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Role of mast cells in male chronic pelvic pain

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Abstract

Purpose—Chronic pelvic pain syndrome (CPPS) accounts for 90% of all chronic prostatitis but has an unknown pathogenesis. In this study, we sought to understand the role of mast cells and nerve growth factor (NGF) in chronic pelvic pain.

Materials and Methods—Expressed prostatic secretions in men with CPPS and controls were tested for the presence of mast cell tryptase and NGF. Mast cell numbers, activation status and NGF expression were examined in the NOD/ShiLtJ experimental autoimmune prostatitis model (EAP) as well as in mast cell deficient Kit^{W-sh}/Kit^{W-sh} mice. Tactile allodynia was quantified using Von Frey filaments as a measure of pelvic pain behavior. Inhibitors of mast cell degranulation, histamine receptor antagonists and anti-NGF neutralizing antibodies were tested for reducing pelvic pain behavior.

Results—Men with CPPS demonstrated increased mast cell tryptase and NGF in expressed prostatic secretions. In the EAP model, increased total and activated mast cells were observed in the prostate. Mast cell deficient Kit^{W-sh}/Kit^{W-sh} mice showed attenuated pelvic pain behavior but no difference in inflammatory infiltrates in the prostate from controls. EAP mice also demonstrated increased intraprostatic NGF expression compared to Kit^{W-sh}/Kit^{W-sh} mice. Treatment of EAP with a mast cell stabilizer in combination with a histamine 1 receptor antagonist resulted in a synergistic reduction in chronic pelvic pain. In contrast, neutralization of NGF *in vivo* did not result in pain relief.

Conclusions—These results suggest that mast cells are important mediators of chronic pelvic pain in EAP and may be potential targets for therapeutic intervention in CP/CPPS.

Keywords

pelvic pain; prostatitis; mast cells; autoimmunity; nerve growth factor

INTRODUCTION

Prostatitis accounts for approximately 2 million outpatient visits per year in the United States, including 1% of those to primary care physicians. Chronic pelvic pain syndrome (CPPS) accounts for 90% of all chronic prostatitis but has no well-defined etiology and limited treatment options available¹. Alterations in the central and/or peripheral nervous system are believed to be involved in CPPS pathogenesis.

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Mast cells are multifunctional immune cells that express high-affinity immunoglobulin E receptors, and release potent inflammatory mediators including histamine, leukotrienes, serotonin, and proteases such as tryptase². Mast cells contain preformed stores of immune mediators, such as histamine and nerve growth factor (NGF) and can be activated to release these stores by chemokines such as MCP-1 and MIP-1 α ^{3,4}. Mast cells have been found in rats with experimental autoimmune prostatitis⁵ as well as in spontaneous prostatitis⁶, suggesting a possible relationship between mast cells and the development of chronic prostatitis.

Mast cells contribute to rapidly occurring peripheral sensitization mediated by NGF⁷. In CPPS, NGF levels have been demonstrated to be elevated in seminal plasma and directly correlated with pain severity⁸. This result suggests the existence of underlying peripheral sensitization mechanisms in CPPS with NGF as the potential mediator. Increased NGF expression is known to increase neuronal excitability as well as enhance neuronal density in peripheral tissues, a feature previously noted in the experimental autoimmune prostatitis (EAP) model^{6,9}. We therefore hypothesized that chronic pelvic pain in EAP involves mast cells and is mediated by the actions of NGF.

MATERIALS AND METHODS

Clinical samples

The criteria utilized for sample collection has been previously described¹⁰. Following approval from Institutional Review Boards, expressed prostatic secretions (EPS) samples were collected by digital rectal examination from urology clinic patients who had no urological disease, or a diagnosis of CPPS IIIB (less than 10 WBC/hpf in EPS). Controls included men with no history or symptoms of urinary tract inflammation, palpably normal prostates and normal PSA values (less than 4.0 ng/ml) for patients 50 years old or older. Men with CPPS had a history of pelvic or perineal pain with or without inflammation for at least 3 months. EPS samples were maintained at -70°C until use in individual assays, at which time samples were thawed and used completely.

Mast cell tryptase and NGF determination

Mast cell tryptase was measured in EPS (200 μg protein) from CPPS and control subjects using the mast cell degranulation kit (Millipore) following manufacturer's protocol. NGF levels in EPS from patients with CPPS, or control subjects were equalized for protein concentrations and 50 μg of protein was blotted and probed with a rabbit anti-NGF polyclonal antibody (M-20, Santa Cruz). NGF levels in EPS were normalized to recombinant NGF (10ng) as a positive standard and analyzed using ImageJ.

Animals and EAP induction

NOD/ShiLtJ and C57BL/6 (B6), (5–7 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Kit^{W-sh}/Kit^{W-sh} mice (5–7 weeks old) were a gift from Dr. David Klumpp, Northwestern University. All experiments were performed using protocols approved by Northwestern University Animal Care and Use Committee. EAP was induced in mice using rat prostate antigen injected subcutaneously in the shoulder as described⁹.

Behavioral testing

Mice were tested prior to immunization (baseline), 20 days, 1 hour after treatment, and 21 days after PAG. Referred hyperalgesia and tactile allodynia were tested on the lower abdomen using Von Frey filaments with forces of 0.04, 0.16, 0.40, 1.00, and 4.00 grams (Stoelting, USA) as previously described⁹. Specificity of response was confirmed by examining the plantar region of the hind paw.

Histochemistry and Immunofluorescence

Mouse prostates were collected from mice and 5 μ m sections were processed for H&E staining by the mouse histology and phenotyping laboratory (MHPL) at Northwestern University. Non-serial sections were scored in a blinded manner using previously established criteria¹¹. Mast cells were stained using 0.02% toluidine blue for 5 minutes and stained cells in non-serial triplicate sections were counted in a blinded manner. For NGF staining, sections were incubated with a rabbit anti-NGF polyclonal antibody (M-20, Santa Cruz) with or without a neutralizing peptide (M-20 (P), Santa Cruz). and detected with streptavidin-Alexa Fluor 594 (Invitrogen) or streptavidin-Alexa Fluor 488. Nuclei were counterstained by incubation with 4',6-diamidino-2-phenylindole (DAPI).

In vivo treatment of chronic pelvic pain

Male NOD mice were treated at post-infection day (PID) 10 or 20 with varying doses of cetirizine dihydrochloride (H1 receptor inhibitor), ranitidine (H2 receptor inhibitor) cromolyn sodium salt (mast cell stabilizer) (Sigma, St. Louis USA), or a combination of these drugs as per experimental design. For antibody neutralization experiments, 100 μ g of anti- beta NGF polyclonal antibody (AB-256-NA, R&D systems) or goat IgG (Jackson ImmunoResearch) was administered intraperitoneally at PID 20.

Statistical analyses

Results were expressed as mean $\pm$ SEM and analyzed for statistical significance by unpaired *t* tests or two-way ANOVA with matching. Post-test analysis of multiple groups was performed using the Tukey-Kramer test and a value of $p < 0.05$ was considered statistically significant.

RESULTS

Mast cell tryptase and NGF are elevated in human CPPS

We postulated that mast cells are present in the prostates of patients with CPPS and undergo activation, releasing bioactive molecules such as mast cell tryptase and nerve growth factor (NGF) into prostatic secretions. As proof of principle, we utilized clinical samples of EPS from patients with CPPS IIIb and compared it to control subjects. Samples were assayed for the presence of mast cell tryptase and NGF as surrogate markers of mast cell degranulation. EPS samples from CPPS patients ($n=7$) demonstrated a significant increase in tryptase levels when compared to controls ($n=5$, $p=0.0063$) (Fig. 1A). We next examined EPS from CPPS ($n=6$) and control patients ($n=5$) for the presence of NGF using an immunoblot assay (Fig. 1B). Our results show that NGF is expressed in the majority of CPPS samples (5/6) while control samples show reduced expression (3/5) (Fig. 1B). Quantification of NGF levels using densitometry shows significantly elevated levels of NGF in the CPPS EPS samples compared to controls ($p=0.0024$) (Fig. 1B).

EAP is associated with increased mast cell density and mast cell activation

We studied mast cells in a murine autoimmune prostatitis model of chronic pelvic pain⁹. Mast cells were identified by toluidine blue staining and individually counted as total, resting, partially activated or fully activated depending on the dispersal of toluidine blue stained granules (Fig. 2A). Total mast cells were increased 5 days after induction of EAP with the majority of cells in the resting stage (Fig. 2C). By day 10 there was significant activation of mast cells that was not observed at 20 and 30 days. However, the apparent reduction in resting cells at 20 and 30 days combined with a lack of any increase in activated cells suggests that a portion of late stage activated cells are not detected because of degranulation. In addition, we also examined prostate sections from 0 (baseline), 10, 20 and

30 days after the initiation of autoimmunity for the expression of NGF, a product of mast cell degranulation (Fig. 2B). NGF was observed to increase at days 10 and 20 with maximal expression being present at day 30 corresponding to the potential period of maximal mast cell degranulation.

Mast cell deficit is associated with an attenuation of pelvic pain in EAP

We hypothesized that mast cells were important in EAP pathogenesis and therefore studied its role by utilizing adult Kit^{W-sh}/Kit^{W-sh} mice (5–7 weeks of age) that were deficient in tissue mast cells due to a mutation in the c-kit receptor. Autoimmune prostatitis was induced in Kit^{W-sh}/Kit^{W-sh} and wild-type C57BL/6 (B6) mice followed by study of pelvic pain development as indicated by enhanced tactile allodynia of the suprapubic region. B6 mice developed robust pelvic pain five days after antigen administration that persisted up to thirty days (Fig. 3A). In contrast, Kit^{W-sh}/Kit^{W-sh} mice did not show any increase in pelvic pain behavior at 5 days and showed inhibited pain responses at 10, 20 and 30 days after antigen administration (Fig. 3B). To assess the specificity of EAP-induced tactile sensitivity, we also quantified the 50% threshold sensitivity in the paw. EAP induced no significant changes in tactile sensitivity of the plantar region of the hind paw in either strain of mice (data not shown). There was also no prolonged change in weight suggesting that EAP in the B6 and Kit^{W-sh}/Kit^{W-sh} mice is not associated with changes in gross physiology (data not shown).

We quantified the extent of inflammation in the prostates of B6 and Kit^{W-sh}/Kit^{W-sh} mice following EAP induction. Inflammation in both groups of mice was chronic with multiple foci of inflammatory cells distributed predominantly in the periglandular regions and stroma of the prostate (Fig. 3D, top panels). Both groups of mice developed chronic inflammation that was not statistically different when quantified using standard inflammation scoring (Fig. 3C). In contrast, toluidine blue staining for mast cells revealed large numbers of mast cells in various stages of activation in the stroma of C57BL/6 but not Kit^{W-sh}/Kit^{W-sh} mice (Fig. 3D, lower panels). Thus, mast cells do not appear to be a requirement for the development of inflammation per se but appear to play a critical role in the establishment of chronic pain.

NGF is increased in EAP

We next compared the expression of NGF in B6 and Kit^{W-sh}/Kit^{W-sh} mice following EAP induction. At 30 days following EAP induction B6 mice showed expression of NGF in the stroma of the prostate that was abrogated in the presence of an anti-NGF neutralizing peptide (Fig. 4A, top panels). In contrast, Kit^{W-sh}/Kit^{W-sh} mice showed lower levels of NGF expression with much of the expression confined to the lumen of the prostatic glands and a lack of expression in the prostatic stroma (Fig. 4A, lower panels).

Given the well-known role for NGF in peripheral sensitization and neuropathic pain¹² we sought to identify whether inhibition of NGF would inhibit pelvic pain in the EAP model. Single administration of an anti-NGF polyclonal antibody 20 days following EAP induction in NOD mice resulted in a trend towards reduced pelvic pain response (Fig. 4C and D) that was however not statistically significant, when compared to control animals with EAP administered a control antibody (Fig. 4B and D).

Inhibitors of mast cell function are therapeutic for chronic pelvic pain

We hypothesized that inhibition of mast cell degranulation or function of bioactive effectors such as histamine would favorably affect the pain status of NOD mice with EAP-induced chronic pelvic pain. Following EAP induction pelvic pain behavior was measured at baseline, 10 days after induction and one hour following intraperitoneal administration of the drug of choice. Mice were administered a mast cell stabilizer, cromolyn sodium; a histamine receptor 1 antagonist, cetirizine; a histamine receptor 2 antagonist, ranitidine; and

a combination of all three pharmacological agents or saline as control. Our results indicate that treatment with H1 and H2 receptor antagonists reduced pain behavior by $13.8 \pm 8.3\%$ and $6.1 \pm 16.7\%$ respectively while controls exhibited no reduction in pain behavior (Fig. 5A–C, F). Treatment with cromolyn sodium alone reduced pain behavior by $31.4 \pm 25.9\%$ while the combination of all three pharmacological agents reduced pain behavior by $69.3 \pm 25.9\%$ ($p < 0.05$) (Fig. 5D&E, F). In additional studies we utilized combinations of the cromolyn sodium and the H1R antagonist or the H2 receptor antagonist. The synergism noted with cromolyn sodium was observed only with the H1 receptor antagonist (data not shown).

We performed dose-response studies in NOD mice 20 days following EAP induction to identify the optimal dose of cromolyn sodium and the H1 receptor antagonist that would be efficacious for the reduction of chronic pelvic pain. Cromolyn sodium was found to be most effective at 24 hours after single administration and at 0.5mg/kg of body weight (Fig. 6A). The effective concentration was statistically significant from controls and did not differ in efficacy from 100mg/kg. The H1 receptor antagonist, in contrast, was not sufficient at all tested doses to achieve statistically significant benefits on pain relief. We also studied the effect of the drug combination (cromolyn sodium and H1 receptor antagonist) using two different routes of administration. The combination showed statistically significant benefit over cromolyn sodium alone in both oral and intraperitoneal routes 24 hours following a single administration (Fig. 6C and D).

DISCUSSION

CP/CPPS is a disease syndrome that has an undefined etiology but is characterized by the presence of chronic pelvic pain. In recent years there has been an increasing appreciation of the role for neuronal mechanisms in CPPS and the closely related pelvic pain condition interstitial cystitis (IC). In both these disease syndromes there exists evidence of immune dysfunction within the target organ, the prostate in CPPS¹³ and the bladder in IC¹⁴. Sterile inflammation of the bladder in IC patients has been associated with the presence of mast cells and mast cell mediators¹⁵. Mast cells are often seen in close apposition to nerves and there is experimental evidence that nerves and mast cells may constantly communicate in an NGF-dependent manner¹⁸. In this study we postulated that mast cells are key mediators of chronic pelvic pain in CPPS. We addressed our hypothesis by examining mast cells in experimental autoimmune prostatitis, a well-characterized murine model of male chronic pelvic pain⁹.

Experimental evidence for mast cells in CPPS has been scant given the difficulty in acquiring prostate tissue to perform immunopathological studies¹⁹. However, secretions of the prostate have been previously evaluated for the presence of inflammatory markers and for the presence of NGF, a protein that can be both released and can contribute to mast cell degranulation^{8, 20}. In agreement with these studies we observed an elevated level of NGF in the EPS of men with CPPS compared to controls. We also examined the EPS sample for mast cell tryptase, a component of mast cell granules that is released during degranulation and has been demonstrated to be elevated in IC urines^{22, 23}. The significant elevation in mast cell tryptase in CPPS compared to controls suggests mast cells are present in the prostate and are in a state of activation. Despite the small sample size of these studies, the consistent presence of markers of mast cell activation in normal clinical specimens, suggests the utility of these surrogates as potential biomarkers for CPPS. In this regard, two previous studies examining NGF in men with CPPS have shown that the levels of NGF directly correlate with the pain severity as measured using the NIH-Chronic prostatitis symptom index^{8, 21}. We are presently performing studies in larger cohorts to validate the use of these mast cell markers as biomarkers for CPPS.

Mast cells were observed to increase in number in the stroma of the prostate of mice with EAP. These results are in agreement with the spontaneous prostatitis model in wistar rats^{6, 24} and the 17 beta-estradiol induced prostatitis model²⁵ where mast cell numbers were increased and were in the process of degranulation. In contrast to the early elevation in mast cell numbers, expression of NGF was low until 30 days, suggesting that NGF release occurs late in the mast cell activation pathway. Alternatively, NGF expression may be from other cell types in the prostatic stroma that are induced as a result of the inflammatory milieu in the prostate. In contrast to the negligible effect that the lack of mast cells had on the presence of inflammatory infiltrates in the prostate, the deficiency of mast cells in the Kit^{W-sh}/Kit^{W-sh} mice resulted in a significantly lower level of pelvic pain behavior. Inflammation in the Kit^{W-sh}/Kit^{W-sh} mice was not abrogated, suggesting that mast cells are not principal players in recruitment and activation of immune cells to the prostate. However, we do not rule out the possibility that the nature of the immune cell types recruited may be influenced by the absence of mast cells in the Kit^{W-sh}/Kit^{W-sh} mice.

NGF mediates pain through the sensitization of nociceptive neurons and is important in the generation and potentiation of pain following tissue injury and inflammation²⁶. Therapeutic inhibition of NGF has been demonstrated to reduce pain-like behavioral responses in a number of animal models of visceral pain²⁷ and more recently in interstitial cystitis in humans²⁸. We therefore examined the utility of anti-NGF therapy to treat chronic pelvic pain in the EAP model. The absence of any significant reduction in chronic pelvic pain suggests that anti-NGF therapy may either need to be sustained or is not sufficient by itself to check the pain behavior in mice with chronic pelvic pain.

Previous studies in a neurogenic animal model of interstitial cystitis have demonstrated the efficacy of targeting the histamine receptors for achieving pain relief²⁹. Histamine metabolites released from mast cells are capable of acting on nociceptive nerve fibers to mediate pain symptoms. In contrast to studies with the neurogenic cystitis model²⁹, we did not observe any relief with the H1 or H2 receptor inhibitor and a modest but statistically insignificant benefit with the mast cell stabilizer alone. Serendipitously, we observed a synergistic benefit to combining the mast cell stabilizer and the H1 receptor inhibitor to produce a profound reduction in pelvic pain. The decrease in pelvic pain behavior was significant at a low dose and therapeutic benefit appeared to largely depend on the concentration of the mast cell stabilizer and less so on the H1 receptor inhibitor.

CONCLUSION

In summary, in this study we have demonstrated a role for mast cells in the development of chronic pelvic pain. In preliminary human studies, we show evidence of elevated mast cell tryptase and NGF in expressed prostatic secretions of CPPS patients. NGF was associated with the development of EAP but NGF neutralization did not inhibit pelvic pain behavior in EAP. In contrast, stabilization of mast cells and inhibition of the histamine 1 receptor provided significant benefit for reduction of pelvic pain behavior. These findings suggest that mast cells and their intermediates play an important role in the pathogenesis of chronic pelvic pain. Future studies in CPPS patients will examine whether mast cells are present in CPPS and will assess the efficacy of anti-mast cell therapies in clinical trials.

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Abbreviations and Acronyms

CP/CPPS	Chronic prostatitis/Chronic pelvic pain syndrome
EPS	Expressed prostatic secretions
EAP	Experimental autoimmune prostatitis
NGF	Nerve growth factor

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FIGURE 1. Trypsase and NGF levels in EPS are higher in patients with CPPS

(A) EPS samples from CPPS patients (n=7) and controls (n=5) were normalized with regard to protein concentration (200 μ g) and analyzed for mast cell tryptase levels. Results are expressed relative to activity of a tryptase positive standard (B) EPS (50 μ g protein) from CPPS patients (CP1-4) and control subjects (control 1–3) as well as recombinant NGF as positive control were subjected to SDS-PAGE separation followed by detection with an anti-NGF polyclonal antibody. Immunoblot images were analyzed using ImageJ and intensity of NGF protein bands relative to the NGF standard is displayed. The immunoblot image shown is representative of two separate experiments but the densitometric analysis represents data from both experiments with CPPS (n=6) and control (n=5) subjects. Significance is indicated by absolute p values.

FIGURE 2. Mast cell numbers and NGF expression increase in EAP

(A) Mast cell in toluidine blue stained sections of mouse prostate harvested at 0, 10, 20, and 30 days after EAP induction. Note increased degranulation with time denoted by less intracytoplasmic granular staining of mast cells. The arrows point to mast cells in different stages of activation. (B) Fluorescent stained sections of mouse prostates harvested at 0, 10, 20, and 30 days after EAP induction. The sections were stained with anti-NGF antibody (red) and DAPI (blue). The arrows point to areas of increased NGF expression. (C) Mast cell numbers were determined in non-serial sections of the mouse prostate stained with toluidine blue. The total number of mast cells in EAP were categorized as activated, partially activated, and resting. (I) Shows the total amount mast cells during the time course. (II, III, IV) Show the amount of activated, partially activated, and resting mast cells respectively. Scale bar represents 50 μ m.

FIGURE 3. Chronic pelvic pain is dependant on mast cells

Male B6 (n=10) (A) or Kit^{W-sh}/Kit^{W-sh} mice (n=8) (B) were tested for tactile allodynia using Von Frey filaments at baseline and 5, 10, 20 and 30 days after EAP induction. (C) Serial sections of the prostate of B6 and Kit^{W-sh}/Kit^{W-sh} mice following EAP induction were stained using hematoxylin and eosin and scored in a blinded manner for inflammation using standardized criteria. (D) B6 and Kit^{W-sh}/Kit^{W-sh} prostate sections show foci of inflammation but only B6 mice show the presence of mast cells. Arrows in upper panel indicate inflammatory infiltrates and in lower panels, the presence of mast cells in B6 and its absence in Kit^{W-sh}/Kit^{W-sh} prostate sections. Scale bar represent 50µm.

FIGURE 4. NGF mediates chronic pelvic pain

(A) Prostate sections of B6 and Kit^{W-sh}/Kit^{W-sh} mice with EAP were stained with anti-NGF antibody (green) or anti-NGF antibody (green) with blocking peptide and DAPI (blue) as nuclear stain. Arrows indicate areas of strong NGF staining appreciable in B6 prostates and less so in the Kit^{W-sh}/Kit^{W-sh} prostate. Male B6 (B, n=7) or Kit^{W-sh}/Kit^{W-sh} mice (C, n=7) were tested for tactile allodynia using Von Frey filaments at baseline and at day 20 prior to initiating treatment with an anti-NGF neutralizing antibody or an isotype control antibody. Pain behavior was tested 1, 2, 3, 4 and 5 days following injection of antibody. (D) NOD mice with EAP were treated at PID 20 with 100 μ g of anti-NGF neutralizing antibody (Anti-NGF) or control IgG (Control) followed by daily testing for tactile allodynia.

FIGURE 5. Simultaneous stabilization of mast cells and inhibition of H1 receptor attenuates chronic pelvic pain

EAP was induced in NOD mice (n=5 in all groups) for 10 days followed by treatment with saline (A), cetirizine (10mg/kg, H1 receptor antagonist) (B), ranitidine (10mg/kg, H2 receptor antagonist) (C), cromolyn sodium (10mg/kg, mast cell stabilizer) (D) or a combination of all three drugs (E) administered intraperitoneally. Mice were tested for tactile allodynia with Von Frey filaments at baseline, day 10, and 1 hour after treatment. (F) Pain behavior expressed as percent decrease from pretreatment levels. Combination therapy was statistically significant from control ($p<0.05$).

FIGURE 6. A mast cell stabilizer and H1 receptor inhibitor show synergistic inhibition of chronic pelvic pain

Dose response of cromolyn sodium (CS) (A) and cetirizine (H1R) (B) in the reduction of chronic pelvic pain at 1 hour and 24 hours following intraperitoneal therapy in EAP (n=5 in all groups). Administration of cromolyn sodium at 0.5mg/kg (C and D) in combination with cetirizine at 2.5 mg/kg (C and D) shows significant synergism for reduction in pain behavior when administered orally (n=6, $p < 0.05$) or delivered intraperitoneally (n=10, $p < 0.05$) in mice with EAP.