

Differential effects of exogenous and endogenous hyaluronan on contraction and strength of collagen gels

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Abstract

The addition of exogenous hyaluronan to biomaterial scaffolds has been an important area of investigation for many decades. The ability to manipulate endogenous production of hyaluronan via the hyaluronan synthases has offered another mechanism to study the effect of hyaluronan. While the literature suggests that exogenously added hyaluronan and endogenously produced hyaluronan will have varying impacts on extracellular matrix organization and function, no studies have directly shown this phenomenon. In this investigation, we demonstrate that the addition of exogenous high molecular weight (~1 MDa) hyaluronan and hyaluronan oligosaccharides have a distinct impact on both contraction and strength of smooth muscle cell-seeded collagen gels when compared to the effects of hyaluronan that is endogenously produced by the hyaluronan synthases. More specifically, the addition of exogenous high molecular weight hyaluronan resulted in more compact collagen gels with a higher ultimate tensile strength, whereas the endogenous overproduction of hyaluronan resulted in the opposite effect. We suggest that the addition of exogenous HA to collagen gels represents a model for the therapeutic administration of HA, whereas the addition of excess HA to a tissue via the endogenous overexpression of *has* represents a model for the pathological accumulation of HA.

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1. Introduction

Hyaluronan (hyaluronic acid, HA) is a long, unbranched polysaccharide component of the extracellular matrix (ECM) consisting of repeating disaccharides of *N*-acetylglucosamine and glucuronic acid. Due to the unique properties of HA, the exogenous addition of HA to cells and/or biomaterial scaffolds has been investigated for decades and has shown promise in inducing angiogenesis, stimulating cell growth and migration, and even promoting scarless wound healing [1–3]. For these reasons, HA has been used in tissue engineering and biomaterials applications for many decades [4]. Additionally, cellular responses to exogenous HA are highly dependent upon molecular weight

(reviewed in Ref. [5]). For example, HA oligosaccharides ($M_w < 150$ kDa) are often associated with increased cytokine production and migration of inflammatory cells in cardiovascular injury [6] and can also induce angiogenesis [7]. In contrast, high molecular weight HA (>1 MDa) is associated with cell quiescence and the inhibition of angiogenesis (reviewed in Ref. [8]).

Endogenously, HA is produced by three membrane-bound enzymes, the hyaluronan synthases (*has*). Three *has* isozymes (*has-1*, *has-2*, *has-3*) have been identified, each of which is responsible for producing high molecular weight HA [9,10]. It has been demonstrated that these isozymes react independently to growth factors [11] and have distinct roles in developmental biology [12]. Interestingly, the *has* isozymes produce HA that behaves differently than exogenously added HA. For example, two different studies showed that the addition of *has-2* antisense to cultures of

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an osteosarcoma cell line reduced HA accumulation, resulting in decreased cell proliferation, motility and invasiveness [13], but that the exogenous addition of high molecular weight HA failed to restore the properties observed with native *has* expression [14].

Several previous reports have demonstrated that the cellular retention and function of HA depends on whether it was produced endogenously via *has* or added exogenously. In monolayer cultures, exogenous HA oligosaccharides can displace the resident extracellular HA from the CD44 receptors [15,16]. However, others have suggested that if one end of the endogenously produced HA is still tethered by the *has* isozyme, it could not be displaced by the addition of exogenous HA oligosaccharides [17]. Furthermore, another investigation demonstrated that the large HA-dependent pericellular matrix and associated microvilli protrusions produced by *has*-overexpressing cells were unaffected by the addition of either high or low molecular weight HA. This same study demonstrated that surface retention of HA on *has*-overexpressing cells was independent of CD44 [18]. Early evidence of this variation between endogenously produced HA and exogenously added HA was provided by Itano et al., who demonstrated that endogenous overexpression of the HA synthases resulted in cells with visibly increased pericellular matrices, altered cell adhesion molecules and the capacity to grow in stratified layers [19]; exogenously added HA has not been reported to induce such changes. These studies demonstrated the different influences of endogenously produced and exogenously added HA on cell behavior in monolayers, but did not address the effects of these different methods of adding HA on tissue organization and material behavior. Previously, we have demonstrated that smooth muscle cells (SMCs) overexpressing the *has* isozymes, when seeded within three-dimensional collagen gels, will produce a less compacted and greatly weakened construct [20], whereas others have reported that exogenous addition of HA will cause greater contraction of a collagen gel by unmodified SMCs (with basal *has* expression) [21]. It is unclear how the exogenous addition of HA will influence the material behavior of cell-seeded matrices.

Thus, there exists a need for a direct comparison of the effects of excess HA, whether this is by endogenous overproduction via *has* or by exogenous addition, within the same model system in order to determine any impact on ECM organization and tissue function. The purpose of this study was to characterize the contraction, material behavior and distribution of HA cell surface receptors of three-dimensional collagen gels, seeded with *has*-overexpressing or control SMCs, when treated with either high molecular weight or oligosaccharide HA.

2. Materials and methods

2.1. Cell culture

Male Fisher 344 rats were killed and aortic SMCs were isolated and cultured under standard culture conditions

using culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g l⁻¹ glucose, 1% non-essential amino acids, 10% fetal bovine serum and 1% antibiotic/antimycotic/antifungal solution (all from Mediatech, Herndon, VA), as previously described [22]. Stable transfection was performed within the laboratory of Dr. Thomas Wight using published protocols [22,23] with the LXS_N retroviral vector and PE501/PA317 packaging cell system to produce a replication-deficient retrovirus to be selected via G418. Cells transfected to overexpress one of the three isozymes (*has-1*, *has-2*, *has-3*), or the empty vector control (LXS_N), were used in all experiments.

2.2. Formation of collagen gels

The cell-seeded collagen gels were prepared as outlined previously [20,24]. Briefly, cells overexpressing one of the *has* enzymes or the empty vector were grown to approximately 80% confluence, then added to a solution containing BD Rat Tail Type I Collagen (BD Biosciences, Franklin Lakes, NJ) and DMEM to obtain a final collagen concentration and cell concentration of 2.0 mg ml⁻¹ and 1.0 × 10⁶ cells ml⁻¹, respectively. Two-thirds of the gels were also mixed with exogenous HA as described in the next section. The mixtures were then pipetted under sterile conditions into Flexcell[®] Tissue Train[®] (Flexcell[®] International, Hillsborough, NC) culture plates. The wells within these customized six-well plates had a silicone elastomer membrane base with two nylon mesh anchors located 180° from each other to hold the collagen gels at each end, as described in detail in Garvin et al. [25]. These plates were then placed on top of the Flexcell[®] baseplate, which applied vacuum to the individual wells, created a 5 × 25 mm trough shape in the elastomeric membrane, and allowed the cell/collagen mixture to pool and gel within the trough to form a cohesive uniaxially aligned collagen gel. The entire system was then incubated in a humidified environment at 37 °C with 5% CO₂. After 24 h, the culture medium was changed and the six-well plate was placed on top of a different Flexcell[®] baseplate consisting of loading posts that allowed for uniaxial stretching of the collagen gel when vacuum was applied. The collagen gels were subjected to uniaxial cyclic strain (5%, 0.25 Hz) continuously for another 7 days within a humidified incubator. The culture medium was changed every 48 h.

2.3. Addition of exogenous HA

The experiment was designed for three treatment groups per cell type for each of the overexpressing vectors (i.e. *has-1*, *has-2*, *has-3*) and a control empty vector (LXS_N). Group 1 samples were untreated, Group 2 samples were supplemented with HA oligosaccharides (~1.74 kDa from Hyalose, Oklahoma City, OK), and Group 3 samples were supplemented with high molecular weight HA (~1.6 MDa from Fluka/Sigma Aldrich, St Louis, MO). Concentrations of HA oligosaccharides [26,27] and high molecular weight HA [21] were selected from the literature from studies previ-

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