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Hyaluronic acid decreases IL-6 and IL-8 secretion and permeability in an inflammatory model of interstitial cystitis ☆

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ABSTRACT

Hyaluronic acid (HA) has received a lot of attention recently as a biomaterial with applications in wound healing, drug delivery, vascular repair and cell and/or gene delivery. Interstitial cystitis (IC) is characterised by an increase in the permeability of the bladder wall urothelium due to loss of the glycosaminoglycan (GAG) layer. The degradation of the urothelium leads to chronic pain and urinary dysfunction. The aetiology of the degradation of the GAG layer in this instance is currently unknown. At a clinical level, GAG replacement therapy using a HA solution is currently utilised as a treatment for IC. However, there is a significant lack of data on the mechanism of action of HA in IC. The current study investigates the mechanistic effect of clinically relevant HA treatment on an *in vitro* model of IC using urothelial cells, examining cytokine secretion, GAG secretion and trans-epithelial permeability. This study demonstrates that HA can significantly decrease induced cytokine secretion (4–5 fold increase), increase sulphated GAG production (2-fold increase) and without altering tight junction expression, decrease trans-epithelial permeability, suggesting that the HA pathway is a clinical target and potential treatment vector.

1. Introduction

Interstitial cystitis (IC) is a chronic bladder disease clinically characterised by pelvic pain, pressure, or discomfort, typically associated with the persistent urge to void (increased urinary frequency) in the absence of infection or other pathology [1]. IC can have a significant impact on a patient's quality of life with those failing to respond to treatment reporting a lower quality of life score than patients in end-stage renal failure [2]. The early symptoms of IC share features with many other bladder disorders such as overactive bladder (OAB) or bladder cancer and diagnosis is primarily through exclusion of these diseases [3]. Lack of a clinical biomarker hampers a true measure of prevalence of IC. Several recent studies have indicated a large population suffering with related symptoms, including a 2005 study that reported a 12% occurrence in women in the US, representing a large untreated population [4].

A key problem in the treatment of IC is a poor understanding of its pathogenesis: however, in recent years advances have been made in identifying potential key players. The bladder wall lining (urothelium) forms an impermeable barrier to urinary solutes. This high resistance barrier with its high proteoglycan concentration prevents urinary solutes from interacting with or damaging the underlying urothelial cells (Fig. 1). Alterations in proteoglycan composition have been proposed as the primary pathological characteristic of IC [5]. Multiple small-scale studies have shown that proteoglycans and glycosaminoglycans (GAGs), particularly hyaluronic acid (HA), are lost from the bladder lining of patients with IC [5–7]. A number of limited studies have attempted to quantify alterations in bladder wall composition by testing for the presence of GAGs in the urine of IC patients [6–9]. The efficacy of several clinical treatments currently available on the market is based on the ability to repair the urothelium by replacing lost proteoglycans. These treatments include high molecular weight HA (Cystistat™), low molecular weight HA (Hyacyst™), and chondroitin sulphate (Gepan™). Treatment is typically administered weekly; as the patient's functional capacity improves, treatment frequency is reduced [10–12].

Disruption of the urothelium GAG layer results in alterations in bladder wall permeability. Specifically, the loss of GAG and/or alteration in GAG surface expression leads to increased trans-urothelial permeability [13]. This increased permeability results in urinary solutes infiltrating the underlying stoma, leading to inflammation, mast cell invasion and sensitisation of neural nerve endings. Neural sensitisation due to potassium leakage from the

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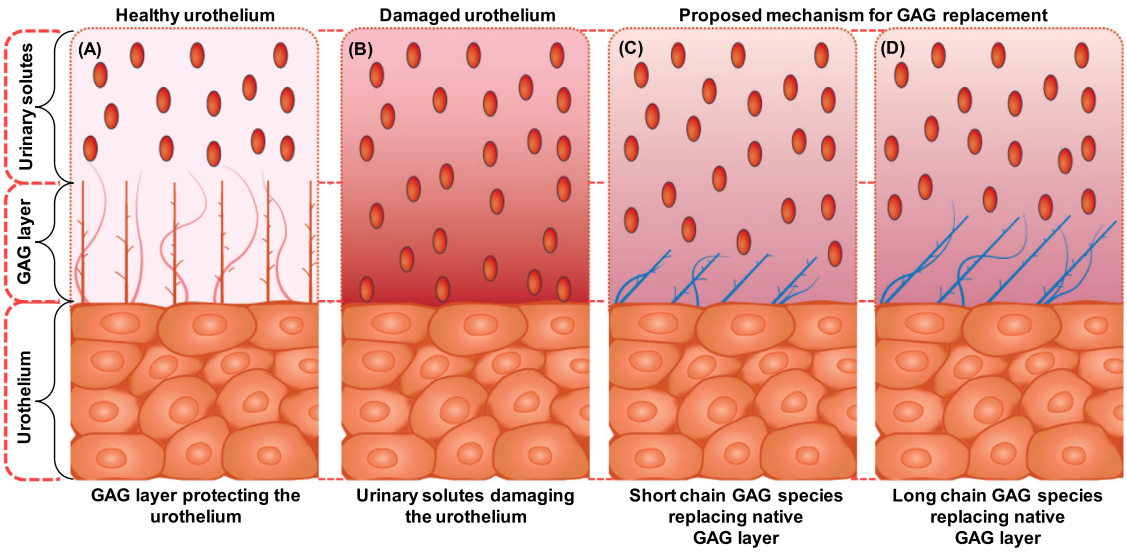

Fig. 1. Potential mechanism for GAG replacement therapy repair in IC. (A) Normal urothelium is composed of several different types of GAG species on the surface separating the urothelial cells from the urinary solutes. (B) IC damaged urothelium has a decreased amount of GAG species on the urothelium surface, increasing permeability, leading to inflammation. (C) Repair of the damaged urothelium has been proposed via GAG replacement therapy. Addition of GAG solutions through intravesical delivery coats the urothelium, separating the urinary solutes from the urothelium, allowing repair and regeneration, (D) with longer chains increasing the distance of urinary solutes from the surface.

bladder lumen results in the classical features of pain and altered bladder reflexes. One of the most important regulators of epithelial permeability is the anastomosing tight junctions, a feature that is altered in the bladder of IC patients [14]. HA has previously been shown to increase expression of tight junction proteins Zo1 and Claudin-1 in human skin keratinocytes and human bladder epithelium. These proteins are highly expressed in the bladder and are closely associated with tight epithelia, i.e., low paracellular permeability [15,16]. A CD44 knockout model has demonstrated that reduced CD44 expression results in decreased expression of tight junction proteins [17]. Therefore, the hypothesis is that the effect of HA on the expression of tight junction proteins is mediated through the CD44 receptor. Anti-proliferative factor (APF) has recently been identified as an agent in the pathogenesis of IC. APF has been proposed as a potential biomarker that is linked to permeability changes via alteration in tight junction protein expression patterns [18,19]. While tumour necrosis factor alpha (TNFα) is known to increase permeability across epithelial layers [20,21], it is better known for its wide-ranging effects on cytokine production [22,23]. Several cytokines have been found at altered levels in the urine and bladder wall of IC patients. These include nerve growth factor and (NGF) interleukin 6 (IL-6) in the urine [24,25] and nitric oxide (NO) in the bladder wall [26]. Altered levels of both IL-6 and IL-8 have been found in the urine of IC patients. However, these alterations have not been shown to correlate directly with disease severity or with the presence of bladder wall ulceration. lL-8 does correlate positively with mast cell infiltration into the bladder wall [27]. It has been suggested that the presence of IC can be predicted by analysing the urine of patients for the presence of IL-6, histamine and methylhistamine [28] however, this remains to be validated by a larger study.

In this study the acute (over 24 h) effect of HA was investigated in an inflammatory *in vitro* model of IC. As the specific pathogenic cascade initiator in IC is currently unknown, TNFα was used in generating a model inflammatory insult. This study aims to determine the mechanistic role of high molecular weight HA in the treatment of IC. In order to compare the *in vitro* mechanistic study to that seen clinically, HA with a molecular weight range of 500–800 kDa, with a concentration of 0.4 mg/ml, was used. Specifically, the effect of HA on inflammatory cytokine expression, tight junction alteration, GAG expression and urothelial permeability was examined. Detailed mechanistic knowledge of the therapeutic effect of HA on inflammation and permeability is crucial for the development of future HA-based treatments for a range of disorders and inflammatory diseases.

2. Materials and methods

2.1. Cell culture

Human urothelial cells HTB4 (ATCC, Manassas, VA) were grown in basal media consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. In all cases cells were grown until 80–90% confluent and washed three times by rinsing with Hanks' Balanced Salt Solution (HBSS) before all experiments. In experiments where it was desirable to remove the GAG layer, cell monolayers were chemically stripped using protamine sulphate (100 ng/ml) (Sigma–Aldrich, Arklow, Ireland) for 30 min before time zero (Fig. 2). After stripping the cells were washed by rinsing three times with HBSS and then allocated to treatment groups. The treated groups, unless otherwise stated, were basal media (control), HA (0.4 mg/ml) (Mylan, Galway, Ireland), TNFα (10 ng/ml) (Sigma–Aldrich, Arklow, Ireland) or HA (0.4 mg/ml) and TNFα (10 ng/ml) in combination. In experiments where the GAG layer was not removed (Figs. 3–5) no pre-treatment with protamine sulphate was carried out. Passages between 3 and 10 were used for all experiments.

2.2. Inflammatory protein array on HA-stimulated urothelial cells demonstrates alteration of cytokine expression by HA and TNFα

HTB4 cells were seeded into T75 plates, grown to confluency and treated with basal medium, HA (0.4 mg/ml) or TNFα (10 ng/ml) for 24 h. At the end of the 24-h treatment period the cells were trypsinised and pellets collected by centrifugation. The cells were then lysed and protein extracted in ice-cold radio-immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, Arklow, Ireland) containing 10 μg/ml phosphatase inhibitor cocktail and 10 μg/ml