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Effect of functionalized polycaprolactone on the behaviour of murine preosteoblasts

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ABSTRACT

The efficiency of biomaterials used in bone repair depends greatly on their ability to interact with bone cells. Hence, we have functionalized polycaprolactone (PCL) films by peptides derived from the bone sialoprotein containing RGD sequence (pRGD), to increase their ability to interact with murine MC3T3-E1 preosteoblasts, and favour cell response to recombinant human bone morphogenetic protein-2 (rhBMP-2). RGE peptides (pRGE) were used as negative controls. The PCL films were hydrolyzed with NaOH and then carboxylic acid groups were activated to allow chemisorption of the peptides. Alkaline treatment increased the hydrophilicity of PCL films without significantly change their roughness. Peptide immobilization on PCL was checked by X-ray photoelectron spectroscopy. Hydrolyzed PCL films (Hydro PCL), which adsorbed fibronectin and vitronectin from serum after 1 h incubation, prevented the spreading of MC3T3-E1 preosteoblasts, while films bearing pRGD or pRGE did not. In contrast, MC3T3-E1 preosteoblasts attached to pRGD and incubated for 1 h in serum-free medium spread better than cells on Hydro PCL or pRGE. Only cells on pRGD had organized cytoskeleton, phosphorylated focal adhesion kinase on Y^{397} and responded to rhBMP-2 by activating Smad pathway. Thus, pRGD PCL may be used to favour bone cell cytoskeletal organization and response to rhBMP-2.

1. Introduction

Biomaterials are widely used in the repair of bone tissue but their efficiency depends greatly on their ability to interact with bone cells [1,2]. Specific adhesion peptides, that mimic the proteins of the extracellular matrix (ECM), have therefore been developed to favour the adhesion of cells to biomaterials [3]. Peptides used to functionalize biomaterials are less expensive than ECM and are more readily available and purer [4–6]. The tripeptide Arg-Gly-Asp (RGD), which is present in proteins like fibronectin, vitronectin and bone sialoprotein is the most commonly used because it interacts with integrins, heterodimeric αβ transmembrane receptors [7]. For example, Ac-CGGNGEPRGDTYRAY-NH_2 peptides (pRGD) derived from bone sialoprotein are recognized by α_v integrin subunits and $\alpha_v\beta_3$ integrins [4,8]. Several studies have demonstrated that RGD peptides grafted onto materials such as quartz, titanium, or hydroxyapatite can increase the adhesion of human osteoblastic cells and the differentiation of rat calvarial osteoblasts *in vitro* and can stimulate bone formation *in vivo* [4,9,10].

Polycaprolactone (PCL) is a semi-crystalline aliphatic polyester, that has been used recently in tissue engineering and bone repair applications because of its properties of biocompatibility and slow degradation [11]. However, cells attach poorly to PCL, so that active molecules such as RGD must be grafted onto it to improve cell-PCL interactions [12–14]. Karakecili et al. have demonstrated that RGD tripeptides favour the initial attachment of murine L929 fibroblasts to a PCL film after incubation for 2 h or 4 h in the presence of 10% foetal bovine serum (FBS) [14].

However, little is known about the influence of such adhesion peptides on the ability of bone cells to respond to growth factors such as the bone morphogenetic proteins (BMPs) or their derived peptides [8,15]. BMPs play a crucial role in the differentiation of mesenchymal stem cells into osteoblasts *in vitro* [16]. Recombinant human BMP-2 (rhBMP-2) is the BMP most frequently used in

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clinical bone healing studies [17]. We have recently shown that adhesion peptides adsorbed onto polystyrene (PS) greatly impair the response of MC3T3-E1 preosteoblasts to a peptide derived from BMP-9 (pBMP-9) [8]. Early cell differentiation in the presence of the pBMP-9 only occurs in MC3T3-E1 preosteoblasts attached to pRGD-coated PS. A better understanding of the crosstalk between adhesion peptides and BMP is therefore required in order to develop new biomimetic materials that allow BMP signalling and promote bone formation.

We therefore functionalized PCL films with pRGD or pRGE (negative control) to verify their influence on cell cytoskeleton organization and response to rhBMP-2. We first characterized the PCL films by scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and water contact angle measurements. We also determined their capacity to non-specifically adsorb serum proteins, especially fibronectin and vitronectin. We then determined the influence of functionalized PCL on the cytoskeleton organization of MC3T3-E1 preosteoblasts, particularly their ability to form focal adhesion points and actin stress fibres. We also determined the cell spreading area and analysed the phosphorylation of focal adhesion kinase (FAK). Lastly, we examined the responses of cells on pRGD to rhBMP-2 by determining Smad pathway activation.

2. Materials and methods

2.1. Materials

The pRGD peptide (Ac-CGGNGEPRGDTYRAY-NH_2) derived from the bone sialoprotein and its negative control pRGE (Ac-CGGNGEPRGETYRAY-NH_2) were synthesized by Celtek Peptides (Nashville, TN, USA) with a final purity of 98%. Recombinant carrier-free human BMP-2, synthesized in *Escherichia coli*, was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Preparation of PCL films

PCL pellets (Sigma–Aldrich, St. Louis, MO, USA) were dissolved in (5% w/v) 1,4-dioxane (Fluka, St. Louis, MO, USA) for 6 h at room temperature under gentle stirring. Glass Petri dishes (19.62 cm^2; Fisher Scientific, Ottawa, ON, Canada) were flooded with this PCL solution (0.1 mL/cm^2) and the solvent was evaporated at 80 °C overnight. The PCL films were functionalized by a modification of the method of Sun and Önneby [18]. Briefly, the films were hydrolyzed (0.25 mL/cm^2) by incubation with 0.5 mol/L NaOH (Sigma) for 2 h at room temperature under gentle agitation, washed once with distilled water for 5 min and then flooded with 0.01 mol/L HCl (Fisher Scientific) for 30 min at room temperature. The films were again washed with distilled water for 5 min and functionalized with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.904 mg/mL, Fluka)/N-hydroxysuccinimide (NHS) (0.143 mg/mL, Fluka) for 20 min at room temperature. The films were then rinsed with phosphate-buffered saline (PBS) (0.15 mL/cm^2) and covered with 2-(2-pyridinyldithio) ethaneamine hydrochloride (PDEA) (0.15 mg/mL, Biacore AB, Uppsala, Sweden) for 1 h at room temperature. The films were washed once more with PBS (0.15 mL/cm^2) for 5 min and placed in pRGD or pRGE solution (0.33 μg/mL, 0.15 mL/cm^2) for 15 min at room temperature.

2.3. Characterization of PCL films

2.3.1. SEM

The films were examined in a Hitachi S-3000N SEM with an accelerating voltage of 5 kV at a magnification of 1000 for surface analysis and at 700 to measure the thickness.

2.3.2. AFM

The surface topography of the PCL films was measured with an AFM (Nanoscope III, Veeco Instruments Inc., Santa Barbara, CA, USA) in contact mode in air. Data were acquired on 5 μm × 5 μm squares. Images were processed using Nanoscope® III software V5.31r1 (Veeco Instruments Inc.).

2.3.3. XPS

The XPS analyses were performed with an ESCALAB 3 MKII (Thermo VG scientific, East Grinstead, UK) with a Magnesium Kα source (206 W). The sample size was 2 × 3 mm and the layer analysed was 50–100 Å. Elements were identified from the survey spectra. The high resolution spectrum of each peak was determined at a passage energy of 20 eV. The value of the C1s component was corrected by applying 285 eV.

2.3.4. Water contact angle measurements

Static contact angles were measured at room temperature using a goniometer (Ramé-Hart, Netcong, NJ, USA) equipped with a microliter syringe. A drop of liquid (2 μL of ultrapure water with an electrical resistance of 17.8 MΩcm) was placed on the surfaces using the syringe. The water contact angles were estimated on the plain surface by optical image analysis on both sides of at least three droplets. This was done twice for each material.

2.4. Adsorption of serum proteins onto PCL films

2.4.1. SDS-PAGE electrophoresis

The PCL films were sterilized by flooding with ethanol 70% (v/v) for 30 min and washed 3 times with 2 mL PBS. The films were then incubated for 1 h with 10% (v/v) FBS in PBS (Invitrogen, Burlington, ON, Canada) at 37 °C under a humidified atmosphere containing 5% CO_2. The PCL films were then washed 3 times with PBS and the proteins were desorbed using Laemmli buffer (NuPAGE® LDS Sample Buffer 4×, Invitrogen). The resulting samples (20 μL) were loaded onto 10% or 7.5% polyacrylamide gels. Proteins were revealed by silver nitrate staining according to the manufacturer's instructions (Bio-Rad Laboratories, Mississauga, Canada). The molecular weights of the major protein bands and their densitometric analyses were determined using FluorChem™ 5500 with AlphaEaseFC™ Software, version 4.0.1. (Alpha Innotech, San Leandro, CA, USA).

2.4.2. Western blot analysis

The desorbed proteins were separated by SDS-PAGE electrophoresis as described above and transferred to nitrocellulose membranes using the Trans-blot®Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories). The nitrocellulose membranes were stained with Ponceau red (Sigma) to confirm transfer efficiency and then incubated overnight in 3% (w/v) bovine serum albumin (BSA, Sigma) in PBS plus 0.1% (v/v) Tween 20. The membranes were washed twice with PBS/0.1% (v/v) Tween 20 and incubated at room temperature for 75 min with rabbit primary antibodies directed against fibronectin (diluted 1/500, Sigma) or rabbit primary antibodies directed against vitronectin (diluted 1/100, Abcam, Cambridge, MA, USA). The membranes were again washed three times with PBS/0.1% (v/v) Tween 20 and the bound antibodies were revealed by incubation with peroxidase-conjugated anti-rabbit IgG secondary antibodies (diluted 1/40,000, Sigma). All antibodies were diluted in PBS containing 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA. Immunoreactive bands were visualized by chemiluminescence (ECL + Plus™, GE Healthcare, Buckinghamshire, UK) and exposure to X-ray film (Thermo Scientific, Rockford, IL, USA). Densitometric analyses were determined using FluorChem™ 5500 with AlphaEaseFC™ Software, version 4.0.1.

2.5. Cell experiments

2.5.1. Cell culture

Murine calvarial preosteoblasts MC3T3-E1 subclone 14 (CRL-2594™, ATCC®, Manassas, VA, USA) were grown at 37 °C in Minimum Essential Medium (MEM) alpha medium (α-MEM, Gibco®, Grand Island, NY, USA) without ascorbic acid, supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) under a humidified 5% CO_2 atmosphere until they reached 70% confluence. Then, the cells were deprived of FBS for 24 h and removed by trypsinization (Invitrogen). The trypsin was neutralized with trypsin inhibitor (Gibco). The cells were washed once with α-MEM, collected by centrifugation, and suspended in α-MEM with or without 10% FBS. The cells were seeded (10,000 cells/cm^2) on 9.62 cm^2 cell culture treated PS or ethanol-sterilized PCL films (19.62 cm^2) and incubated for 1 h at 37 °C under a humidified 5% CO_2 atmosphere to allow them to become attached. Cells were used for experiments between passages 2 and 13.

2.5.2. Focal adhesion and cytoskeleton organization evaluated by immunofluorescence

Attached cells were fixed with 3% (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilized for 5 min with 0.5% (v/v) Triton X100 in PBS. Non-specific binding sites on the PCL films were blocked by incubation with 1% (w/v) BSA in PBS for 30 min at 37 °C under a humidified 5% CO_2 atmosphere. Cells were immunostained by incubating them with mouse monoclonal anti-vinculin antibodies (diluted 1/50, Sigma), or mouse primary antibodies against phosphorylated FAK on Y^{397} (diluted 1/25, Millipore, Temecula, CA, USA). Bound primary antibodies were visualized by incubation with FITC-conjugated anti-mouse immunoglobulin (diluted 1/150, Sigma). All antibodies were diluted in PBS containing 0.1% (w/v) BSA and cells were incubated with antibodies for 30 min at 37 °C under a humidified 5% CO_2 atmosphere. Filamentous actin (F-actin) was also stained using rhodamine–phalloidin diluted 1/200 with 0.1% (w/v) BSA in PBS (Invitrogen). The PS or PCL films were then washed, mounted on glass microscope slides and examined with an Eclipse TE2000-S microscope equipped with a 60× objective and a Retiga 1300R camera (Nikon). The area of cell spreading was evaluated using SigmaScan Pro V5 (Systat Software, San Jose, CA, USA).