins cooperate to regulate osteogenic differentiation [26,27]. However, little is known about the crosstalk between adhesion peptides and growth factor pathways [28]. We have therefore examined the effect of adhesion peptides derived from bone sialoprotein (pRGD) [15,29] and collagen type I (pDGEA) [15], which, respectively, interact with $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins [16,30] on the responses of murine MC3T3-E1 preosteoblasts to pBMP-9. For this purpose, we analyzed cell proliferation, differentiation and apoptosis. Murine MC3T3-E1 preosteoblasts were used because their developmental sequence is typical of osteoblasts. These cells produce specific bone markers like alkaline phosphatase (ALP) and collagen type I [31]. We also analyzed the impact of pBMP-9 on the production and distribution of specific integrin subunits. Lastly, we evaluated the effect of polystyrene (PS) dishes coated with pRGD or pDGEA on the early differentiation of cells incubated with pBMP-9.

2. Materials and methods

2.1. Materials

All peptides were synthesized by Celtek Peptides (Nashville, TN, USA) with a final purity >98%. pBMP-9 was dissolved in ultrapure water (pH 6.3), while the positive and negative peptides were dissolved in ultrapure water (pH 7.3). The positive peptides had the following sequences: Ac-CGGNGERPRGDTYRAY-NH_2 (pRGD) [29], found in bone sialoprotein, and Ac-CGGDGEA-NH_2 (pDGEA) [15], found in collagen type I. The negative peptides were Ac-CGGNGERPR-GETYRAY-NH_2 (pRGE) and Ac-CGGDGAA-NH_2 (pDGAA). Twenty-four-well plates ($2\,cm^2$/well) were incubated with pRGD or pDGEA (10 μg/mL) in phosphate buffered saline (PBS) for 1 h at 37 °C. The wells were then washed (2×10 min) with PBS and peptide adsorption was assessed by measuring optical density of the supernatant at 205 nm. All peptides were adsorbed at a density of $0.98\pm0.02\,\mu g/cm^2$ obtained using standard curves prepared with peptide concentrations of 0–10 μg/mL in PBS.

2.2. Cell culture

Murine calvarial preosteoblasts MC3T3-E1 (CRL-2594™, ATCC®, Manassas, VA, USA) were grown at 37 °C in minimum essential medium (MEM) alpha medium without ascorbic acid (α-MEM, Gibco®, Grand Island, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma®, Oakville, ON, Canada), 2 mM L-glutamine (Invitrogen™, Burlington, ON, Canada), 100 U/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) under a humidified 5% CO_2 atmosphere. Cells were used for experiments between passages 2 and 15. All experiments were performed in serum-free medium following a 24 h serum starvation, except for the proliferation assays. Trypsin inhibitor (Invitrogen) was used instead of FBS to neutralize trypsin (Invitrogen) in all experiments.

2.3. Focal adhesion and integrin immunolabelling in serum-free medium

Peptide solutions were incubated on PS ($9.6\,cm^2$ cell culture petri dishes) for 1 h at 37 °C. These dishes were washed twice with PBS. Cells were seeded (1×10^4 cells/cm^2) and incubated for 1 or 24 h at 37 °C. The cells attached to pRGD- or pDGEA-coated PS were fixed by immersion in 3% (w/v) paraformaldehyde in PBS for 15 min and permeabilized for 5 min with 0.5% (v/v) Triton® X100 (Sigma) in PBS. Non-specific binding sites were blocked by incubating in PBS containing 1% non-fat dried milk for 30 min. Cells were immunostained by incubating them with rabbit polyclonal antibodies raised against vinculin (diluted 1:50, Sigma) or with rabbit polyclonal antibodies raised against α_v integrin subunits (diluted 1:30, Chemicon®, Temecula, CA, USA) or rat polyclonal antibodies raised against β_1 integrin subunits (diluted 1:40, Chemicon). Bound primary antibodies were visualized by incubation with fluorescence-labelled secondary antibodies, FITC-conjugated anti-rabbit or anti-rat immunoglobulins (diluted 1:200, Chemicon). Negative controls were prepared using the secondary antibodies alone. All antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA, Sigma) and cells were incubated with antibodies for 30 min at room temperature. Filamentous actin (F-actin) was also stained using rhodamin–phalloidin diluted 1:200 in PBS (Sigma). The cells attached to PS were then washed, mounted with glass slides and examined with an Eclipse TE2000-S inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a 60× objective and a Retiga™ 1300R digital CCD camera (QImaging®, Burnaby, BC, Canada).

2.4. Integrin subunits in cell membrane extracts

Cells attached to uncoated, pRGD- or pDGEA-coated PS ($28.3\,cm^2$ cell culture petri dishes) after incubation for 1 or 24 h in serum-free medium with or without pBMP-9 (400 ng/mL) were washed twice with ice cold PBS and lysed at 4 °C in 300 μL 20 mM Tris–HCl (pH 7.4) containing 20% glycerol (v/v) and a tablet of complete, Mini protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). The lysed cells were homogenized and centrifuged at 20,000*g* for 30 min. The resulting pellets were resuspended in the lysis buffer. Similar amounts of membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Mississauga, ON, Canada). The nitrocellulose membranes were stained with Ponceau red (Sigma) to confirm transfer efficiency and then incubated overnight in a solution of BSA (3% w/v) in PBS Tween® 20 (0.1% v/v, Sigma). The nitrocellulose membranes were washed three times with PBS containing Tween 20 (0.1% v/v) and incubated for 2 h at room temperature with a primary rabbit antibodies against α_v (diluted 1:1000, Chemicon) or a primary rat antibodies against β_1 (diluted 1:1000, Chemicon). The membranes were again washed three times with PBS containing Tween 20 (0.1% v/v) and bound specific antibodies were revealed by incubation with a peroxidase-conjugated anti-rabbit or anti-rat second antibodies (diluted 1:20,000, Sigma).