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Delivery of a vector encoding mouse hyaluronan synthase 2 via a crosslinked hyaluronan film

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Abstract

We have developed a crosslinked hyaluronic acid (HA) film with DNA incorporated within its structure and have characterized this system for its efficacy in sustained transferring of a vector encoding mouse hyaluronan synthase 2 (Has2). Analysis of the DNA release kinetics indicated that the HA films degraded when treated with hyaluronidase and that they released DNA over a prolonged period of time. Gel electrophoresis revealed that this DNA was intact and immunohistochemical analysis verified the transfection capabilities of DNA release samples. The ability of released DNA encoding Has2 to promote HA synthesis was confirmed by quantifying the amount of HA produced by COS-1 cells that were transfected with release samples. The intended future application of the HA films is in prevention of post-operative peritoneal adhesions. In addition to serving as a physical barrier, the film would function as a vehicle for sustained delivery of DNA encoding Has2, which would promote the synthesis of HA in transfected tissues.

Keywords: Hyaluronic acid; HA synthase; Post-operative adhesions; Gene delivery

1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan that is composed of repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine [1–4]. It is a major component of the extracellular matrix that provides stability and elasticity and is also present in connective tissues, synovial fluid of the joints, and the vitreous humor of the eyes [1–5]. HA makes a good candidate for biomedical applications because it is highly conserved and is biocompatible, biodegradable, and non-immunogenic [6–9]. Thus far, it has been utilized in such applications as viscosurgery, viscosupplementation, and wound healing [10–12]. A crosslinked dihydrazide derivative of HA, in which adipic dihydrazide is the crosslinker, has also been proven effective as a gene delivery vehicle with sustained release capabilities [6,13].

Crosslinked HA can be structured into many different forms such as hydrogels, matrices, scaffolds, microspheres, and films. In the form of a film, the crosslinked HA has the potential to serve as a barrier to prevent post-operative peritoneal adhesions, which occur in more than 50% of patients that undergo pelvic surgery [14]. Post-operative abdominal adhesions are the leading cause of small bowel obstructions and of secondary infertility in women [15,16]. They also have a great economic impact [17]. Several different approaches to prevent the formation of post-operative adhesions have been attempted, two of which have been approved in the US for clinical use: Interceed™, which is a form of oxidized regenerated cellulose, and Seprafilm™, a mixture of modified HA and carboxymethylcellulose [1,18,19]. However, the clinical results have so far been less than optimal [1,20–22]. Alternative methods such as an auto-crosslinked polysaccharide HA derivative and a polyethylene glycol product (SprayGel™) that crosslinks upon the mixing of two precursors of the gel have also been attempted with moderate results [23,24].

HA makes a good candidate for preventing post-surgical adhesions because of its lubricating properties

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and additionally, it plays a role in wound healing. Exogenously injected HA has been demonstrated to improve wound healing; however, it is known to be rapidly eliminated [25,26]. The dihydrazide crosslinked HA could overcome this dilemma because it is insoluble and its breakdown requires prolonged hyaluronidase degradation. In addition, the crosslinked HA has the potential to serve as a gene delivery vehicle, providing a sustained release of DNA over a prolonged period of time [6,13].

In this article, we propose the notion of utilizing a HA film in the prevention of post-operative adhesions by serving as a physical barrier. In addition, it has the capability of sustaining delivery of DNA encoding HA synthase 2, thereby promoting HA production upon gene transfer. Recently, extensive research on HA synthase has emerged, leading to characterization and identification of three different isoforms (Has1, Has2, and Has3), all of which are evolutionary conserved and differ from one another in stability, the rate in which they facilitate HA elongation, and in the size of the final product [27,28]. However, use of HA synthase as a therapeutic agent and the application of gene therapy for the prevention of post-surgical adhesions have not been explored. Has2 promotes the synthesis of HA at the plasma membrane and the HA is subsequently extruded to the outside of the cell [27,28]. Thus, the crosslinked HA film could serve not only as a physical barrier that has an extended lifetime, but it could also serve as a gene delivery vehicle for introducing DNA encoding Has2 to provide additional HA in post-surgical peritoneal adhesion prevention.

2. Materials and methods

2.1. Gene construct

Plasmid DNA encoding a pCMV β-galactosidase reporter gene construct was purchased from Clontech (Palo Alto, CA).

A vector encoding mouse Has2 was selected because it promotes synthesis of a larger HA molecule than Has1 and Has3. It has been found to have a higher rate of synthesis, longer duration of activity, and greater stability than Has1 [30]. In addition, Has2 has shown to be up-regulated in wounded peritoneal mesothelial cells (PMCs) [31]. The plasmid encoding mouse Has2 was prepared containing a pCMV promoter and *N*-terminal FLAG fusion tag (Sigma-Aldrich, St. Louis, MO). The PCR template consisted of a pCIneo vector (Promega Corporation, Madison, WI) encoding the entire Has2 gene ORF that was previously cloned into the Xma I site, as described by Spicer et al. [32]. This vector also included an optimized Kozak consensus sequence A--ATGG initiation signal, where the first base of the second codon was altered from C to G so as to avoid duplicate initiation signals. Primers containing Hind III and Kpn I restriction sites were created that restored the original sequence and allow for subcloning. All reactions were carried out using *pfu* Turbo high fidelity polymerase (Stratagene, La Jolla, CA). PCR products were purified, digested with Hind III and Kpn I, and were ligated into purified, similarly digested vector. The ligation products were then transformed into competent XL-1 Blue *E. coli* (Stratagene) and grown on selective media. Colonies were chosen for DNA minpreps (Qiagen, Alameda, CA), examined by gel electrophoresis, and confirmed by sequencing. Both gene constructs (β-galactosidase and Has2) were transformed into NovaBlue competent *E. coli* cells, and isolated in large quantities (in milligram range) using a Qiagen Giga Prep® plasmid isolation kit.

2.2. DNA–HA film preparation

HA films with incorporated DNA were prepared using the DNA plasmids described above. Briefly, DNA was blended into a 1% HA solution. The DNA–HA mixture was deposited into 4 cm diameter aluminum molds, each receiving 8 ml of mixture. They were then allowed to air dry under sterile conditions. After complete dehydration, each DNA–HA film was partially hydrated in a solution of 80% isopropyl alcohol (IPA)/20% water (100 ml) containing 10 mg of adipic dihydrazide (ADH, Sigma-Aldrich). After a 30 min incubation period, 1 ml of a 12 mg/ml aqueous solution of ethyl-3[3-dimethyl amino] propyl carbodiimide (EDCI, Sigma-Aldrich) was added. The reagent solution was adjusted to a pH of 5 to initiate the crosslinking reaction. The reaction was terminated by transferring the films from the crosslinking solution to a solution of 80% IPA/20% water. Subsequently, residual reagents were removed by several washes in 80% IPA/20% water. The DNA–HA films were then air dried under sterile conditions.

2.3. DNA–HA film mechanical and morphological characterization

The mechanical properties of HA films were determined using a Tyron 250 Micro Bionix Testing System (MTS Systems, Minneapolis, MN) fitted with a 50 N force transducer. All testing protocols followed the guidelines established in ASTM D3039/D3039M-00. The films, sectioned into strips (dog bone shaped with the width of the midsection at $\sim$2.6 mm), were mounted onto stainless-steel spring-loaded grips (MTS) leaving a gauge length of $\sim$14 mm. The load–deformation relationship was recorded while the films were stretched under constant displacement at a rate of 10%/s.