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Artificial extracellular matrix composed of collagen I and highly sulfated hyaluronan interferes with TGFβ$_1$ signaling and prevents TGFβ$_1$-induced myofibroblast differentiation

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ABSTRACT

Sulfated glycosaminoglycans are promising components for functional biomaterials since sulfate groups modulate the binding of growth factors and thereby influence wound healing. Here, we have investigated the influence of an artificial extracellular matrix (aECM) consisting of collagen I (coll) and hyaluronan (HA) or highly sulfated HA (hsHA) on dermal fibroblasts (dFb) with respect to their differentiation into myofibroblasts (MFb). Fibroblasts were cultured on aECM in the presence of aECM-adsorbed or soluble transforming growth factor β1 (TGFβ1). The synthesis of α-smooth muscle actin (αSMA), collagen and the ED-A splice variant of fibronectin (ED-A FN) were analyzed at the mRNA and protein levels. Furthermore, we investigated the bioactivity and signal transduction of TGFβ1 in the presence of aECM and finally made interaction studies of soluble HA or hsHA with TGFβ1. Artificial ECM composed of coll and hsHA prevents TGFβ1-stimulated αSMA, collagen and ED-A FN expression. Our data suggest an impaired TGFβ1 bioactivity and downstream signaling in the presence of aECM containing hsHA, shown by massively reduced Smad2/3 translocation to the nucleus. These data are explained by *in silico* docking experiments demonstrating the occupation of the TGFβ-receptor I binding site by hsHA. Possibly, HA sulfation has a strong impact on TGFβ1-driven differentiation of dFb and thus could be used to modulate the properties of biomaterials.

1. Introduction

The development and application of functional biomaterials is necessary to support dermal wound healing in acute and chronic skin wounds, like trauma or chronic ulcerations [1]. Cutaneous wound healing is a multistep process that involves several cell types and events, beginning with the inflammatory response, migration and proliferation of dermal and epidermal cells and ending with matrix synthesis in order to fill the wound gap and re-establish the mechanical barrier of the skin [2–4]. During these processes dermal fibroblasts (dFb) are crucially involved, rapidly adhering to the wound matrix, proliferating and subsequently synthesizing extracellular matrix (ECM). To obtain an ECM synthesizing phenotype dFb have to differentiate into myofibroblasts (MFb) [5,6].

Myofibroblasts are key players in the physiological reconstruction of connective tissue after injury [5]. They synthesize high amounts of ECM components like collagen and during non-pathological wound healing they are removed by apoptosis when epithelialization occurs [7]. The transition from dFb to MFb requires stimulation with transforming growth factor β$_1$ (TGFβ$_1$) and mechanical tension [5]. Myofibroblasts express the ED-A splice variant of fibronectin (ED-A FN) and α-smooth muscle actin (αSMA) [8], which is integrated into stress fibers and thereby generates contractile forces. Despite the synthesis of collagen and the expression of ED-A FN, αSMA expression and fibrous organization represent the most reliable marker of differentiated MFb [6]. The formation and contraction of granulation tissue is essential to maintain tissue integrity, to reduce the size of the wound and close the defect, resulting in a scar. But when apoptosis of MFb is lacking, pathological changes result in hypertrophic scars or fibrosis [7].

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$TGF\beta_1$ is the key cytokine in the evolution of lesions characterized by MFb formation [6].

$TGF\beta_1$ is a disulfide bonded homodimeric cytokine [9] that is secreted as a part of the large latent complex, which consists of the latency-associated protein, latent $TGF\beta_1$ binding protein 1 and the growth factor itself [10,11] and, thereby, can be stored in the ECM [12,13]. The protease-independent release and activation of latent $TGF\beta_1$ requires integrin-mediated MFb contraction in the presence of a stiff growth substrate [14]. Binding of active $TGF\beta_1$ to TGFβ receptor II (TGFβRII) leads to the recruitment and phosphorylation of TGFβ receptor I (TGFβRI). This heteromeric complex phosphorylates cytoplasmic Smad2 and Smad3, resulting in the heteromeric Smad2/3 complex that binds to Smad4 and is translocated to the nucleus to regulate gene transcription of a variety of genes [5,12]. However, alternative signaling pathways are known, as discussed later.

A promising approach for the development of functional biomaterials is the combination of native ECM components with chemically modified substances to selectively modulate physiological processes like wound healing. Here we have investigated an artificial ECM (aECM) consisting of collagen I (coll) as a structural protein and several hyaluronan (HA) derivatives. Hyaluronan is a natural, polyanionic, linear heteropolysaccharide, consisting of repeating non-sulfated disaccharide units of D-glucuronic acid (β-1–3) and *N*-acetyl-D-glucosamine (β-1–4) [15,16]. In skin this glycosaminoglycan (GAG) represents a major component of the ECM and is mainly produced by fibroblasts [17]. For this study HA was chemically modified by the introduction of sulfate groups to obtain highly sulfated derivatives in comparison with native HA. This chemical modification aims to increase the binding of cell-derived growth factors like $TGF\beta_1$. GAGs are interesting molecules for artificial biomaterial design because of their low immunogenicity and their property to bind, concentrate and prevent the diffusion of growth factors. Furthermore, they can improve and stabilize the presentation to relevant receptors and protect growth factors from proteolytic degradation [18–20]. The main GAG types differ only slightly in the basic sugar backbone of the GAG chain. However, subsequent sulfation, deacetylation and epimerization modifications distinguish individual GAG chains and are critical for their specific activity [21]. Protein–GAG recognition is driven by ionic interactions because of the high negative charge provided mainly by sulfate but also by carboxylate groups of the GAG and basic amino acids of the protein. The topology and distribution of basic amino acids in the GAG-binding site influence the specificity of molecular recognition of GAG sequences [20,22,23]. Despite the degree of sulfation, GAG–mediator interactions are also affected by the position of the sulfate groups within the anhydrosugar repeating unit and by the linkage of sulfate group to the sugar ring (O- or N-sulfation) [24].

The introduction of sulfate groups along the carbohydrate backbone of GAGs has been generated by non-template driven processes and thereby provides heterogeneity in the fine structure of these molecules. It is known that the interaction of different GAGs with different proteins is dictated by the pattern and orientation of the sulfate groups along the carbohydrate backbone [25,26]. A certain protein can also interact with different sulfation patterns with different binding affinities and kinetics, whereby the kinetics affect the functions of cytokines or chemokines and the biological outcomes [27]. Moreover, the site on the protein recognised by the GAG structure contributes to whether GAG binding supports or inhibits a signaling process [28]. Thus, guided modifications to GAG structures may support or inhibit ligand–receptor interactions, providing a special mechanism for regulatory control.

Our previous work with sulfated GAGs integrated in collagenous matrices showed that initial adhesion and cell proliferation of dFb progressively increased with the degree of GAG sulfation, while synthesis of the ECM components coll and HA was decreased on aECM containing highly sulfated GAG. The MFb differentiation marker αSMA was not significantly affected by aECM within 48 h at the mRNA level in resting fibroblasts [29].

Since we hypothesize that $TGF\beta_1$ interactions with sulfate groups could influence its bioavailability and thereby could regulate MFb differentiation, the present study has investigated the impact of aECM with highly sulfated HA (hsHA) on the $TGF\beta_1$-driven differentiation of dFb to MFb by analyzing the induction of MFb marker genes αSMA, coll and ED-A FN in dFb within 72 h in the presence of aECM-adsorbed or soluble $TGF\beta_1$. Furthermore, we provide data on the underlying mechanisms concerning the bioactivity and signal transduction of $TGF\beta_1$ in the presence of highly sulfated aECM and, finally, we provide an in silico interaction model of non-sulfated HA and highly sulfated derivatives with $TGF\beta_1$, which supports our data on the influence of HA sulfation on the bioactivity of $TGF\beta_1$.

2. Materials and methods

2.1. Preparation and characterization of aECM

2.1.1. Materials

Native high molecular weight HA from *Streptococcus* was obtained from Aqua Biochem (Dessau, Germany). The synthesis and analysis of hsHA derivatives were described in Kunze et al. [15] and Hintze et al. [18]. Low molecular weight HA (LMW-HA) was prepared by controlled thermal degradation of native HA according to Moeller et al. [30]. The characteristics of the HA derivatives used are summarized in Table 1.

2.1.2. Artificial extracellular matrices

The preparation, analysis and stability of aECM were described previously [29,31]. In brief, the coll/GAG matrices were produced by *in vitro* fibrillogenesis of rat collagen I (BD Bioscience, Heidelberg, Germany) in the presence of different GAGs in tissue culture plates (Nunc, Langenselbold, Germany). 1 mg ml^{-1} acid-solubilized coll was mixed 1:1 with the same concentration of HA or the appropriate same molarity of disaccharide units for hsHA according to the molecular weight of the disaccharide units (Table 1). This approach was chosen to compare the same number of possible binding sites, since these compounds are heterogeneous in length and differ significantly in the molecular weight of their disaccharide units.

Table 1
Nomenclature and characteristics of native, LMW and sulfated HA.

Sample	Description	S content (%)	DS_S	M_w (Da)	*PD*	M_w disaccharide unit (g mol^{-1})
HA	Native HA			1,174,865	4.80	401.3
hsHA	Highly sulfated HA	13.4/13.5	3.1/3.4	49,725/51,145	1.70/1.73	717.6/748.3
LMW-HA	Thermally degraded HA			47,785	2.37	401.3

DS_S, average number of sulfate groups per disaccharide repeating unit (possible DS_S range $0 \leqslant DS_S \geqslant 4.0$); M_w, weight-averaged molecular weight determined by GPC according to Salek-Ardakani et al. [24]; *PD*, polydispersity index (molecular weight distributions) determined by GPC according to Salek-Ardakani et al. [24].

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