Universidade do Minho

Escola de Engenharia

Semana da Escola de Engenharia
October 24 - 27, 2011

ATTACHMENT AND SPREADING OF HUMAN DERMAL FIBROBLASTS ON SELF-ASSEMBLED BIOACTIVE MATRICES

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KEYWORDS
Hyaluronan, peptide amphiphiles, skin regeneration

INTRODUCTION

Extracellular matrix (ECM) plays a key role in wound healing as ECM components are known to have the ability to regulate cellular processes, such as adhesion, growth and migration in the different phases of the healing process. The design of synthetic biomaterials capable of functioning as natural ECM analogs have received exquisite attention for application in tissue engineering in order to form functional and structural tissues (Patterson et al. 2010, Lutolf et al. 2005). Several approaches have been reported to include native ECM proteins, like fibronectin or collagen, to mediate cellular processes (Shroff et al. 2010). Generally, these systems use full length proteins for scaffold functionalization, which require mild reactions as proteins may easily become denaturated and ineffective after heat or chemical treatment. An alternative approach to the use of full length proteins, are small peptides that offer advantages such as increased stability, controlled orientation, simple synthesis, and their ability to incorporate multiple biological signals. Cell adhesion to native ECM is mediated through binding of integrin proteins on the cell surface to specific epitopes present on proteins of the ECM, creating focal adhesions, anchoring the cell and allowing for communication with the surrounding environment (Collier et al. 2010). For example, fibronectin, binds to integrins through a domain containing Arg-Gly-Asp-Ser (RGDS) (Ruoslahti and Pierschbacher 1986). This sequence, first developed by Pierschbacher and Rouslahti, functions as a general adhesive sequence and has been widely used for functionalization of biomaterials improving cell adhesion (Hersel et al. 2003). Therefore, bioactive matrices that can mimic multiple aspects of native ECM (biochemical and physical signals) would be of great benefit in skin regeneration strategies.

Towards this challenge, we report here the development and characterization of bioactive membranes that result from the instant self-assembly between peptide amphiphiles and the glycosaminoglycan hyaluronic acid (HA), a major component of skin ECM. To foster cell adhesion and proliferation on the self-assembling membranes, the fibronectin-derived RGDS epitope was incorporated into the peptide structure. Due to their ability to recapitulate biochemical signals of skin tissue niche, these molecules offer many unique advantages as starting materials for skin regeneration applications.

MATERIALS AND METHODS

Synthesis and characterization of peptide amphiphile (PA)

All peptides possessed the same basic construction, consisting of a sequence of 3 valine (V), 3 alanine (A) and 3 lysine (K) residues, attached to an alkyl tail of 16 carbons. In this work, three different peptides were synthesized: C_{16}-$V_3A_3K_3$, C_{16}-$V_3A_3K_3$RGDS (RGDS) and C_{16}-$V_3A_3K_3$DGSR (scrambled).

The PAs were synthesized following a standard solid phase Fmoc chemistry in an automated peptide synthesizer. After cleavage of PA from the resin, the crude product was isolated by precipitation with cold diethyl ether and further purified by reverse phase high performance liquid chromatography (RP-HPLC).

Peptides were characterized by electrospray ionization mass spectrometry (ESI-MS), transmission electron

microscopy (TEM) and circular dichroism (CD) spectroscopy.

Membrane production and characterization

Membranes were prepared by casting 1 wt% HA and 2 wt% PA aqueous solutions onto individual wells of a 48-well plate, followed by incubation at 60 ºC for 4 h and then rinsed with sterile deionized water to remove excess of HA and peptide.

Cell adhesion assay

For cell adhesion assays, membranes incorporating the RGDS epitiopte at different percentages were produced. For that, 2% (w/v) peptide solutions were prepared consisting of 100% diluent peptide (C_{16}-$V_3A_3K_3$), 1%, 10% and 50% RGDS, and 10% scrambled peptide (DGSR). Membranes were then produced following the procedure described previously. Confluent hDFb at passage 4 were harvested from monolayer cultures using trypsin-EDTA. Cell *pellet* was resuspended at a density of 5.0 x 10^4 cells/mL in serum-free DMEM without phenol red (Sigma, Germany) supplemented with 1% (v/v) antibiotic/antimycotic solution (final concentration of 100 units/mL penicillin and 100 mg/mL streptomycin; Gibco, UK). Cells were cultured in 48 well plates at 37 ºC in a humidified atmosphere of 5% CO_2 in air. hDFb were cultured on tissue culture polystyrene (TCPS) coverslips as controls. At different culture times (2, 6, 12 and 24 h), samples were collected for further analysis including cell proliferation (DNA quantification) and morphology (Scanning Electron Microscopy, SEM) and organization of actin cytoskeleton and focal adhesion points.

RESULTS

Although the hyaluronan-peptide membranes integrate components of natural skin ECM, they do not promote cell attachment. To support integrin-mediated cellular adhesion and migration on the biomimetic membranes, we have included the RGDS sequence in the peptide structure, which is a well-known ligand for integrin receptors.

The results from DNA quantification showed higher number of cells when fibroblasts were grown on 50% RGDS-HA, thus indicating better adhesion of cells to the membranes containing 50% RGDS when compared to the ones with lower concentration of RGDS, 1 and 10% RGDS-HA, or without RGDS, V_3A3K_3-HA and DGSR-HA ($p<0.01$). This behavior was observed shortly (2 hours) after seeding and up to twenty four hours. The condition 10% RGDS-HA showed higher adhesion than V_3A3K_3-HA and DGSR-HA, ($p<0.05$). We further examined fibroblast morphology on the matrices. The cell morphology observed by SEM was similar to what has been observed by other authors on RGD-coated glass substrates. However, after 24 hours only the conditions 10% and 50% RGDS-HA showed fully adherent cells and the presence of filopodia. Immunostaining results confirmed higher adhesion in the condition 50% RGDS-HA, as observed by the abundance of stained nuclei, although no vinculin or high organized actin stress fibers were seen after staining.

CONCLUSIONS

These studies indicated that human dermal fibroblasts show lower adhesion when grown on membranes without the cell recognition epitope RGDS in comparison to the ones containing the RGDS sequence. We expect that the proposed biodegradable hybrid matrices could offer significant potential in skin tissue engineering strategies, as a bioactive supportive matrix for promoting wound regeneration, and also as model systems for fundamental studies in wound healing.

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ACKNOWLEDGEMENTS

Daniela S. Ferreira acknowledges the financial support received from Fundação para a Ciência e a Tecnologia (PhD scholarship SFRH/BD/44977/2008