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Intravesical Chondroitin Sulfate Inhibits Recruitment of Inflammatory Cells in an Acute Acid-Damage “Leaky Bladder” Model of Cystitis

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Abstract

OBJECTIVES—Replacement of the glycosaminoglycan (GAG) layer with intravesically-administered GAGs is an effective therapy for interstitial cystitis in at least some patients. Intravesically-administered chondroitin sulfate was previously shown to bind to and restore the impermeability of surface-damaged (“leaky”) urothelium to small ions. This study investigated whether a physiologic effect of “GAG replenishment therapy” altered recruitment of inflammatory cells in an acute bladder damage model.

METHODS—Rat bladders were damaged with 10mM HCl. Negative control bladders were treated with PBS. On the following day, the animal bladders were treated with 20mg/mL chondroitin sulfate in PBS, while the negative and positive controls were treated with PBS alone. Two and four days after treatment with chondroitin sulfate, animals were euthanized, and sections of their bladders were analyzed by Toluidine Blue staining for mast cells immunohistochemical labeling using antibodies against CD-45 for lymphocytes, and myeloperoxidase for neutrophils.

RESULTS—Chondroitin sulfate treatment statistically significantly reduced recruitment of inflammatory cells including neutrophils and mast cells to the suburothelial space but did not alter recruitment of CD-45-positive lymphocytes.

CONCLUSIONS—For the first time we demonstrate that intravesical GAG replenishment therapy also produces a physiological effect of decreasing recruitment of inflammatory cells in an acute model of damaged bladder. These findings support use of intravesically administered GAG for bladder disorders that result from a loss of impermeability, including interstitial, radiation and chemical cystitis, and possibly others as well.

Keywords

Interstitial Cystitis; Inflammation; Glycosaminoglycans; Chondroitin Sulfate

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Introduction

Interstitial cystitis (IC) is a disorder that presents with various combinations of a triad of symptom complexes—lower abdominal pain, urgency and frequency^{8, 10}. Although the disorder was thought to be solely a problem of the bladder, modern research investigating the prevalence of co-morbidities strongly suggests IC may be the bladder manifestations of a wider, systemic visceral problem that is being labeled chronic pelvic pain^{2, 17, 26} (CPP). Animal models have shown that acute colonic inflammation alters bladder smooth muscle function¹⁹ and that communication occurs through nerves via the dorsal root ganglion¹⁶ thus demonstrating that “organ cross talk” could be responsible for diffuse manifestations of IC/ CPP.

Although the cause is unknown, the bladder symptoms seem to be derived from a loss of the impermeability barrier that is localized in the apical cell layer^{9, 21}. Normally the bladder urothelium is the least permeable of mammalian membranes¹³, but in IC the urothelium is dysfunctional with partial loss of umbrella cells with their associated defenses that include tight junctions and glycosaminoglycan (GAG) layer^{7, 9, 12, 15, 24} and multiple changes in expression of differentiation and barrier-related proteins, as we showed earlier^{12, 24}. Whether the morphologic and biochemical changes in the urothelium is due to failure to differentiate properly or to urinary cytotoxins²³ that damage the urothelium similarly to the effects of acid, protamine sulfate or stress²⁹ (or both) is not clear. Other than the cited structural studies, remarkably little direct evidence for loss of permeability has been presented. The strongest evidence is indirect; a high proportion of patients respond with pain to instillation of an 0.1 to 0.2 M solution of potassium ions, but not of sodium ions²². Ruggieri and coworkers⁴ showed a difference in permeability to ^{99m}Tc-diethylenetriaminepentaacetic acid that was significant at $p=0.07$, but whether this represents a type II error due to the sample size of 10 patients and 9 controls is unclear. Ericksson showed a four-fold increase in permeability over controls following bladder distention of patients, and for the first time unambiguously demonstrating a difference in permeability⁷. The most unambiguous demonstration was a small study from Buffington³ showing altered kinetics of excretion of fluorescein by normals and IC patients due to recycling of fluorescein due to absorption of the excreted fluorescein back into the bladder.

Few therapies are effective in treating IC, but among the most effective has been intravesical administration of glycosaminoglycans (GAGs)^{18, 20} to replace the GAG layer that is missing on patient bladders. Animal studies using the acute acid-damaged mouse and rat bladder as a model for a leaky bladder showed that chondroitin sulfate bound specifically to areas lacking apical (“umbrella”) cells and restored the bladder impermeability to the K^+ mimetic ⁸⁶Rb⁺ to control levels¹¹. However, restoration of the barrier function using exogenous GAG has not been shown to have a physiologic effect that suggests efficacy of “GAG replenishment therapy.”

In this paper we showed that in the acute acid-damaged bladder that treatment with chondroitin sulfate sharply inhibited the recruitment of neutrophils and mast cells to the suburothelial space. Chondroitin sulfate was used rather than pentosan polysulfate, heparin or the non-sulfated hyaluronan because chondroitin sulfate has negligible effect on the clotting system¹ as well as minimal activity as an effector of signaling systems, as is the case with heparin¹⁴ or hyaluronan²⁷. The effect on restoring barrier function will therefore more likely represent the physical action of restoring the barrier function. We conclude that not only does treatment of a “leaky” urothelium with intravesical chondroitin sulfate restore the impermeability to ions, this results in a depression of the acute inflammatory response that would normally occur.

Materials and Methods

Rat model for testing restoration of the permeability barrier by chondroitin sulfate

The animals were treated using the model previously described¹¹ in an IACUC-approved protocol. Briefly, Sprague-Dawley rats (300 g) with a cannula surgically implanted in the dome of the bladder (Charles River Laboratories, Cambridge, MA) were anesthetized and treated by injecting 400 μ L of 10 mM HCl through the cannula into the bladder. The negative control received 400 μ L of PBS instead of the HCl solution. The solutions remained in the bladders for 10 min. The acid was then aspirated, the bladders were washed once with 0.15 M sodium bicarbonate to neutralize any remaining acid and then washed with PBS. On the following day, animals in the ChS group were treated with 400 μ L of 20 mg/mL chondroitin sulfate in 0.154 M NaCl provided as a sterile solution for intravesical use (trade name Uracyst from Stellar Pharmaceuticals, London, Ontario). The negative control and the positive control animals both received 400 μ L of PBS alone. The solutions were allowed to remain in the bladder for 20 minutes, at which time the solutions were removed. Two and four days following administration of chondroitin sulfate, two rats each from the positive and negative controls and chondroitin sulfate-treated groups were euthanized. The bladders were partially inflated with PBS containing 1% formalin and then were soaked in the 1% formalin solution overnight prior to mounting in paraffin and sectioning as 5 micron sections. Several sections at different levels in the bladder were cut from each bladder.

Immunohistochemical staining

Sections were selected at random from different levels of the bladder and immunohistochemical (IHC) staining was performed as previously described⁵. Sections were de-waxed with a graded xylene and ethanol series and re-hydrated with a graded ethanol water series. Anti-CD45RC antibody, anti-Myeloperoxidase antibody, and Toluidine Blue staining were used to detect lymphocytes, neutrophils, and mast cells, respectively. IHC labeling was performed with two primary antibodies, anti-CD45RC (AbD Serotec, MCA53Ga, mouse monoclonal, no retrieval 1:50), and anti-Myeloperoxidase (Dako, A0398, rabbit polyclonal, no retrieval, 1:600). Two secondary antibodies were used, pre-diluted anti-rabbit (Zymed Histostain-Plus Kit) and goat anti-mouse (Calbiochem, 401216, 1:100). Toluidine Blue (Polysciences Inc., #1234, 1g/100ml 70% ethanol stock) was performed by incubating rehydrated sections for 2 minutes with a working solution of stock Toluidine Blue diluted 1:10 with 1 g/ml aqueous NaCl followed by 3 rinses with deionized water and coverslipping.

Slides were examined by microscopy, and stained cells located in the lamina propria were scored as positive. To avoid bias, the circumference of the entire bladder of each section was analyzed. Two rats were analyzed for each condition. Individual cells of interest were counted without knowledge of how the animals had been treated. Data were analyzed by one-way ANOVA to identify whether statistically significant differences existed in the mast cell and neutrophil datasets, and Mann-Whitney analysis was performed to test for statistical significances of differences between individual pairs at day 2 and day 4 using a one-tailed analysis because our hypothesis is that the PBS treatment will always produce higher counts than the chondroitin sulfate treatment.

RESULTS

Fig. 1 illustrates representative examples of sections from bladders that were damaged with acid and then treated with chondroitin sulfate or PBS the following day and harvested either 2 or 4 days after treatment with chondroitin sulfate (3 and 5 days post damage). As has been

shown previously, the acid-damage model removed most of the “umbrella cell” layer, exposing the intermediate cell layer. This produced leakiness, edema, and inflammation in the area underlying the urothelium. Mast cells (Fig. 1A) and neutrophils (Fig. 1D) were present at low levels in the undamaged control bladder. After treatment of the bladder with HCl, the numbers of mast cells (Fig. 1B) and neutrophils (Fig. 1E) were dramatically increased. The amount of edema and inflammation appeared less pronounced in the chondroitin sulfate-treated bladders, and the numbers of mast cells (Fig. 1C) and neutrophils (Fig. 1F) were significantly reduced by treatment with chondroitin sulfate to restore barrier function.

The counts of mast cells and neutrophils are shown in Figure 2. At two and at four days post administration of chondroitin sulfate (three and five days post damage with HCl), the number of mast cells per section increased four- and six-fold respectively in the absence of chondroitin sulfate. The increase in neutrophils was actually larger (7-fold at day 3 and 11-fold at day 5), but was more variable per section. The differences within the data sets were highly significant ($p < 0.0001$) by ANOVA. All of the cell counts for treated animals were significantly different from the controls (p at least < 0.05). Treatment with chondroitin sulfate immediately inhibited the recruitment of mast cells, and the difference was significant even 2 days following treatment with chondroitin sulfate (p -values are listed in the figure caption). Four days after treatment with chondroitin sulfate, the mast cell counts were reduced by 43%. The effect of chondroitin sulfate on recruitment of neutrophils was also significant, showing a trend at day 2 and statistical significance at day 4; neutrophils were reduced by 35% four days after treatment. There were no differences in the numbers of lymphocytes (CD 45+)(data not shown).

DISCUSSION

Although interstitial cystitis does not generally manifest with a robust immune cell response, evidence of inflammation is still present in the bladder as compared to healthy individuals^{12, 15}. The morphologic features actually suggest physical loss of the umbrella cell layer and variable loss of intermediate cells as well up to and including complete denudation of the urothelium^{12, 15}, which are features partially duplicated by the acute acid damage model used here or the protamine sulfate model used by other investigators²⁵. The presence of urine is necessary for eliciting an immune response from these physical damage models²⁵, indicating that loss of impermeability is a key factor in producing an inflammatory response. Because interstitial cystitis also involves loss of impermeability, it is likely that the immune effector cell response observed in interstitial cystitis also has its origins in penetration of the bladder by urinary solutes. Since similar responses are seen in these models and human interstitial cystitis, it appears that these physical damage models can serve as a useful model, at least for the acute response to loss of impermeability. However, the model is not intended to duplicate IC itself, and only provides a means to assess the effects of correcting bladder permeability in an acute model.

The inflammatory response is complex and is modulated by several sets of effector cells. Neutrophils are associated with tissue injury as well as pathogen invasion and have been identified in elevated numbers in the urine of IC patients⁶. Mast cells are generally associated with an allergic-type response and also have been strongly implicated in IC²⁸. However, the mast cells in this study are not degranulated, as shown by their metachromatic reaction of the Toluidine Blue with intracellular heparin, indicating that histamine and other vasoactive amines are intact within the granules. At two and at four days post treatment, the number of mast cells per section was increased four- and six-fold respectively in the absence of GAG replenishment therapy. The increase in neutrophils was actually larger, but was more variable per section.

Although administration of chondroitin sulfate intravesically restored the impermeability of the bladder surface to control levels¹¹, the effector cell response was muted but not abolished completely. One reason for this is that the chondroitin sulfate was not administered until 24 hours later because cells killed by the acid treatment slough from the urothelium during the first 24 hours, thereby exposing the urothelium to urinary solutes. If chondroitin sulfate is administered within the first 24 hours it will be lost along with these sloughed cells. Alternately, the effector cell response could still have been triggered by the tissue damage through other mechanisms. Nonetheless, “GAG replenishment therapy” with chondroitin sulfate clearly diminished the inflammatory response due to loss of impermeability and presumably would with other GAGs as well. Interestingly, the response rate for GAG replenishment therapy is only about 60%¹⁷, whereas 91% of patients had a positive potassium sensitivity test in one study with intravesical chondroitin sulfate¹⁸. The optimal dosing interval is not known, and once weekly may be insufficient in some patients to maintain a functional GAG layer. Also, the prevalence of “leakiness” is unknown and is inferred from potassium sensitivity test data. Clearly the role of loss of permeability in the bladder and how it might relate to comorbidities (e.g. bowel symptoms) is unknown and in need of further study.

In summary, these results demonstrate a clear muting of the inflammatory response induced by loss of the permeability barrier of the urinary bladder and support the use of intravesical chondroitin sulfate or other GAG for treatment of bladder disorders that involve the loss of the full impermeability barrier of the bladder. In addition to interstitial cystitis, other disorders such as radiation cystitis and overactive bladder could involve this etiologic factor because the potassium sensitivity test is positive as well in over 60% of patients^{5, 22}.

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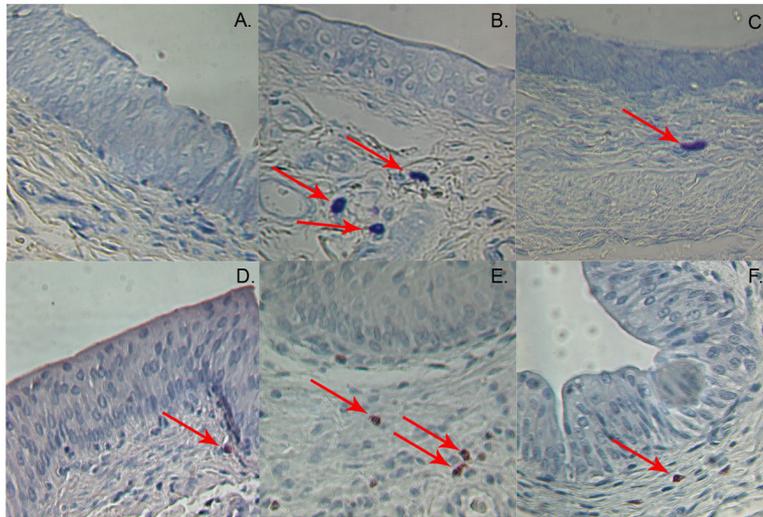


Figure 1. Photographs illustrating examples of positive cells and their distribution as a function of treatment. (A) Control slide showing rare mast cells in untreated bladder. (B) Increased number of mast cells 4 days after HCl treatment of bladder to induce leakiness. (C) The number of mast cells is decreased 4 days following intravesical chondroitin sulfate treatment (D) Control slide showing relatively rare neutrophils in untreated bladder. (E) Increased number of neutrophils 4 days after HCl treatment of bladder to induce leakiness. (F) The number of neutrophils is decreased 4 days following intravesical chondroitin sulfate treatment. Magnification is 400X.

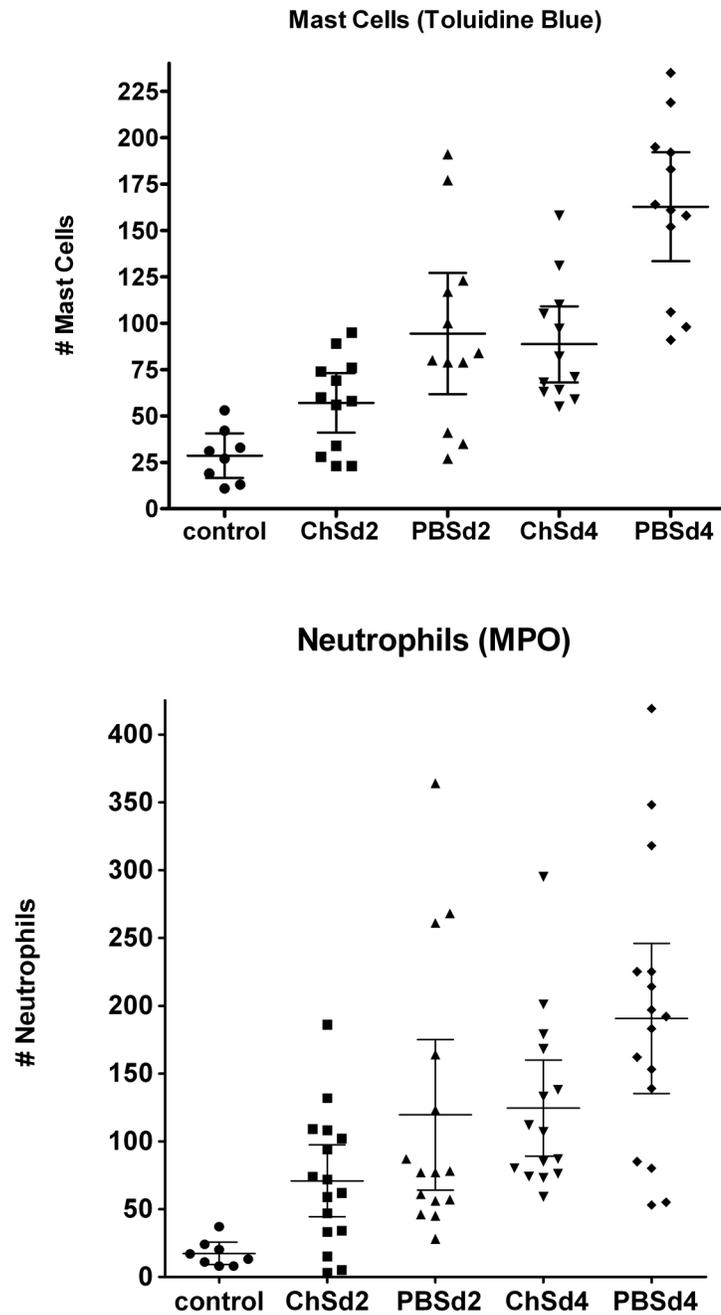


Figure 2. Comparison of the effect of intravesical chondroitin sulfate on recruitment of mast cells (top) and neutrophils (bottom) two and four days following treatment with acid to increase bladder permeability. Each symbol represents the number of cells seen in the lamina propria of a single, entire cross section. A total of two rats was sampled for each condition. Shown are the means and the 95% confidence intervals. Testing for statistical significance of differences showed the decrease in neutrophils produced by chondroitin sulfate was not significant ($p = 0.13$) at two days but was significant ($p = 0.024$) at four days. For mast cells, the difference was significant ($p = 0.015$) at two days and highly significant at four days ($p = 0.0004$).