Acta Biomaterialia 31 (2016) 241–251

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Modulation of MAPK signalling by immobilized adhesive peptides: Effect on stem cell response to BMP-9-derived peptides

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ARTICLE INFO

Article history:
Received 3 July 2015
Received in revised form 18 November 2015
Accepted 2 December 2015
Available online 8 December 2015

Keywords:
Integrin
Bone morphogenetic proteins
Cell signalling
Runx2

ABSTRACT

Biomimetic materials were developed to regulate stem cell behaviour. We have analyzed the influence of polycaprolactone (PCL) films, functionalized with adhesive peptides derived from fibronectin (pFibro) or bone sialoprotein (pBSP), on the response of murine multipotent C3H10T1/2 cells to bone morphogenetic protein-9 (BMP-9) and its derived peptides (pBMP-9 and SpBMP-9). PCL-pFibro promoted better cell cytoskeleton organization and faster focal adhesion kinase activation than did PCL-pBSP. PCL-pFibro also promoted MAPK signalling to improve the cell response to BMP-9 by inactivating ERK1/2 and stimulating p38 and JNK. BMP-9, pBMP-9 and SpBMP-9 induced greater phosphorylation of Smad1/5/8 in cells attached to PCL-pFibro than in cells on PCL-pBSP. These phosphorylated Smad1/5/8 were translocated to the nucleus. BMP-9 and its derived peptides restored the phosphorylation of JNK in cells on PCL-pBSP, but it remained less phosphorylated than in cells on PCL-pFibro stimulated with pBMP-9 and SpBMP-9. Cells attached to PCL-pFibro contained more Runx2, essential for stem cell commitment to become osteoblasts, than did cells on PCL-pBSP when incubated with BMP-9 and its derived peptides. Runx2 was no longer detected when the cells were pre-treated with JNK inhibitor. Therefore pFibro plus BMP-9 and its derived peptides may be a promising strategy to develop biomimetic materials.

Statement of significance

Biomaterials functionalized with adhesive peptides to favour bone repair have generated a great interest over the past decade. However, the effect of these materials on the ability of cells to respond to growth factors remains poorly known. One major growth factor subfamily involved in bone formation is the bone morphogenetic protein (BMP). However, these BMPs are expensive. We therefore developed less costly derived molecules. We showed how adhesive peptides derived from bone matrix proteins grafted onto polymer films affect the intracellular signalling and thus the ability of stem cells to be activated by BMP and its derived molecules. We have therefore identified a combination of bioactive polymers and BMP molecules that direct the stem cells towards bone forming cells.

1. Introduction

Biomimetic materials that promote bone cell adhesion, survival and differentiation have generated a great interest over the past decade for the development of new bone substitutes and tissue engineering strategies [1–5]. For example, polycaprolactone (PCL) functionalized with adhesive peptides that mimic extracellular matrix proteins favours the adhesion of bone marrow stromal cells or preosteoblasts to its surface [6,7]. The most commonly used adhesive peptides contain the cell binding motif Arg-Gly-Asp (RGD), which is found in proteins like bone sialoprotein (BSP), vitronectin and fibronectin [3,5,8]. The RGD motif is recognized by several cellular heterodimeric αβ transmembrane receptors, including the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [9,10]. In addition, the binding of $\alpha_5\beta_1$ integrins to the RGD motif of the fibronectin FIII-10 domain can be modulated by a PHSRN motif located in the FIII-9 module [11,12].

However, the effect of materials functionalized by adhesive peptides on the intracellular signalling and their impact on the subsequent cell response to growth factors like bone morphogenetic proteins (BMP) remain poorly understood [2,13]. BMPs,

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http://dx.doi.org/10.1016/j.actbio.2015.12.005
1742-7061/

which are cytokines of the transforming growth factor-β family, act on cells by inducing the oligomerization of type I and type II Ser/Thr kinase receptors by binding to them, or activating preformed heterodimeric- or homodimeric receptor complexes [14,15]. Tetrameric complex with two type I and two type II Ser/Thr kinase receptors activates the phosphorylation of type I receptors by type II receptors, leading to stimulation of the intracellular Smad1/5/8 cascade (also called the canonical pathway). BMP binding to the receptors can also activate a pathway involving TGF-β1-activated tyrosine kinase 1 (TAK1) and the mitogen-activated protein kinase (MAPK)-like ERK1/2, p38 and JNK pathways [16]. 20 BMPs have been identified to date. Only recombinant human BMP-2 and BMP-7 are presently approved by the Food and Drug Administration for commercial use [17,18]. Nevertheless, BMP-9 had a stronger osteoinductive potential than BMP-2 *in vitro* in murine model cell lines such as C2C12 and C3H10T1/2 and *in vivo* [19–21]. For example, Luu et al. [21] used adenovirus encoding 14 BMPs (AdBMP) to show that C3H10T1/2 infected with AdBMP-9 or AdBMP-6 contained greater alkaline phosphatase activity and more osteocalcin than did cells infected with AdBMP-2.

However these BMPs are expensive to produce. Several teams have therefore developed less costly peptides that contain the sequence recognized by the type II BMP receptor [22–24]. This sequence, called the knuckle epitope, differs slightly in BMPs like BMP-2, BMP-7 and BMP-9 [25]. We therefore developed two peptides derived from BMP-9 (pBMP-9 and SpBMP-9) [26,27] based on the studies of Suzuki et al. [24] and Saito et al. [23] on peptides derived from the knuckle epitope of BMP-2. The SpBMP-9 was obtained by replacing the ACC motif of pBMP-9 with ASS. Indeed, Saito et al. demonstrated that a peptide KIPKASSVPTELSAISTLYL, corresponding to residues 73–92 of BMP-2, in which the cysteine residues were replaced by serine and the methionine by threonine, enhanced the ALP activity in murine multipotent C3H10T1/2 cells [23]. We have previously shown that pBMP-9 promotes the differentiation of murine MC3T3-E1 preosteoblasts attached to polystyrene [28]. It also induces some woven bone formation when injected, together with chitosan, into the quadriceps of mice [27]. However, the early differentiation of murine MC3T3-E1 preosteoblasts induced by pBMP-9 depends on the type of integrins involved in cell adhesion. Marquis et al. [5] found that murine MC3T3-E1 preosteoblasts incubated with pBMP-9 and adhering to polystyrene coated with BSP-derived peptide (pBSP), developed more alkaline phosphatase activity while cells on pDGEA-coated polystyrene targeting $\alpha_2\beta_1$ integrins showed no such increase. Tian et al. [29] also found that fibronectin coating, that targets both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, increased the BMP-9-induced phosphorylation of Smad1/5/8 in human microvascular endothelial cells.

We have studied the effect of PCL functionalized with peptide derived from BSP (pBSP) or peptide containing fibronectin RGD and PHSRN motifs (pFibro) on the organization of focal adhesions and the activation of focal adhesion kinase (FAK) and MAPK proteins in murine multipotent C3H10T1/2 cells. We then verified the impact of this signalling on the ability of C3H10T1/2 cells to respond to BMP-9 and its derived peptides, pBMP-9 and SpBMP-9.

2. Materials and methods

2.1. Materials

The peptide pBSP (Ac-CGGNGEPRGDTYRAY-NH_2) derived from bone sialoprotein was synthesized by Celtek Peptides (Celtek Bioscience, TN, USA) and the peptide pFibro (Ac-CGGPHSR NGGGGGGRGDG-NH_2) derived from fibronectin motifs was synthesized by EZBiolab (Carmel, IN, USA), both with a final purity of 98%. Recombinant carrier-free human BMP-9, synthesized in Chinese Hamster Ovary cells, was purchased from R&D Systems (Minneapolis, MN, USA), while pBMP-9 and SpBMP-9 were synthesized by Celtek Peptides (Celtek Bioscience, TN, USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). 2-(2-Pyridinyldithio) ethaneamine hydrochloride (PDEA) was purchased from GE Healthcare (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Primary rabbit antibodies against phosphorylated FAK (Y^{397} or $Y^{576/577}$), phosphorylated Smad1 ($Ser^{463/465}$)/Smad5 ($Ser^{463/465}$)/Smad8 ($Ser^{426/428}$), phosphorylated Smad1 ($Ser^{463/465}$)/Smad5 ($Ser^{463/465}$), total Smad1/5/8, total ERK1/2, total p38, phosphorylated ERK1/2 and phosphorylated p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary murine antibodies against phosphorylated JNK and primary rabbit antibodies against integrin subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary rabbit antibodies for immunostaining of phosphorylated FAK (pFAK, Y^{397}), vinculin and FITC-conjugated anti-rabbit IgG secondary antibodies were purchased from Sigma (St. Louis, MO, USA). Peroxidase-conjugated anti-rabbit IgG secondary antibodies were purchased from GE Healthcare. JNK inhibitor SP600125 was purchased from Millipore EMD (Billerica, MA, USA).

2.2. Methods

2.2.1. Preparation of PCL films and characterization of peptide density

PCL films were prepared and functionalized by adhesive peptides as previously described [7]. They were washed with distilled water and covered with a solution of EDC/NHS (0.149 mg/mL) for 20 min. These films were washed with phosphate-buffered saline (PBS), and covered with PDEA for 1 h. The films were washed with PBS once more, and covered with pBSP or pFibro solution for 30 min at room temperature with agitation. The density of peptides grafted onto PCL was determined indirectly by quantifying the concentration of peptides remaining in the supernatant after the grafting procedure (three independent experiments). Briefly, the supernatant peptide solutions removed after incubation with three PCL surfaces for 30 min were pooled and freeze dried. Their contents were suspended in ultra-pure sterile water and the peptide concentrations were determined by analytical high performance liquid chromatography (HPLC) (Model 335, Varian, Agilent Technology) coupled to a fluorescence detector (excitation 274 nm, emission 310 nm, Varian model 363, Agilent Technology) and/or measurement of absorbance at 205 nm by UV spectrophotometer (Biotek synergy HT with take3 plate). The HPLC elution gradient was (A) acetonitrile containing 0.1% (v/v) TFA and (B) water containing 0.1% (v/v) TFA. The flow rate was 1 mL/min. Data were analyzed with the Galaxie Chromatography Data System v 1.9.301.220. Sample concentrations were calculated by comparing the area under the eluted peaks with those of a standard curve of peptides. The concentrations of the pBSP and pFibro remaining in the supernatant after the grafting procedure were similar and the densities of pBSP and pFibro grafted onto PCL films were evaluated at about 86 ± 5 pmol/cm^2.

2.2.2. Cell experiments

2.2.2.1. Cell culture. Murine multipotent C3H10T1/2 cells (clone 8; CCL-226, ATCC, Manassas, VA, USA) were used between passages 10 and 15. Cells were grown at 37 °C under a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) without ascorbic acid, supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Wisent, Saint-Bruno, QC, Canada), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco®, Grand Island, NY, USA). Cells were detached

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