Biomaterials 32 (2011) 8938–8946

Contents lists available at ScienceDirect

Biomaterials

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

Growth promoting substrates for human dermal fibroblasts provided by artificial extracellular matrices composed of collagen I and sulfated glycosaminoglycans

Anja van der Smissen [a,d,*], Vera Hintze [b,d], Dieter Scharnweber [b,d], Stephanie Moeller [c,d], Matthias Schnabelrauch [c,d], Annett Majok [a,d], Jan C. Simon [a,d], Ulf Anderegg [a,d]

[a] Department of Dermatology, Venereology and Allergology, Leipzig University, 04103 Leipzig, Germany
[b] Max Bergmann Center for Biomaterials, Technische Universität Dresden, 01069 Dresden, Germany
[c] Biomaterials Department, INNOVENT e.V. Jena, 07745 Jena, Germany
[d] Collaborative Research Center (SFB-TR67), Matrixengineering Leipzig and Dresden, Germany

ARTICLE INFO

Article history:
Received 5 July 2011
Accepted 9 August 2011
Available online 27 August 2011

Keywords:
Wound healing
Collagen I
Sulfated glycosaminoglycan
Hyaluronan
Chondroitin sulfate
Human dermal fibroblasts

ABSTRACT

The application of native extracellular matrix (ECM) components is a promising approach for biomaterial design. Here, we investigated artificial ECM (aECM) consisting of collagen I (coll) and the glycosaminoglycans (GAGs) hyaluronan (HA) or chondroitin sulfate (CS). Additionally, GAGs were chemically modified by the introduction of sulfate groups to obtain low-sulfated and high-sulfated GAG derivatives. Sulfate groups are expected to bind and concentrate growth factors and improve their bioactivity. In this study we analyzed the effect of aECM on initial adhesion, proliferation, ECM synthesis and differentiation of human dermal fibroblasts (dFb) within 8–48 h. We show that initial adhesion and cell proliferation of dFb progressively increased in a sulfate dependent manner. In contrast, synthesis of ECM components coll and HA was decreased on high-sulfated aECM coll/HA3.0 and coll/CS3.1. Furthermore, the matrix metallo-proteinase-1 (MMP-1) was down-regulated on coll/HA3.0 and coll/CS3.1 on mRNA and protein level. The fibroblast differentiation marker α-smooth muscle actin (αSMA) is not affected by aECM on mRNA level. Artificial ECM consisting of coll and high-sulfated GAGs proves to be a suitable biomaterial for dFb adhesion and proliferation that induces a "proliferative phenotype" of dFb found in the early stages of cutaneous wound healing.

1. Introduction

The development of functional biomaterials is essential for the support of dermal wound healing. Tissue-engineered skin substitutes represent a therapeutical option for the treatment of acute and chronic skin wounds including trauma and chronic ulcerations secondary to diabetes mellitus or venous stasis. Skin replacements are not only supposed to replace the defect and reconstruct tissue functionality but also should be easily applicable under clinical conditions [1,2].

Cutaneous wound healing is a multistep process that involves several cell types and events. The initial step is the inflammatory response, followed by proliferation and migration of dermal and epidermal cells and matrix synthesis in order to fill the wound gap and re-establish the mechanical barrier of the skin [3–5]. Dermal fibroblasts (dFb) are crucial cells during these processes. Due to a fast adhesion to the wound matrix, followed by proliferation and extracellular matrix (ECM) synthesis they fill the wound defect. To gain the ECM synthesizing activity dFb have to differentiate into myofibroblasts (MFb). MFb are characterized by a contractile activity due to α-smooth muscle actin (αSMA) expression and integration into stress fibers [6]. Furthermore, MFb synthesize high amounts of ECM components like the structural protein collagen I (coll) and the glycosaminoglycan (GAG) hyaluronan (HA) [7].

The stimulation of either proliferation or differentiation of dFb is provided by cell–cell and/or cell-matrix interactions or by soluble mediators and growth factors. For example, fibroblasts differentiate to MFb phenotype under the control of keratinocyte-derived transforming growth factor β_1 (TGFβ_1), whereas fibroblasts stimulate keratinocyte proliferation in a paracrine manner by release of

Abbreviations: GAGs, glycosaminoglycan; Coll, collagen I; dFb, dermal fibroblasts; MFb, myofibroblasts; HA, hyaluronan; HA1.0 (3.0)sulfate groups per disaccharide, HA with a sulfation degree of 1.0 (3.0); CS, chondroitin sulfate; CS1.8 (3.1)sulfate groups per disaccharide, CS with a sulfation degree of 1.8 (3.1).

* Corresponding author. Leipzig University, Department of Dermatology, Venereology and Allergology, 04103 Leipzig, Germany. Tel.: +49 341 9725932; fax: +49 341 9725878.
E-mail address: anja.vandersmissen@medizin.uni-leipzig.de (A. van der Smissen).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.08.025

interleukin-6 (IL-6) or keratinocyte growth factor (KGF) [5]. For tissue engineering and biomaterial design initial cell-matrix interactions are a major point of interest. dFb are influenced by the existing cell-surrounding ECM and are able to sense the surrounding ECM with the cell surface and especially cell surface receptors like integrins. Binding to native type I collagen by integrins involves mainly $\alpha1\beta1$ and $\alpha2\beta1$ forms [8–10]. The recognition of the glycosaminoglycan HA is mediated by the receptors CD44, the receptor for hyaluronic acid-mediated motility (RHAMM) or LYVE [11–13]. The presence of several receptors permits the sensing of biochemical and physical properties of the ECM like the recognition of single chemical components, matrix roughness and stiffness [14–16]. The importance of integrins in mediating the cell-matrix interactions of fibroblasts under various culture conditions has been shown by Eckes et al. [17]: While relaxed collagen lattices induce an inflammatory fibroblast phenotype, mechanically stressed lattices refer to a stiff matrix and induce an activated ECM synthesizing phenotype. Moreover, it was shown that the stiffness of the cell-surrounding ECM has a strong influence on latent $TGF\beta_1$ activation and therefore on fibroblast to MFb differentiation [16]. An appropriate functional biomaterial should lead to direct interactions with cells and especially signal molecules to induce initial cell proliferation, followed by repair of the connective tissue and cellular differentiation finally supporting wound healing processes.

A promising approach for biomaterial design is the application of combined native ECM components. In this study we investigated artificial ECM (aECM) consisting of the structural protein coll I and the GAGs HA or chondroitin sulfate (CS). GAGs are natural, negatively charged, linear heteropolysaccharides, consisting of repeating disaccharide units [18,19]. Recently, biomaterial design focused on GAGs because of their low immunogenicity and the property to interact with growth factors thus enhancing their biological activity. Extracellular GAGs can bind, concentrate and prevent the diffusion of growth factors, partially improve and stabilize the presentation to their relevant receptors and protect them from proteolytic degradation [14,20,21]. Protein-GAG interactions are principally governed by ionic interactions because of the high negative charge provided by sulfate and carboxylate groups of GAG and basic amino acids of the protein. Topology and distribution of the basic amino acids of the GAG binding site influence the specificity in molecular recognition of GAG sequences [21–23]. Despite the degree of sulfation GAG-mediator interactions are also affected by the position of the sulfate groups within the anhydrosugar repeating unit and the linkage of sulfate group to the sugar ring hydroxyl or amino substituents (O- or N-sulfation) [24]. Gama et al. [25] provide evidence for the existence of a "sulfation code" whereby the precise position of the sulfate groups along the carbohydrate backbone permits GAGs to encode information in a sequence-specific manner. Precise modifications to GAGs could facilitate or inhibit ligand–receptor interactions, providing a special mechanism for regulatory control. Here, GAGs are chemically modified by the introduction of sulfate groups to obtain low-sulfated and high-sulfated derivatives in comparison to the native GAGs. CS, the most abundant sulfated GAG and HA, the only non-sulfated GAG, represent both ideal starting materials for this purpose [19]. The charge densities of GAGs are strongly increased with each additional sulfate residue. The introduction of sulfate groups into GAG backbone by chemical modification aims to improve binding of cell-derived growth factors. In this way the native spatio-temporal growth factor pattern could be utilized and aECM might cause a self-adjusting, improved wound healing process.

The aim of the present study was to investigate the effect of aECM containing sulfated GAGs on the biology of dFb since these cells are efficiently regulated by their surrounding matrix and are crucial cells during dermal wound repair. In this paper we analyze processes relevant to wound healing like initial adhesion, cell proliferation, ECM synthesis and differentiation of dFB to MFb.

2. Materials and methods

2.1. Preparation and characterization of aECM

2.1.1. Materials

Native, high-molecular weight HA (HA-HMW), from *Streptococcus*, was obtained from Aqua Biochem Dessau, Germany. Chondroitin sulfate, from porcine trachea (CS-A/C; a mixture of 70% chondroitin-4-sulfate and 30% chondroitin-6-sulfate) was purchased from Kraeber & Co GmbH, Ellerbek, Germany.

2.1.2. Synthesis of modified GAGs

Low molecular HA (HA-LMW) and sulfated HA derivatives were synthesized and characterized as described in Kunze et al. and Hintze et al. [18,20]. The sulfation of CS was performed via its tetrabutylammonium salt as follows:

General procedures: 5.0 g (9.93 mmol) of CS-A/C (sodium salt) were dissolved in distilled water (250 ml) at room temperature for 1 h. The solution was stirred with 50 g of Dowex WX 8 ion exchanger (tetrabutylammonium-form) over night and after filtration, the polymer solution was lyophylized and dried under vacuum at 40 °C providing the tetrabutylammonium salt of CS-A/C (TeBA-CS). Yield: 98%. After sulfation products were isolated from the reaction mixture by precipitation into acetone (3.0 l) and neutralized using ethanolic NaOH solution. The formed sodium salts of the sulfated CS were washed several times with acetone and purified by dialysis against distilled water followed by lyophilization of the aqueous solutions and drying of the resulting polymers under vacuum.

High-sulfated CS-A/C (CS3.1): 5.0 g (6.92 mmol) of TeBA-CS were suspended under argon at room temperature in DMF (200 ml). 21.19 g (138.35 mmol) of the SO_3-DMF complex dissolved in 60 ml DMF, were added (polymer:SO_3 ratio = 1:20), and the reaction solution was stirred for 1 h at room temperature. Work-up was performed as described above. Yield: 76% (related to CS-A/C-Na).

Low-sulfated CS-A/C (CS1.8): 5.0 g (6.92 mmol) of the SO_3-Pyridin complex, dissolved in 40 ml DMF, was added to a suspension of 5.50 g (34.59 mmol) of TeBA-CS in DMF (400 ml; polymer:SO_3 ratio = 1:5) under argon at room temperature. The reaction solution was stirred for 20 min. Work-up was performed as described above. Yield: 77% (related to CS-A/C-Na). Characteristics of the used GAGs are given in Table 1.

2.1.3. Artificial extracellular matrices

The preparation of aECMs is described elsewhere (submitted manuscript Hintze, V et al.) In brief, the coll/GAG matrices where produced by *in vitro* fibrillogenesis of rat collagen I (BD Bioscience, Heidelberg, Germany) in the presence of different GAGs in well-tissue-culture-plates (Nunc, Langenselbold, Germany). 1 mg/ml of acid-solubilized coll was mixed 1:1 with the same concentration of HA-HMW or the appropriate same molarity of disaccharide units for all other GAGs. This approach was chosen to compare the same amount of possible binding sites. Collagen was allowed to fibrillize for 16–18 h at 37 °C and then dried on the well plates. After two washing steps with 0.5 ml deionized water the coatings were dried again.

2.1.4. Analysis of aECM composition and stability

The characterization of aECMs is described elsewhere (submitted manuscript Hintze, V et al.). In brief, the coll content of the dried aECM containing coll and GAG derivatives was determined from the protein amount in the washing fractions by the method of Lowry [26]. After washing and 1 h incubation at 37 °C in PBS 91–97% of the original amount of collagen could be detected in the aECM coatings and did not markedly decrease further over 8 d of desorption in PBS at 37 °C. The quantity of sulfated GAG derivatives associated to the coll matrix was determined by analyzing the dried coatings directly by dimethylmethylene blue (DMMB) assay after digestion with papain at 60 °C [27]. The quantity of unmodified HA-LMW and HA-HMW associated to the coll matrix was determined by analyzing the dried coatings

Table 1
Nomenclature and characteristics of native and chemically sulfated GAGs.

Sample	Description	S content (%)	DS_S	M_W (Da)	PD
HA-HMW	Native HA	–	–	1,174,865	4.80
HA1.0	Low-sulfated HA	5.98	1.0	31,056	4.47
HA3.0	High-sulfated HA	12.31	3.0	47,835	1.72
HA-LMW	HA - thermically degraded	–	–	48,785	2.37
CS-A	Native CS	5.40	0.9	19,763	1.56
CS1.8	Medium-sulfated CS	9.02	1.8	23,215	1.52
CS3.1	High-sulfated CS	12.63	3.1	20,675	1.46

DS_s – average degree of sulfation per dianhydro sugar unit; M_w - weight-average molecular weight, determined by gel permeation chromatography (GPC) according to [20]; PD - polydispersity index (molecular weight distributions) determined by GPC [20].