Sodium cromolyn reduces expression of CTGF, ADAMTS1 and TIMP3 and modulates post-injury patellar tendon morphology

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Abstract

Objectives—The purpose of this study was to determine whether administration of a mast cell inhibitor (sodium cromolyn, SC) would influence tendon repair and extracellular matrix gene expression following acute injury.

Methods—CD1 mouse patellar tendons were unilaterally injured and mast cell prevalence was determined. The effect of SC injection on tendon hypercellularity, cross-sectional area, collagen organization, and expression of extracellular matrix-related genes was examined.

Results—Mast cell prevalence was markedly increased in injured patellar tendons ($p=0.009$), especially at 8 weeks post injury ($p=0.025$). SC injection increased collagen organization compared to uninjected animals at 4 weeks and attenuated the development of tendon hypercellularity and tendon thickening post-injury. Expression of CTGF, ADAMTS1 and TIMP3 in injured tendon was reduced in the SC group.

Conclusion—SC injections moderated the structural alterations of healing tendon in association with downregulation of several genes associated with tendon fibrosis. This work corroborates previous findings pointing to a role of mast cells in tendon repair.

Keywords

patellar tendon injury; mast cells

INTRODUCTION

Tendon repair follows a prolonged time course, with acutely injured tendons demonstrating structural abnormalities indicative of ongoing remodeling up to 10 years following the initial trauma [1]. Chronic alterations in injured tendons include fibrosis, thickening of the tendon

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and associated tissues with accelerated turnover of the extracellular matrix, and increased presence of nerves and vessels within fibrotic and/or load bearing regions of the tendon [2–4]. These abnormalities are associated with pain and reduced functional performance, and thereby contribute to the significant socioeconomic impact of chronic tendon injuries.

Currently, the therapeutic options for improving tendon repair are limited, and often consist of generalized approaches aimed at decreasing inflammation (e.g. corticosteroid injections or NSAIDs), or paradoxically, enhancing a deficient inflammation-repair response (e.g. prolotherapy, extracorporeal shockwave therapy). Clearly the extent of the inflammatory response plays a crucial role in determining the final outcome of tendon repair [5]. Histological findings in chronic tendon pathology are similar to those found in scar tissue, with a preponderance of disorganized extracellular matrix and a noticeable increase in the presence of fibroblasts, myofibroblasts, endothelial and smooth muscle cells [1]. The possible roles of other cell populations in injured tendon have not been thoroughly examined.

In a recent clinical study, a substantial increase in the number of mast cells present in the patellar tendons of men and women with chronic patellar tendinopathy was identified [6]. Mast cells, in addition to their well known role in mediating allergic reactions, also contribute to the repair and remodeling phases of soft tissue healing by releasing factors which promote proliferative and fibrogenic activity (e.g. TGF β , PDGF) as well as potent enzymes known to influence tissue repair in a variety of ways (e.g. mast cell tryptase, CPA3). A previous study reported an increased number of mast cells following acute tendon injury [7], but to our knowledge there has not been an experimental approach to examine the implications of mast cell inhibition following tendon injury. Mast cells have been suggested to have potential roles in regulating vessel function following injury [8] and additionally, are known to play a role in the connective tissue response to immobilization and reloading [9]. In other models of connective tissue injury or inflammation, mast cell stabilizing drugs have shown potential as anti-fibrogenic agents [10, 11].

Given the above, we hypothesized that sodium cromolyn injections could lead to reductions in the expression of genes associated with fibrosis, and would reduce the extent of post-injury tendon thickening. We employed an *in vivo* model to gain insight into the potential role of mast cells following tendon injury, and as proof-of-principle concerning the ability of mast cell stabilizers to modulate tendon repair.

MATERIALS AND METHODS

All animal procedures were carried out with the approval of the local animal ethics committee. 40 CD-1 mice were obtained at 8 weeks of age (Charles River) and used at 10 weeks of age. All mice underwent the surgical procedure, and the contralateral uninjured limb served as a control. The decision to use the contralateral limb as a control was based on a previous study with this model which showed no significant changes in blood flow or in the expression of all examined genes in the uninjured side (JOR, in press). Mice were placed under isoflurane, and buprenorphine (0.1mg/kg) and saline (0.5 ml) were injected subcutaneously. Using sterile surgical preparation, a 5 mm incision was made over the

shaved medial knee in order to avoid injuring the skin directly over the tendon. The tendon was exposed by laterally shifting the skin opening, and a number 11 blade was passed through the lateral and medial retinacula and directly behind the patellar tendon. A 0.5mm biopsy punch (Shoney Scientific, Waukesha, WI) was used to create a defect in the central part of the tendon, using the blade as a backing. The skin was apposed with a single sterile wound clip (9mm Autoclips, Becton-Dickinson, Franklin Lakes, NJ) placed medial to the knee. After 1 week, any remaining wound clips were removed.

Sodium cromolyn injections

Sodium cromolyn was obtained from Sigma (C0399), resuspended by vigorous agitation in sterile PBS at a concentration of 9mg/ml, passed through a 22 μ m filter, aliquoted into sterile Eppendorf tubes and stored at -20°C. Cromolyn-injected animals (n=21) received intraperitoneal injections (0.1mg/g) three times per week throughout the course of the study. One animal was lost to the study due to an undetermined adverse reaction to the injection. The other injected animals demonstrated no observable side effects of injection, and maintained equivalent weights and activity levels as uninjected animals.

Histology

Twenty-two tendons (left and right, from 11 animals, 5 injected and 6 controls) were dissected at 4 weeks to examine tendon cell density in the tendon proper (i.e. avoiding paratendinous regions). All injured tendons were macroscopically abnormal on dissection. Tendons were fixed in 4% paraformaldehyde in PBS, paraffin embedded, sectioned at 5 microns thickness, and stained with haematoxylin and eosin (H&E). Tendon cell density was determined for the tendon proper by counting all tendon cells in 3 randomly selected viewing fields using the 40x objective, and expressed as cells/field.

Collagen organization

Collagen structures in the same 22 histological specimens described above were visualized using second harmonic generation (SHG) microscopy. The method used was exactly as previously described (JOR in press) to generate an index of the density of organized (fibrillar) collagen. The total SHG signal intensity values generated by systematically scanning each tissue section using the 20x objective were normalized by the cropped collagen area (μ m²) and expressed in arbitrary units (AU).

Mast cell prevalence

74 cDNA samples (37 right, 37 left) from a previous study of tendon differentiation markers (JOR, in press) were used to conduct qPCR for CPA3, a mast cell specific gene. qPCR for mast cell gene products has previously been reported as a more sensitive method to quantify mast cell prevalence than the traditional histological approach [12]. We used previously prepared cDNA samples from uninjured and injured mouse patellar tendons (not treated with sodium cromolyn) for the ethical reason that it reduced the number of mice needed for the current study. Gene expression was quantitated with probe and primer sets for CPA3 (ABI cat#483940 m1) and 18s (ABI cat#4308329). Expression was determined using the relative

quantitation method and gene expression was normalized to 18S and expressed as percent increase (right vs left) for each time point, as previously reported (JOR in press).

RNA isolation and quantitative PCR

Right (injured) tendons from injected (n=4) and uninjected (n=4) mice were dissected using a sterile blade and flash frozen in liquid nitrogen. Tendons were powdered in a tissue mill (Mikrodismembrator S, Sartorius, Germany) for 30 seconds at 3000 RPM in cryogenic tubes (Nalgene), then placed in 1 ml Trizol. RNA was extracted according to the manufacturer's instructions and purified using RNEasy columns (Qiagen) and RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), as previously reported [13]. SYBR green qPCR arrays (SABiosciences) with primer-probe sets against 84 extracellular matrix related genes were used. For a list of genes encoded on the array, please see http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-013A.html. The qPCR reaction was carried out using an ABI (Applied Biosystems, Foster City, CA, USA) 7500 Fast system.

MicroCT analysis

MicroCT analysis was used to determine the cross-sectional area of the tendon proper as a means to gauge the extent of post-injury tendon thickening. Mice were euthanized by CO₂ inhalation and tendons dissected with bony attachments (patella and tibia) intact. The specimen was placed in 2.5% iodine for 3 hours at 4° C, then pinned flat on styrofoam and submersed in a 2 ml tube filled with sunflower oil. Tendons were scanned for their entire length at 45 kV, for 12.5 minutes (VivaCT, Scanco, Brüttisellen, Switzerland). In optimization studies, the reduced x-ray lucency resulting from a 3 hour incubation in iodine solution was found to occur preferentially in the water soluble components (e.g. tendon, fascia) but was relatively excluded from fatty tissues (e.g. paratendinous tissue), thereby increasing the contrast between these tissue layers and allowing them to be distinguished. The cross-sectional area for tendon proper at the patellar tendon midpoint was traced with the onboard software and calculated for the right (injured) and left (uninjured control) tendons of n=21 mice (10 control, 11 injected) 4 weeks after injury.

Statistical analysis

To examine differences in mast cell prevalence, a 1-way ANOVA was used followed by a Wilcoxon Sign Rank test comparing matched right and left values. Student's t-test for independent samples was used to compare collagen density in the injured tendons of injected vs. control animals. The Wilcoxon Sign Rank test was used to detect differences in tenocyte density and tendon cross-sectional area. Statistical significance was predetermined at p<0.05. To examine changes in gene expression, SABiosciences analysis software (version 3.2) was used with p<0.05. Values are presented as mean +/- standard error.

RESULTS

Mast cell prevalence following injury

In the current study, uninjured patellar tendons demonstrated very low levels of a mast cell specific marker (CPA3) indicating minimal presence in healthy tendon; however, following

injury CPA3 expression increased dramatically (ANOVA $p=0.009$), particularly at 8 weeks ($p=0.025$) (Figure 1). This finding indicates that the presence of mast cells is increased in injured tendon, particularly in the remodeling phase. Histological examination of mast cell distribution in injured tendon indicated that the mast cells were found in association with vessels in the paratendinous connective tissue surrounding the tendon.

Effect of sodium cromolyn on tendon hypercellularity

We found that the tenocyte density was significantly increased in the patellar tendon proper in injured tendon compared to the uninjured contralateral tendon, indicating the development of hypercellularity (Figure 2, $p=0.05$). However, in animals which received SC injections, there was a less pronounced, non-significant trend toward hypercellularity in the injured tendon.

Effect of sodium cromolyn on tendon cross-sectional area and collagen organization

Four weeks after injury, the patellar tendon CSA in the non-injected group was increased by $85.8 \pm 6.84\%$ on the injured side ($p=0.0027$). In contrast, the injured tendons from sodium cromolyn injected animals were also increased but to a lesser extent ($50.0 \pm 6.09\%$, $p=0.0105$). In the non-injected group, all injured tendons experienced $>40\%$ increase in CSA compared to the uninjured side, indicating the presence of tendon thickening; conversely, in the cromolyn-injected group only 5/11 of the injured tendons demonstrated a greater than 40% increase in CSA. These findings suggest that the post-injury increase in tendon CSA may have been modulated by mast cell inhibition. The collagen organization of injured tendons which had been exposed to SC was substantially higher than that of non-injected animals ($p=0.009$, Figure 3).

Gene expression changes mediated by mast cell degranulation

Based on the above morphological improvements seen with SC injections, we conducted qPCR on a panel of genes implicated in extracellular matrix metabolism to examine potential differences in injured tendon from injected vs. non-injected groups. All genes examined were expressed, but only three showed significant changes in expression levels. CTGF (connective tissue growth factor) was significantly downregulated by 2.0 fold ($p=0.043$). CTGF is implicated in the development of fibrosis in a variety of pathological scenarios, acting downstream of TGF β [19]. CTGF has previously been shown to be expressed by tenocytes [20], and undergoes upregulation following injury [21]. CTGF has also been proposed as an attractive target to decrease scarring following tendon injury [22]. Thus, the reduction of CTGF expression in response to SC injections may represent a potential benefit of this treatment. The other two downregulated genes were ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) (by 2.8 fold, $p=0.010$) and TIMP-3 (tissue inhibitor of metalloproteinase 3, by 2.1 fold, $p=0.030$). The ADAMTS enzymes represent a subfamily of metalloproteinases that are present in tendon, with ADAMTS-1 acting as a secreted heparin binding enzyme with degradative activity against versican (the main proteoglycan in tendon) [23]. In cultured tenocytes, ADAMTS-1 is stimulated by the addition of TGF β [24]. Therefore, it is possible that reduced availability of mast-cell derived TGF β in SC injected animals resulted not only in decreased CTGF, but also in decreased ADAMTS1 expression. TIMP-3 is the most highly expressed of a panel of

TIMPs examined in normal and ruptured human tendons [25], and its downregulation SC may affect the balance of extracellular remodeling in injured tendon. TIMP-3 regulation in tenocytes has not been examined to our knowledge, but in other cell types its expression, like CTGF and ADAMTS-1, is induced by TGF β both *in vitro* and in clinical scenarios involving fibrosis [26, 27]. It has been postulated that elevated TIMP-3 expression in fibrotic conditions may contribute to the development of excessive scarring by inhibiting the turnover of reparative collagen in healing wounds [27]. In summary, SC injection significantly influenced several TGF β -induced tendon genes, and further work will be required to explore the biology of these particular pathways.

DISCUSSION

This study demonstrated that administration of SC to acutely injured tendon can moderate post-injury structural abnormalities and significantly down-regulate genes associated with fibrosis (CTGF, ADAMTS-1, TIMP3). This work corroborates previous findings in support of a role of mast cells in tendon repair [7].

This study contributes evidence that tendon repair is physiologically influenced by mast cells and their products which are known to accumulate following injury [6]. Mast cells may be involved in the initial inflammation following injury, and may also influence later events through the secretion or release of soluble factors, through direct intercellular interactions, or through enzyme-mediated effects in extracellular environment. Mast cells release histamine, heparin, growth factors including TGF β , leukotrienes, and cytokines including IL1 and TNF α . Mast cells are found in close proximity to nerve endings and also to fibroblasts, and may directly communicate with these cell types [28, 29]. Mast cells also release CPA3 and mast cell tryptase, both of which have known proliferative effects on fibroblasts [30]. Despite these potential physiological influences on tendon repair, the current study suggests that mast cells are not an absolute requirement for tendon repair to occur. This finding is consistent with wound healing studies in mast-cell deficient (W/W^v) mice in which mast cells were not essential for healing, but did influence the quality of repair[31].

Mast cells clearly play a role in regulating extracellular matrix metabolism during injury repair. Mast cell extracts have previously been shown *in vitro* to stimulate the production of collagen by skin fibroblasts [32], and they are also capable of activating MMP activity, for instance via tryptase-mediated cleavage of MMP3 [33]. An altered balance of collagen synthesis and secretion in response to mast cell activity would clearly affect the quantity and organizational properties of repair tissue. W/W^v mice demonstrated increased hydroxyproline content and tighter collagen structure in the late phase of wound healing[31], similar to the current study in which SC treated tendons appeared more compact on microCT, with increased collagen density observed via SHG microscopy. Mast cells have also been implicated in the process of scarring (fibrosis) [34], a well known negative sequel of tendon repair [35].

Sodium cromolyn is available as a mast cell stabilizer for local or systemic application for conditions including asthma, dermatitis, mastocytosis and rheumatoid arthritis. Although SC treatment influenced the expression of genes related to extracellular matrix metabolism in

the current study, the exact mechanisms of action remain to be elucidated. Indeed, mast cell granules could exert a variety of direct biological effects in tendon which do not require a modulation of mRNA levels. SC inhibited the expression of several genes known to be induced by TGF β ; in other healing musculoskeletal tissues such as ligament and muscle, anti-TGF β therapy has shown promise in improving the final tissue structure and limiting the extent of scar tissue [36, 37]. Conversely, studies in which mast cell recruitment is enhanced during tendon repair, for instance via local administration of stem cell factor, may also provide information on the functions of mast cells in tendon repair processes. Due to the numerous possible influences of mast cells in an injured environment, including the potential for substance-P mediated neurogenic effects [reviewed in 38], further work will be required to uncover the roles of mast cells in injured tendons.

While the results suggest that SC treatment may be effective in modulating CTGF expression and abrogating post-injury tendon structural abnormalities, there are several limitations of this study. First, we were not able to include biomechanical or functional outcome measures in these experiments; clearly the inclusion of biomechanical testing would have provided further insight into the influence of SC on the outcome of tendon healing. While this is an acknowledged limitation, it does not detract from the main conclusions of this study, namely that mast cells play a physiologically relevant role in tendon repair, and that blocking their ability to degranulate reduces the expression of several genes (CTGF, TIMP3, ADAMTS-1) and influenced the structure of healing tendon including the density of collagen and tendon cells. The size of the injured tendon is rather small, thus preventing our ability to assay mast cell enzyme activity *in situ*. The number of samples for the qPCR array (n=8) was necessarily small; however, the advantage of this approach is that it allowed us nevertheless to survey a relatively large number of genes and to identify statistically significant changes in CTGF, TIMP3 and ADAMTS-1. In this study we have not used transgenic or knock-out mouse strains. In future, the use of strains such as W/W^v or CD34^{-/-} mice could provide further insight into the underlying mechanisms of mast cell involvement in tendon repair. For example, transplantation of mast cells from CTGF^{-/-} mice into W/W^v mice could provide definitive data on the role of this mast cell product in tendon repair.

CONCLUSION

Although the potential roles of mast cells in tendons or ligaments have been discussed for many years, the current paper demonstrates a role for this cell type in tendon injury using a relevant *in vivo* model with well defined outcome measures. We found that systemic administration of a mast cell inhibitor influenced tendon gene expression (CTGF, ADAMTS1 and TIMP3) and abrogated post-injury structural abnormalities (tendon thickening, hypercellularity and collagen disorganization). The strategy of mast cell inhibition for tendon injuries warrants further research.

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Figure 1.

Mast cell prevalence as a function of time after injury. Values represent CPA3 expression levels normalized to 18s and expressed as % change relative to the contralateral uninjured tendon. Error bars denote SE. * $p=0.025$. In graph, $n= 8, 6, 7, 8, 8$ (from left to right).

Figure 2.

Tendon cell density in sodium cromolyn (SC) injected vs non-injected animals. Cells were counted on H&E slides, and comprised fibroblast-like cells (i.e. no vascular or inflammatory cells were counted). * $p = 0.05$, Wilcoxon sign rank test, $n=6$.

Figure 3.

Patellar tendons were systematically examined with multiphoton (A,D,G) and second harmonic generation (B,E,H) and merged images were created (C,F,I) – all images at same magnification (63x objective, please see methods for details). Uninjured tendon in SC-injected and non-injected animals was indistinguishable (SC-injected animal shown in panels A,B,C), demonstrating slender tenocytes (arrow). Injured tendons with no SC treatment (D,E,F) were hypercellular (arrow) and the extracellular matrix emitted a reduced SHG signal. SC treatment (G,H,I) resulted in better SHG signal, although the matrix still demonstrates some disorganization compared to uninjured controls.