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Full Length Article

Degradation of extracellular matrix regulates osteoblast migration: A microfluidic-based study

N. Movilla, C. Borau, C. Valero, J.M. García-Aznar *

Multiscale in Mechanical and Biological Engineering, Aragon Institute of Engineering Research, Department of Mechanical Engineering, University of Zaragoza, 50018 Zaragoza, Spain

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ABSTRACT

Bone regeneration is strongly dependent on the capacity of cells to move in a 3D microenvironment, where a large cascade of signals is activated. To improve the understanding of this complex process and to advance in the knowledge of the role of each specific signal, it is fundamental to analyze the impact of each factor independently. Microfluidic-based cell culture is an appropriate technology to achieve this objective, because it allows recreating realistic 3D local microenvironments by taking into account the extracellular matrix, cells and chemical gradients in an independent or combined scenario. The main aim of this work is to analyze the impact of extracellular matrix properties and growth factor gradients on 3D osteoblast movement, as well as the role of cell matrix degradation. For that, we used collagen-based hydrogels, with and without crosslinkers, under different chemical gradients, and eventually inhibiting metalloproteinases to tweak matrix degradation. We found that osteoblast's 3D migratory patterns were affected by the hydrogel properties and the PDGF-BB gradient, although the strongest regulatory factor was determined by the ability of cells to remodel the matrix.

1. Introduction

Cell migration plays a critical and crucial function on many regenerative processes, including bone regeneration. Migration of highly motile cells (such as fibroblasts) has been the focus of intensive experiments and mathematical modeling research [26,32,33,36,39,46], specifically in wound healing applications. However, there are few experimental works in the literature regarding osteoblast migration [10,22,24], in comparison with the more numerous mathematical modeling approaches due to the wide variety of modeling techniques [2,5,19,20,38].

Most of in-vitro experiments of osteoblast migration have been developed with cells of different animal origin, for instance neonatal rat calvarial osteoblasts [10] or murine osteoblastic cell lines [14,45]. However there are not many in-vitro works of 3D migration with human osteoblasts. In fact, as far as authors are concerned, the most relevant work corresponds to the one from [40] that studied 3D migration induced by apatite.

Osteoblast population migratory response to chemo-attractants has been normally studied on chosen substrates or non-porous surfaces (for example, metals used for orthopedic/dental applications) [10] or using commercially available Boyden chamber assays [22]. However, none of these assays allow examination of cellular chemotaxis on 3D environments. The present study adapts the well-documented tumor cell migration assays under a chemical gradient [1,3] to examine osteoblast 3D migration.

To recreate physiological osteoblast 3D migration, the use of biomimetic hydrogels is fundamental. Osteoblast culture using type I collagen gel, which is the major component of bone matrix, was first described by Elsdale and Bard [15]. Osteoblasts in such culturing conditions maintained their function, 3D structure and cell–cell interaction [45]. In fact, they found that osteoblasts migrated into the gel, extended dendritic protrusions towards neighboring cells, and synthesized collagen fibrils, mineralizing around themselves. Nevertheless, Uchihashi et al. [45] focused their work on understanding the transition mechanism from osteoblast to osteocyte, but did not quantify osteoblast migration. Mullen et al. [34] investigated the effect of collagen cross-linking (with glutaraldehyde) in conjunction with substrate thickness on the 2D osteogenic cell behavior finding that MC3T3 cells cultured on a soft fibrous substrate attain the same spread cell area as those cultured on a much higher modulus, but nonfibrous substrate. More recently, Robin et al. [40] showed that osteoblast 3D invasion is enhanced by the presence of apatite bone mineral in the extracellular matrix (ECM), although migration was not quantified in terms of speed and directionality. To date, to the best of authors' knowledge, the migration characteristics of osteoblasts on 3D collagen gels modified with crosslinkers have not been examined.

Different kind of growth factors have been studied in the literature to evaluate the osteoblast chemotactic properties: TGF-β is a chemoattractant used for stimulating rat osteosarcoma cells [29] and human osteoblasts [27]; OP-1 is chemically and structurally similar to

* Corresponding author.
E-mail address: jmgaraz@unizar.es (J.M. García-Aznar).

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bone morphogenetic protein-2, a chemoattractant for human osteoblasts [27]; insulin-like growth factors (IGF-1 and IGF-2) also stimulate the site-directed migration of osteoblasts [27,35]; Robin et al. [40] demonstrated that the presence of bone apatite promotes osteoblast migration, possibly through ion release and/or mediated by the roughness of the apatite or the alteration of the mechanical properties. In any case, the platelet derived growth factor β (PDGF-BB) has been found to be one of the most potent chemotactic factors for primary osteoblasts and osteoblastic cell lines in 2D cell migration [6,22,27,31]. In fact, PDGF has been used as a treatment for improving bone fracture healing in preclinical and clinical stages [12,23].

To migrate in 3D, cells can use either proteolytic (mesenchymal) or non-proteolytic (amoeboid) strategies [13]. In proteolytic migration, cells adhere to matrix secreting active metalloproteases (MMP), which break down the ECM and thus create macroscopic holes that allow their movement. However, in non-proteolytic migration, cells deform the ECM or squeeze through it, without degradation. From the literature, it is not clear whether osteoblast migration in collagen-based matrices presents a proteolytic or a non-proteolytic strategy. Therefore, the main aim of the present study is to quantitatively examine the migration characteristics of human osteoblasts on 3D collagen-based matrices. For that, we used 4 mg/ml collagen hydrogels, eventually adding crosslinkers and/or inhibiting metalloproteases activity. In addition, different PDGF-BB chemical gradients were induced in order to understand their regulatory role.

2. Materials and methods

2.1. Fabrication of microfluidic chips

Microdevices were fabricated in PDMS following the methodology described by Shin et al. [43]. Actually, they were made of polydimethylsiloxane (PDMS-Dow Corning GMbH Sylgard 184, Dow Chemical, Germany) at a 10:1 ratio of base to curing agent. PDMS microdevices were plasma-bonded to 35 mm glass-bottom petri dishes (IBIDI) and treated with poly-D-lysine (PDL) solution (Sigma- Aldrich, Germany) at 1 mg/ml for enhanced surface-matrix attachment.

The geometry of the microfluidic devices (Fig. 1A, B) consists of two media channels running parallel to and located on either side of an extended central channel containing the hydrogel with the cells, which simulates the bone matrix (based on the one used by [17]).

2.2. Collagen-based gels and 3D cell culture

The hydrogel used to simulate bone matrix was collagen type I (BD Biosciences), which was prepared to a final concentration of 4 mg/ml with DPBS (Lonza) following the methodology proposed by Shin et al. [43]. The dilution was brought to pH 7.4 with 0.5 M NaOH. Cells suspended in culture medium were mixed with the collagen hydrogel to a final dilution of 1×10^5 cells/ml. This dilution was then pipetted into the central gel chamber and the hydrogel was confined by surface tension. Once in place, collagen gel solution was polymerized in a humidity chamber at 37 °C and 5% CO_2 for 20 min. After that, the matrix was hydrated with Osteoblast Growth Medium (OGM) and incubated overnight for stabilization of matrix and cell adhesion.

Human Osteoblast (HOB, C-12720, Promocell) cells were cultured using Osteoblast Growth Medium (C-27001, 0.1 ml/ml of Fetal Calf Serum, Promocell) and used in passages 3–6. Cells were maintained in a humidified atmosphere incubator at 37 °C and with 5% CO_2.

2.3. Mechanical and microstructural characterization of collagen-based scaffolds

Collagen hydrogels used for cell culture were characterized by rheology to obtain their mechanical properties using a Haake Rheostress 1 rheometer. Collagen hydrogels were immediately pipetted on the rheometer after being prepared and polymerized in-situ during 24 h at 37 °C. All samples were tested using a cone-plate configuration with a 35 mm diameter and a cone angle of 1°. To avoid dehydration the samples were sealed with low viscosity oil. After 24 h the gels were mechanically loaded applying oscillatory strain sweeps with an excitation frequency of 0.1 Hz.

In addition, microarchitecture of the hydrogels was evaluated by means of scanning electron microscopy (SEM). Images were acquired using a SEM Inspect™ F50 (FEI Company, Eindhoven, The Netherlands) in an energy range between 0 and 30 keV. The hydrogels for the microstructural study were prepared as described before and allowed to stabilize for 24 h in a humidified chamber at 37 °C. The preparation of the samples was made by firstly frozen each sample separately in liquid nitrogen. Subsequently, the tubes containing the samples were submitted to lyophilizer (Telstar cryodos Freeze Dryer) for at least 24 h. Finally, the samples were coated with a carbon film before they were examined with the SEM technique.

To modify the mechanical properties of the collagen-based hydrogels we altered the microstructure via crosslinking with Transglutaminase (TG2) [18], which increases strength and resistance of biopolymers to proteolytic digestion [7,25]. Purified recombinant human Transglutaminase II (TG2) in solution (R&D Systems) at 25 μg/ml was mixed into the hydrogel solution and softly pipetted into the devices. The enzyme was added in the last place to the collagen-reaction mix to minimize any self-imposed crosslinking. The hydrogel was polymerized for 20 min in a humidified chamber, at 37 °C and 5% CO_2. Following polymerization, the matrix was hydrated an incubated overnight in the same conditions.

2.4. Application of a chemical gradient

In order to recreate the local conditions that occur in the environment of a bone fracture or injury, we induce in the microfluidic device a chemical gradient using PDGF-BB as chemoattractant. The concentrations of the recombinant human PDGF-BB (Invitrogen) isoform used in

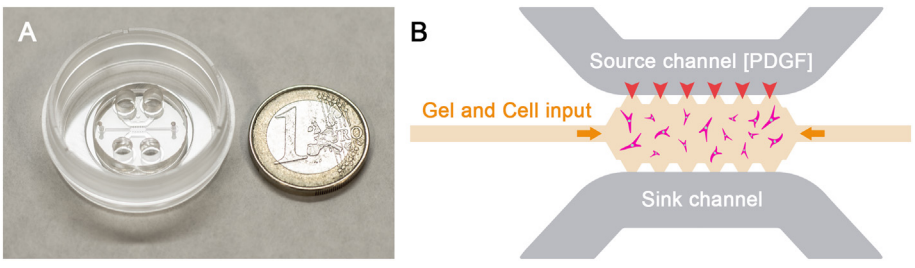

Fig. 1. A) Picture of the microfluidic device placed inside a 35 mm plate. B) Scheme of the central part of the device. The central channel is filled with collagen and cells through the auxiliary channels (orange horizontal arrows). The two main media channels, namely the source channel and the sink channel, ensure the hydration and diffusion of the PDGF-BB through the hydrogel. Red arrow heads indicate the direction of the chemical gradient. Importantly, the height of the channels is 300 μm for all the geometry. Further geometric details can be found at [33]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)