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Research article

Correlation between ECM guidance and actin polymerization on osteogenic differentiation of human adipose-derived stem cells

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ABSTRACT

The correlation between extracellular matrix (ECM) components, cell shape, and stem cell guidance can shed light in understanding and mimicking the functionality of stem cell niches for various applications. This interplay on osteogenic guidance of human adipose-derived stem cells (hASCs) was focus of this study. Proliferation and osteogenic markers like alkaline phosphatase activity and calcium mineralization were slightly increased by the ECM components laminin (LA), collagen I (COL), and fibronectin (FIB); with control medium no differentiation occurred. ECM guided differentiation was rather dependent on osterix than on Runx2 pathway. FIB significantly enhanced cell elongation even in presence of actin polymerization blockers cytochalasin D (CytoD) and ROCK inhibitor Y-27632, which generally caused more rounded cells. Except for the COL surface, both inhibitors increased the extent of osterix, while the Runx2 pathway was more sensitive to the culture condition. Both inhibitors did not affect hASC proliferation. CytoD enabled osteogenic differentiation independently from the ECM, while it was rather blocked via Y-27632 treatment; on FIB the general highest extent of differentiation occurred. Taken together, the ECM effect on hASCs occurs indirectly and selectively via a dominant role of FIB: it sustains osteogenic differentiation in case of a tension-dependent control of actin polymerization.

1. Introduction

Stem cells require an appropriate three-dimensional environment referring as stem cell niche, which guides their differentiation and self-renewal [8]. Thereby, they own the capability for tissue regeneration. Such environment is characterized by a complex network of extracellular matrix (ECM) components serving as anchorage points for the cells. The anchorage points are defined as binding motifs of adhesion ligands such as collagens, fibronectin, and laminin [20], mediating adhesion to ligand specific receptors expressed at the cell surface. Additionally, the ECM storages signaling molecules like growth factors and others. Such signaling molecules, which are also secreted by neighboring cells, induce stem cell responses [9]. Mechanical properties of the ECM were shown to stimulate specifically either neuronal, myogenic, or osteogenic differentiation [10]. It is believed that this control is dependent on actin polymerization and thereby cell morphology, which varies in dependence of anchorage to ECM with different stiffness [13]. Osteogenic differentiation for instance is then classified by large spread cells, high intracellular forces due to well polymerized actin – predominantly observed on stiff interfaces.

The detailed composition of the niche is tissue-specific – and dynamic, but little information is available on how all parameters interplay and control stem cells. Such basic knowledge is not only necessary to understand tissue regeneration. It further gives insights into how stem cell expansion, stem cell-based therapies, and tissue engineering approaches can be improved [15]. All mentioned research fields demand an artificial stem cell niche, which is realized by micro- or nanotechnologies. Beside the fabrication of a three-dimensional environment, however, appropriate materials for this niche are required. The big challenge

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenic proteins; bMSC, mesenchymal stem cells from the bone marrow; Con, uncoated control surface; COL, collagen type I; CytoD, cytochalasin D; DMSO, dimethylsulfoxide; ECM, extracellular matrix; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FIB, fibronectin; hASC, human adipose-derived stem cells; LA, laminin; LDH, lactat dehydrogenase; MLC, myosin light chain; MSC, mesenchymal stem cells; PBS, phosphate buffered saline; p-ERK, phosphorylated extracellular-signal-regulated kinase; p-FAK, phosphorylated focal adhesion kinase; p-Runx2, phosphorylated Runx2; Rel., relative; ROCK, RhoA-Rho kinase; Runx2, Runt-related transcription factor 2; SEM, standard error of mean

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is related to finding biomimetic materials, which combine the correct stiffness, anchorage sides, and growth/differentiation stimuli within the ECM, in order to control stem cell responses. Therefore, basic knowledge on the interplay of all parameters on stem cell guidance is necessary in order to select the right component for the particular application.

Osteogenic differentiation is accomplished by mesenchymal stem cells (MSCs). The source of these cells is basically the bone marrow. However, mesenchymal stem cells can also be derived from other tissues [2]. Adipose-derived stem cells (ASCs), for instance, are more easily available with a higher cell density than mesenchymal stem cells from the bone marrow (bMSCs) [4]. Therefore, they are an attractive cell source for stem cell investigations and biomedical approaches. In the past, several studies have analyzed the impact of ECM components on differentiation of bMSCs. It was shown that the ECM selectively guides their differentiation [19]. However, in what manner ASCs are affected by the ECM is poorly understood. Even though both cell types, bMSC and ASCs, undergo differentiation towards the same lineages, their differentiation pattern differ [5,12]. Concerning osteogenic markers like alkaline phosphatase (ALP), osteocalcin, and calcium mineralization it was demonstrated that the time point of expression, extent and duration varies. For instance, cultivated on zirconium scaffolds the maximum of ALP activity is at day 3 for human ASCs, but at day 7 for bMSCs [14]. Therefore, it cannot be concluded that the ECM plays an identical role for ASC differentiation like for bMSCs.

In this study ECM impact on osteogenic ASC differentiation was analyzed. Thereby, the influence of collagen type I (COL), laminin (LA), and fibronectin (FIB) were compared. The cells were cultivated in control and osteogenic cell culture media. Typical osteogenic markers like alkaline phosphatase activity, and calcification were quantified at different time points of cultivation. Above that cell proliferation was estimated. Due to the described correlation between cell morphology and stem cell differentiation, we further blocked actin polymerization via cytochalasin D (CytoD) in a tension-independent and rho-associated kinase (ROCK) inhibitor Y-27632 in a tension-dependent manner in order to address dependencies between actin, cell shape, osteogenic differentiation, and ECM components.

2. Material and methods

2.1. ECM preparation

Following ECM components were used for comparison: laminin (LA) from Engelbreth-Holm-Swarm murine sarcoma basement membrane, collagen type I (COL) from rat tail, and fibronectin (FIB) from bovine plasma (Sigma-Aldrich, Deisenhofen, Germany: L2020, C3867, F1141). Starting from a sterile stock solution for each ECM component of 0.01% in phosphate buffered saline (PBS), glass slides or the well bottoms of 24 multiwell plates were coated with 5 $\mu g/cm^2$ at room temperature for 30 min [20]. The control referred to uncoated slides or well bottoms. Afterwards, all treatments were washed with PBS. Coating was performed directly before starting the experiments.

2.2. Cell culture, differentiation, and blocking of actin polymerization

Chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) unless otherwise noted. Human adipose-derived stem cells (hASCs) were either purchased from Lonza (Lot numbers 0000399065 and 0000407088; Basel, Switzerland) or isolated and cultivated as previously described in [14]. Cells from passage 4–5 were used for the experiments. Each measurement was performed with control and osteogenic hASCs. Control medium consisted of Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (Biochrom AG, Berlin, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce osteogenic differentiation, the control medium was additionally supplemented with 0.1 μM dexamethasone, 10 mM β-glycerolphosphate, and 50 μM ascorbate-2-phosphate. For each experiment 2×10^4 undifferentiated hASCs per treatment were seeded onto ECM-coated slides or well bottoms: either in control or in osteogenic media. To block actin polymerization, two different antagonists were used for comparison. Both were solved in dimethylsulfoxide (DMSO) accordingly to the instruction protocol (abcam Biochemicals, Cambridge, Great Britain) and then given to the control and osteogenic cell culture media. The first antagonist was cytochalasin D (CytoD), which depolymerizes actin filaments; used with a final concentration of 2 μM. The second was Y-27632 dihydrochloride with a concentration of 5 μM. Last inhibits the Rho-associated kinase (ROCK), which is involved in signaling cascades after cell adhesion to organize actin polymerization. To estimate the effects of both actin polymerization blockers, a DMSO control for control and osteogenic hASCs was additionally prepared. In that case DMSO with a concentration of 0.04% v/v was applied as a solvent control. All cell culture media were changed every third day.

Proliferation and osteogenic differentiation in dependence of ECM components and actin polymerization inhibitors were analyzed after 3, 7, 14 and 21 days of cultivation. Cell staining in terms of morphology and osteogenic transcription factors was estimated after 3 days of cultivation. Typical osteogenic markers were further stained after 21 days cultivation time.

2.3. Cell proliferation

The cell density was determined via lactat dehydrogenase assay (LDH) at different time points, following the online protocol of OPS Diagnostics (Lebanon, USA). After washing the cells with PBS, 300 μl lysis buffer was added and the cell layers were scraped manually. Then the cell lysate was transferred to a microcentrifuge tube and centrifuged at 12,000 g for 10 min. For analyzing the LDH activity, the absorbance of the supernatants was detected at 492 nm wavelength using a microplate reader (Tecan Infinitive M200Pro and Tecan i-controlTM software, Crailsheim, Germany). The measured LDH activity was correlated with the cell number from a cell standard curve prepared under the same conditions [6]. The results were averaged ± SEM, given as cell number/cm^2 and obtained from three independent measurements.

2.4. Staining of actin filaments and transcription factors

Via staining actin filaments, the effects of ECM components and both actin polymerization blockers on actin filament organization and cell elongation of control and osteogenic hASCs were determined. Additionally, Runx2, p-Runx2, and osterix of osteogenic hASCs were visualized via immunostaining. After 3 days in culture ECM-coated glass slides with adherent hASCs were fixed using 4% paraformaldehyde dissolved in PBS for 20 min and permeabilized with 0.3% Triton X-100/PBS. To prevent nonspecific antibody binding, a 2% bovine serum albumin/PBS solution was applied at 37 °C for 2 h. After blocking, the cells were incubated with primary antibodies diluted in 0.3% Triton X-100/PBS at 4 °C overnight. Runx2 was diluted to 1:1500 (Biolabs GmbH, Frankfurt, Germany), p-Runx2 to 1: 100 (Antikörper Online, Aachen, Germany), and osterix to 1:50 (Santa Cruz, Heidelberg, Germany). After several washing steps, the cells were incubated with a fluorescence-conjugated secondary antibody at an appropriate dilution (Alexa Fluor® 488 conjugated Goat anti-rabbit IgG (H+L))/(Alexa Fluor®