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Bone. Author manuscript; available in PMC 2021 January 13.

Published in final edited form as:

Bone. 2018 April ; 109: 259–266. doi:10.1016/j.bone.2017.08.023.

Mast cell inhibition as a therapeutic approach in fibrodysplasia ossificans progressiva (FOP)

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Abstract

Background—Episodic flare-ups of fibrodysplasia ossificans progressiva (FOP) are characterized clinically by severe, often posttraumatic, connective tissue swelling and intramuscular edema, followed histologically by an intense and highly angiogenic fibroproliferative reaction. This early inflammatory and angiogenic fibroproliferative response is accompanied by the presence of abundant mast cells far in excess of other reported myopathies.

Results—Using an injury-induced, constitutively-active transgenic mouse model of FOP we show that mast cell inhibition by cromolyn, but not aprepitant, results in a dramatic reduction of heterotopic ossification. Cromolyn, but not aprepitant, significantly decreases the total number of mast cells in FOP lesions. Furthermore, cromolyn specifically diminishes the number of degranulating and resting degranulated mast cells in pre-osseous lesions.

Conclusions—This work demonstrates that consideration of FOP as a type of localized mastocytosis may offer new therapeutic interventions for treatment of this devastating condition.

Keywords

Mast cells; Fibrodyplasia ossificans progressiva (FOP); Heterotopic ossification; ACVR1; Cromolyn

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Authors' roles

Conception and design of the work were by RJP and FSK. Collection and/or assembly of data were by TAB, CML, CRB, and HW. The manuscript was written by RJP, with revisions by all authors. Data analysis and interpretation were performed by all authors. The manuscript was approved by all authors.

Conflict of interest

The authors declare that they have no competing interests.

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1. Introduction

Mast cells are found within connective tissue and vascularized organs. Their progenitors leave the bone marrow to enter the circulation and mature within target tissue in the presence of the KIT ligand stem cell factor (SCF) [1]. SCF is critical for mast cell expansion,differentiation and survival. Mast cells are activated in response to mechanical stimulation, temperature, snake venom, and antigens via the high affinity receptor for IgE [2]. Their activation results in acute and chronic inflammation, vascular injury, cell recruitment, cell adhesion, and, ultimately, tissue remodeling and angiogenesis [2–4]. Mast cells secrete preformed inflammatory mediators, including serine proteases (i.e. tryptase), histamine, serotonin, proteoglycans (i.e. heparin), and cytokines (e.g., TNF-α), as well as late-phase products such as chemokines and additional cytokines.

Mast cell subtypes cycle among resting granulated (RG), degranulating (DG), and resting degranulated (RD) cells, with RG cells becoming DG cells upon activation and DG cells then becoming RD cells after release of preformed inflammatory and late-phase products [5,6]. Finally, RD cells become RG cells when their inflammatory mediators are reconstituted in cytoplasmic granules [5,6].

In FOP, episodic tissue swelling and intramuscular edema is followed by intense angiogenic and fibroproliferative reactions. The early edematous, preosseous fibroproliferative stages characteristic of FOP flare-ups suggested to Gannon et al. [7] that inflammatory mast cells and their mediators in tissue edema, wound repair, fibrogenesis, angiogenesis, and tumor invasion were potentially involved in the pathology of FOP lesions. They showed that inflammatory mast cells are present at every stage of the development of FOP lesions and are most pronounced at the highly vascular fibroproliferative stage.

Here we test the hypothesis that mast cell inhibitors/stabilizers such as cromolyn (cromogliclic acid) and aprepitant can prevent heterotopic ossification (HO) in a mouse model of FOP. We found that mast cell inhibition by cromolyn, but not aprepitant, results in a dramatic reduction of heterotopic ossification. Cromolyn, but not aprepitant, significantly decreased the total number of mast cells in FOP lesions. In addition, cromolyn specifically diminishes the number of degranulating (“activated”) and resting degranulated mast cells in pre-osseous lesions.

2. Methods

2.1. In vivo testing of mast cell inhibitors

A transgenic mouse model containing a constitutively active (ca)ACVR1 allele flanked by loxP sites (caACVR1 mice) was used in all animal experiments [8,9]. A 50-μL, 0.9% NaCl solution containing adenovirus-Cre (5×10^{10} genome copies per mouse; Penn Vector Core, University of Pennsylvania) to induce expression of caACVR1 and cardiotoxin (10 μM solution; Sigma-Aldrich, St. Louis, MO, USA) to induce an injury/inflammatory response was injected into the hindlimb musculature of mice at 3 weeks of age. Mice were treated via intraperitoneal injection with 800 mg/kg/ day of cromolyn in phosphate-buffered saline(PBS),oral gavage with 20, 100, or 500 mg/kg/day aprepitant in PBS, or vehicle

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control. For all treatment groups, a minimum of four animals was used per group. Mice were treated for 4 days prior to and 14 days following injection in the left hind limb. Tissues were recovered at 2, 5, 7, 10, or 14 days after injections.

Mice were bred in an animal biosafety level (ABSL) 1 facility then transferred to an ABSL2 facility where procedures were performed. Mice were housed as 1 or 2 mothers with litters and then separated at weaning into cages of no more than 5 males or 5 females. Both males and females were used for experiments. Other aspects of animal care and usage (e.g., light/dark cycle) were standard and performed by University. Laboratory Animal Resources (ULAR) in accordance with ULAR policies and protocols.

2.2. Micro computerized tomography

Micro computed tomography (μCT) was performed on the injected leg post mortem using a Scanco VivaCT 40 (Bruettisellen, Switzerland) to determine the volume of heterotopic bone and obtain a two-dimensional image of the medial view of the sagittal plane of the limb. Scanning was performed using a source voltage of 55 kV, a source current of 142 μA, and an isotropic voxel size of 10.5 μm. Bone was differentiated from “non-bone” by an upper threshold of 1000 Housfield units and a lower threshold of 150 Housfield units.

2.3. Histology

For histological analyses, animals were euthanized 2, 5 or 7 days post cardiotoxin muscle injury. The left hind limb was removed and placed in 3.7% paraformaldehyde for 72 h. The tissue was decalcified for 3–5days with ImmunoCal™ (Decal Chemical Corporation) and processed for paraffin embedding. Sequential sections were cut at 8 μm and stained with C.E.M. (Combined Eosinophil-Mast Cell) staining kit® (American MasterTech) or Safranin O. C.E.M. staining was performed according to manufacturer’s protocol. Safranin O staining was performed according to standard protocol.

Approximately 10 consecutive sections were alternately stained with C.E.M. or Safranin-O. The region of interest was defined as the entire section above the ankle, not including bone and its articular cartilage. All mast cells within the region of interest were identified and quantified. Mast cell subtypes were characterized as resting granulated (RG), degranulating (DG), or resting degranulated (RD) and distinguished on the basis of major histological features. RG cells have low nuclear to cytoplasmic ratios, dark staining with nuclei less visible, intact membranes, and large sizes. DG cells have varied nuclear to cytoplasmic ratios and variegated staining, are of medium-large sizes, and have broken membranes. RD cells have high nuclear to cytoplasmic ratios, lighter staining with very visible nuclei, intact membranes, and small sizes. Samples were viewed under a Nikon 50i Eclipse microscope and images were captured using Nikon DS-Fi1 camera and NIS elements F version 4.3 software.

2.4. Statistics

Comparison among treatment doses in the aprepitant group was analyzed by one-way analysis of variance (ANOVA), and between cromolyn-treated and control groups by Student t-test (two-tailed). Effects of time and cromolyn treatment were analyzed by two-

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way ANOVA, followed by the Bonferroni post-test. All statistics were performed using GraphPad Prism 4.0 software (San Diego, CA). Differences were considered statistically significant at a *p*-value of <0.05, and all data is represented as mean ± SEM.

3. Results

3.1. Cromolyn treatment results in diminished heterotopic bone formation in the caACVR1 animal model

We tested the hypothesis that mast cell inhibition can reduce HO in a mouse model of FOP by using two mast cell antagonists that act by different mechanisms. Aprepitant is a substance P (SP)/neurokinin 1 (NK1) receptor antagonist in mast cells. The SP/NK-1 receptor system affects immune function (especially mast cells) through sensory nerves that modulate neurogenic inflammation. Cromolyn inhibits sensitized mast cell degranulation and indirectly blocks calcium ions from entering the mast cell, thereby preventing mediator release.

Fig. 1 shows that over a broad range of doses aprepitant does not inhibit heterotopic bone formation in the caACVR1 mouse model. In contrast, cromolyn dramatically reduces HO by day 14 after injury-induced lesion formation (Fig. 2).

3.2. Mast cells are reduced in cromolyn- but not aprepitant-treated caACVR1 animals

In pre-osseous lesions, mast cells can be detected by two days after injury induction (Fig. 3). The repertoire of mast cell subtypes, including activated DG cells, are present early and are identified by their characteristic appearances (see Material and methods — Histology section and Fig. 3).

Fig. 4A demonstrates that in control lesional tissue sections, mast cells are present throughout the pre-osseous stages (up to day seven after injury). There is a statistically non-significant trend toward higher total mast cell number (Fig. 4A) and specific mast cell subtypes (Fig. 4B) by day seven. There was a statistically significant reduction in the total number of mast cells in the cromolyn-treated animal group compared to the control group, but not in the aprepitant-treated group (Fig. 5).

Further analysis of the cromolyn-treated group revealed that reductions in specific mast cell subtypes are primarily responsible for the decrease in total mast cells compared to the control group (Fig. 6). Both DG and RD cells increased significantly post-injury (p b 0.05). Although all mast cell subtypes declined with cromolyn treatment over time, the most significant effects were with DG (p b 0.01) and RD cells (p b 0.0001). Post-hoc analysis demonstrated that DG cells on day seven after injury and RD cells at all time points after injury were significantly reduced by cromolyn treatment (Fig. 6).

4. Discussion

The episodic connective tissue swelling and intramuscular edema seen with flare-ups in FOP are accompanied by highly angiogenic fibroproliferation seen histologically. Gannon et al. [7] showed that mast cell density at the periphery of FOP lesions is as much as 150-fold

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greater than in normal skeletal muscle or in uninvolved skeletal muscle from patients with FOP and up to 40-fold greater than in any other inflammatory myopathy. These findings demonstrate that mobilization and activation of inflammatory mast cells are pathological hallmarks of FOP lesions and serve as potential targets for pharmacologic intervention in this extremely disabling disease. Mast cell involvement has also been demonstrated in non-hereditary HO, strongly suggesting the cell type is critical to the pathology of HO [10,11].

Mast cells play a key role in the allergy response and anaphylaxis [2], and have been more recently recognized as mediators in tissue edema, wound repair, fibrosis, angiogenesis, pain pathophysiology, and tumor invasion [4,12–21]. Using Nse-BMP4/MOR (−/−) double mutant mice, in which μ-opioid receptor (MOR)-null mice over-express BMP4 in cells with an active Nse promoter, Kan et al. [22] showed attenuated mast cell activation and concomitant reduction in HO formation in response to injury. Opioid signaling may thus play a key role in mast cell activation and the downstream inflammatory responses associated with HO.

Following BMP induction, mast cells are recruited and activated by the potent neuro-inflammatory molecules Substance P (SP) and calcitonin gene related peptide (CGRP) [23]. Inhibition of the SP and CGRP signaling pathways via various genetic and pharmacological methods can reduce HO formation in animal models [24–26]. SP is also highly expressed in early stage lesional tissue from FOP patients [24]. SP acts on mast cells via binding to the NK-1R receptor, which induces mast cell degranulation and release of numerous pro-inflammatory factors [27].

Pharmacological inhibition of mast cells and SP is theoretically possible in two ways. The NK-1R receptor can be directly targeted by the NK-1R antagonist aprepitant, which has been successfully used to inhibit intracellular calcium ion flux, a readout of SP signaling [28]. However, at least in the caACVR1 FOP model, aprepitant was not effective in limiting injury-induced HO. Conversely, direct inhibition of mast cell degranulation and reduction of mast cell number by cromolyn was successful at significantly reducing HO. Our results that mast cells are present in early pre-osseous stages of lesion formation in the caACVR1 mouse model recapitulate the initial observation by Gannon et al. in FOP lesional tissue [7].

Cromolyn may disrupt the activation cycle of mast cells by multiple cellular mechanisms (Fig. 7). Cromolyn may antagonize both the activation of mast cells (formation of DG cells from RG cells) as well as the production of RD cells, preventing the regeneration of RG cells. Based on this latter mechanism, if cromolyn is given after an early FOP lesion has formed, it might potentiate the DG phase (by inhibiting the DG to RD cell transition) and potentially exacerbate a flare-up. This suggests that in its clinical use in FOP patients, as in the proof-of-concept FOP mouse model used here, cromolyn should be given prophylactically to prevent mast cell degranulation. It is also possible that cromolyn indirectly inhibits the DG to RD cell transition; that is, there are fewer RG mast cells because cromolyn effectively reduces the number of DG cells such that there are many fewer cells that can become RG cells. Cromolyn may also act by direct inhibition or removal of DG and RG cells from the regeneration cycle.

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Although its existing FDA indication for asthma may make cromolyn a potential option for FOP patients, its current routes of usual administration result in poor systemic distribution. Spray or aerosolized liquid forms for nasal or oral inhalation represent the best common routes for systemic distribution and cromolyn is currently used at the physician's discretion as a Class II medication in current FOP treatment guidelines [29]. A liquid intravenous form of cromolyn has been reported [30,31]; however, it undergoes rapid elimination with possible hypertension and nausea as potential adverse effects [32]. An alternative option involves using the c-kit tyrosine kinase inhibitor imatinib to induce mast cell apoptosis, which has proven successful at reducing inflammation associated with rheumatoid arthritis [33] and severe refractory asthma [34], as well as reducing HO in an Achilles tendon injury model [35], and importantly, HO in a FOP mouse model [36].

5. Conclusions

Considering FOP as a type of localized mastocytosis may offer new therapeutic opportunities for treatment. Cromolyn is very safe and effective in abrogating HO in an injury-induced mouse model of FOP, but at doses in excess of those currently capable of being delivered in a safe and convenient manner. Improvement in the bioavailability of cromolyn, either by change in formulation or route of administration, may offer enhanced therapeutic benefits. Other mast cell inhibitors may offer similar and/or additional therapeutic benefits.

Acknowledgments

Funding

This work was supported by the Cali-Weldon Preclinical Drug Testing and Biomarker Development Program at the University of Pennsylvania, the Ian Cali Distinguished Clinician-Scientist Award, the Robert and Arlene Professorship in Geriatric Medicine at the Mayo Clinic, and the Radiant Hope Foundation (to RJP); the Center for Research in FOP and Related Disorders at the University of Pennsylvania Perelman School of Medicine; the Ian Cali Endowment for FOP Research; the Whitney Weldon Endowment for FOP Research and the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine (to FSK). This work wasalso supported by thePenn Center for Musculoskeletal Disorders (NIH P30-AR050950), and the National Institutes of Health (NIH R01-AR41916).

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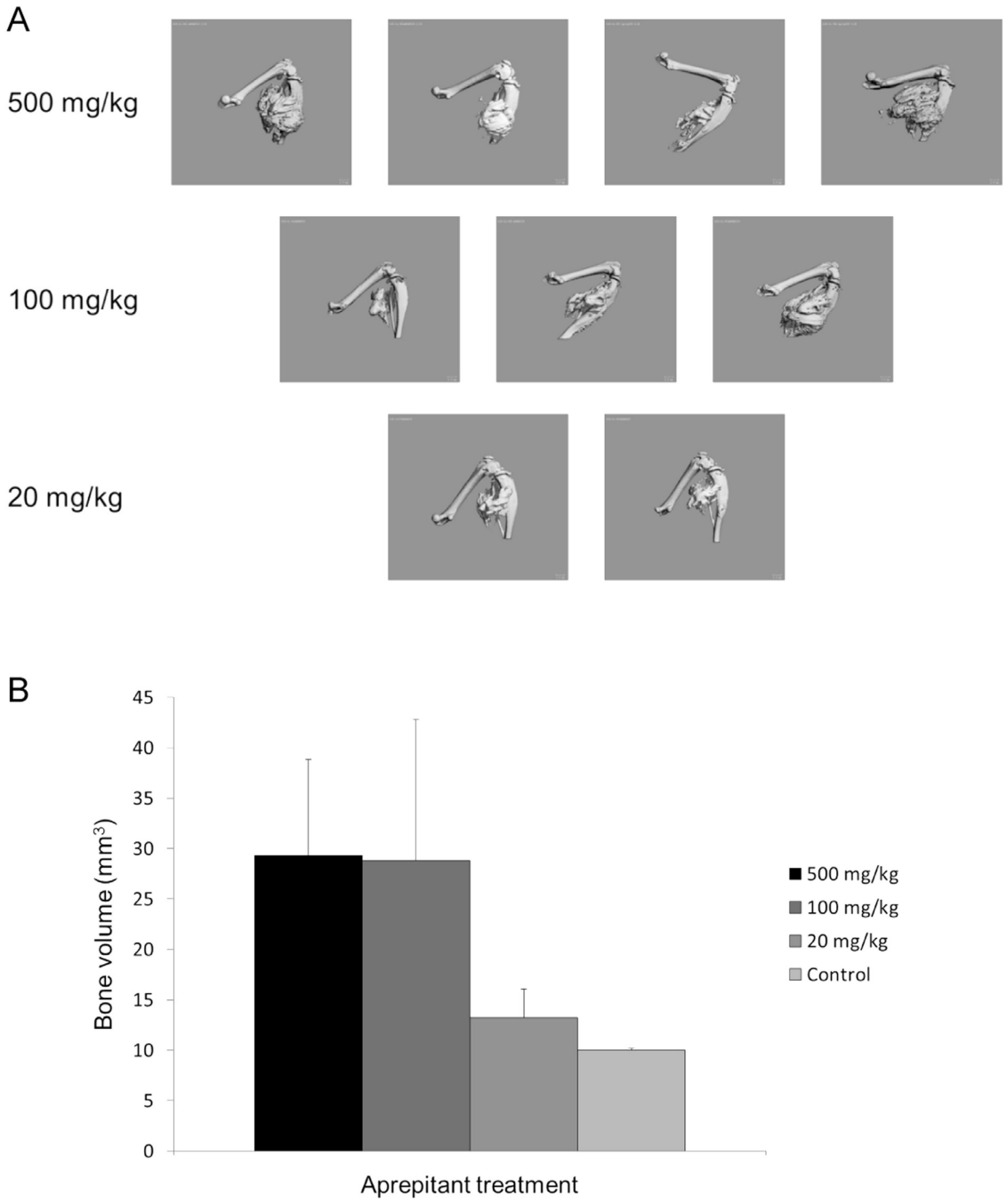

Fig. 1.

Aprepitant does not reduceHOina caACVR1 mouse model of FOP. (A) Representative examples of HO formation by μCT. (B) Quantification of HO volume among the treatment doses of aprepitant.

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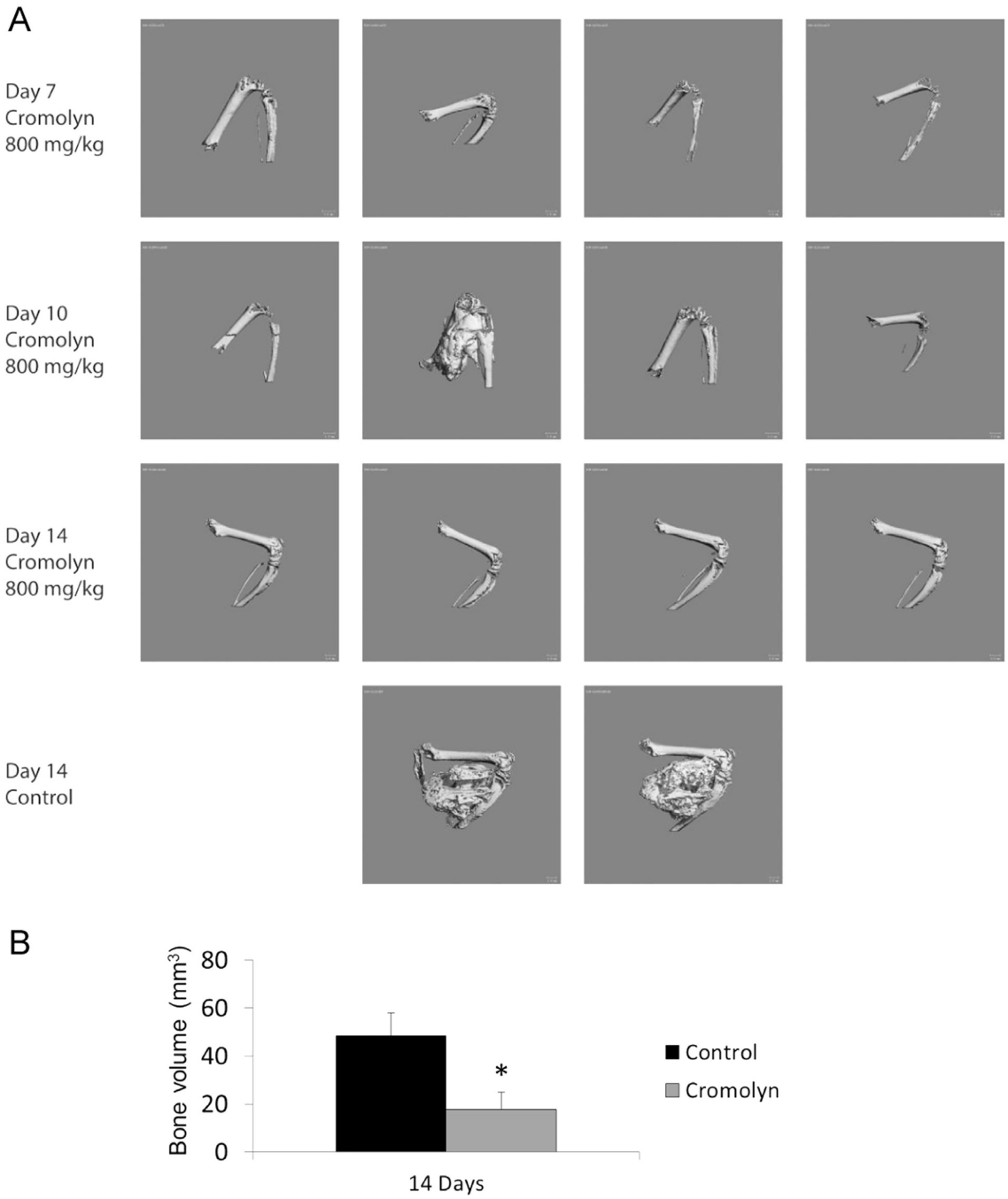

Fig. 2.

Cromolyn effectively reduces HO in a caACVR1 mouse model of FOP. (A) Representative examples of HO formation by μCT. (B) Quantification of HO volume between the treatment groups. *, $p < 0.05$.

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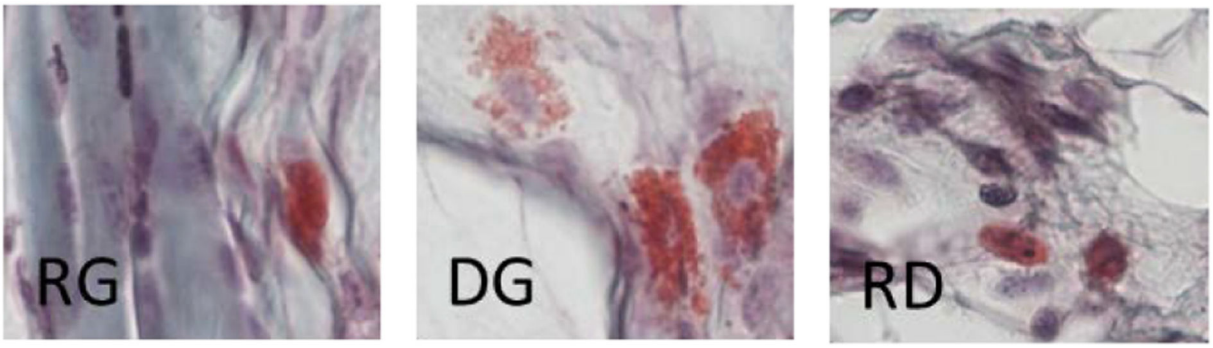

Fig. 3.

Mast cell subtypes in early injury-induced lesions in a caACVR1 mouse model of FOP (day 2 after injury). Note the broken membrane and release of granules in activated (DG) mast cells. RG, resting granulated; DG, degranulating; RD, resting degranulated (RD).

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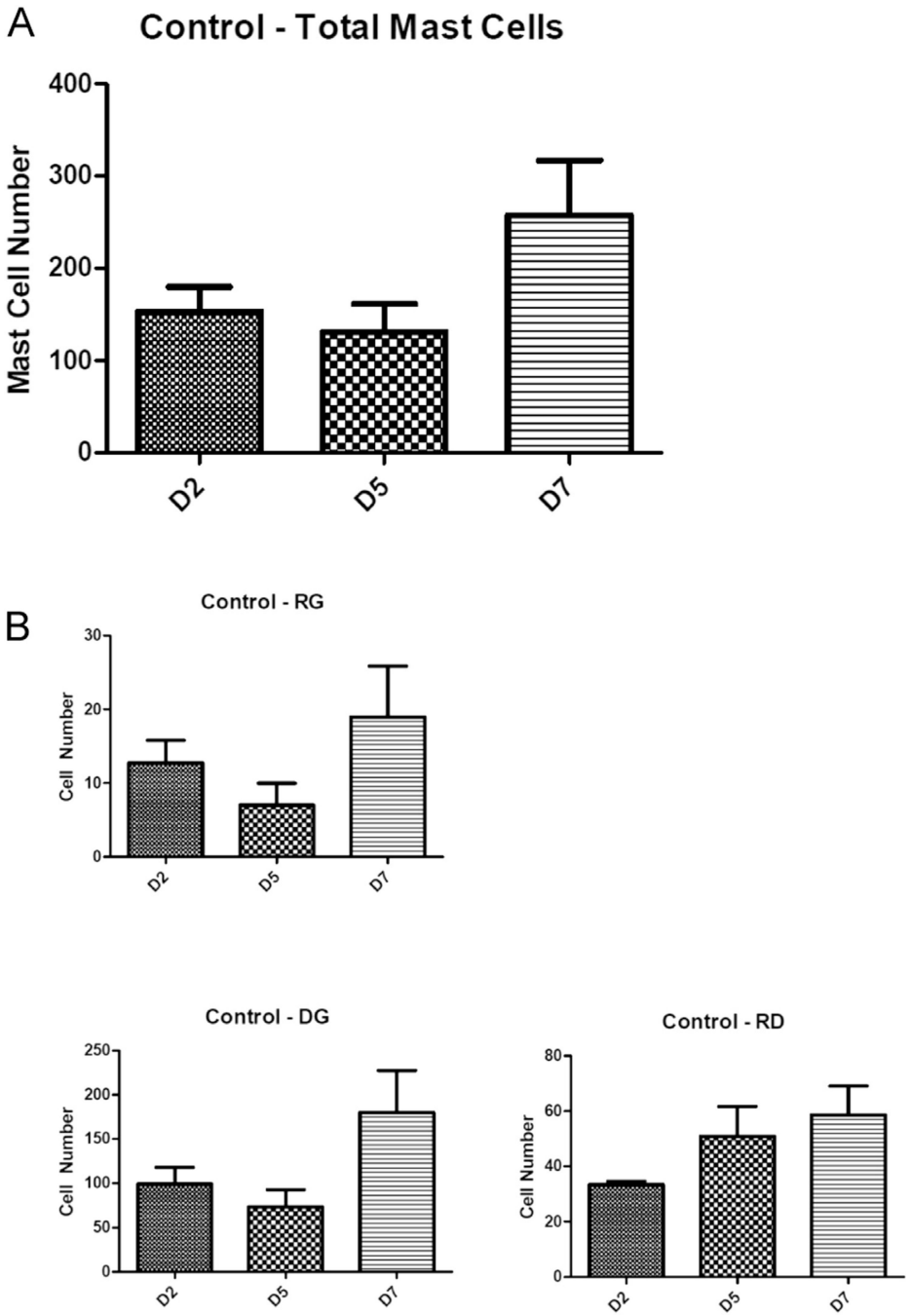

Fig. 4.

Quantification of total mast cells (A) and mast cell subtypes (B) in control caACVR1 mice. Data is shown for days two, five, and seven after muscle injury. D, day.

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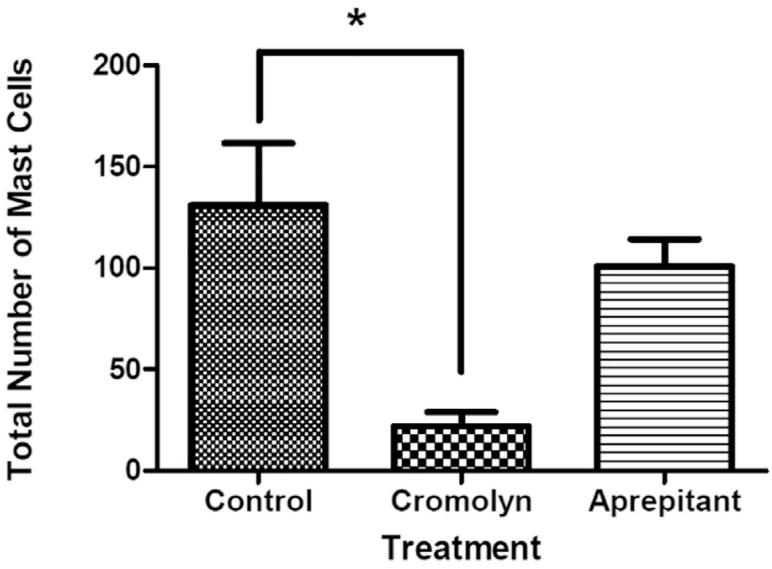

Fig. 5.

Total mast cell number isreduced in cromolyn- but not aprepitant-treated caACVR1 mice. Data shown is for day five after injury induction. *, $p < 0.05$.

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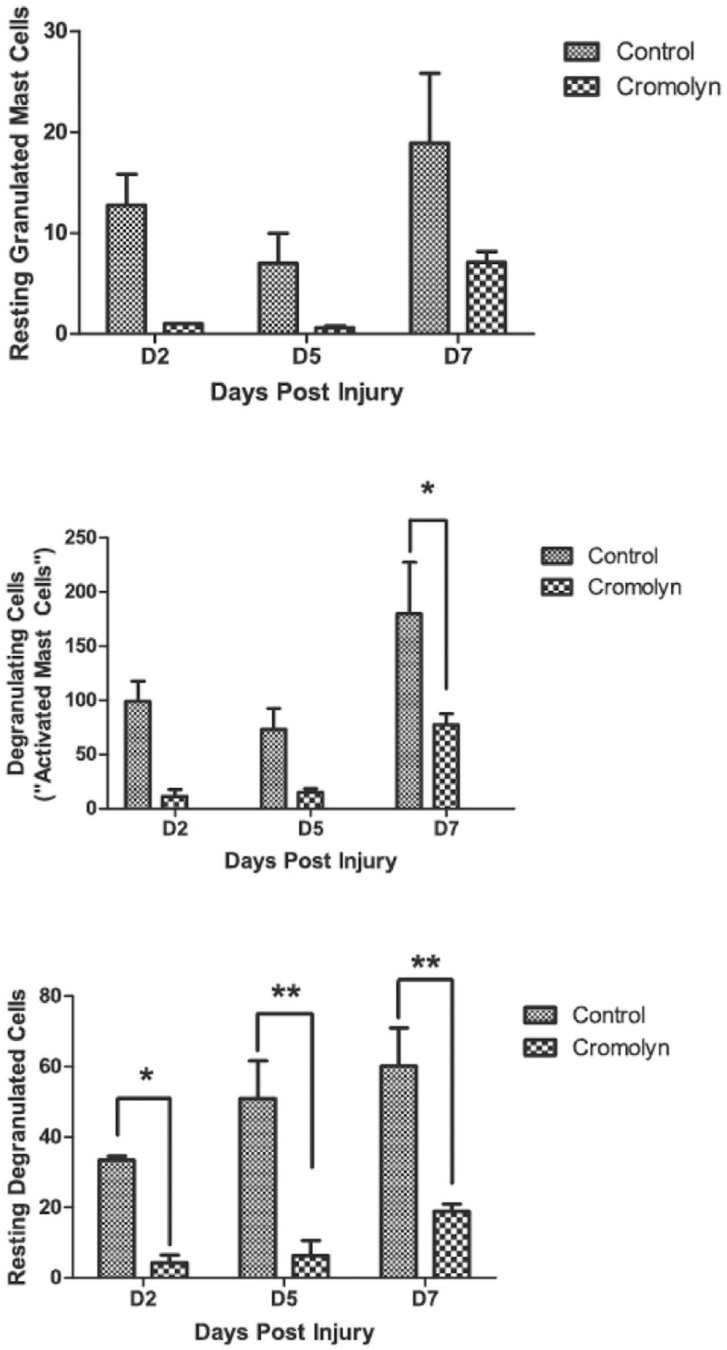

Fig. 6.

Quantification of mast cell subtypes in cromolyn-treated caACVR1 mice. Data is shown for days two, five, and seven after muscle injury. *, $p < 0.5$; **, $p < 0.01$; D, day.

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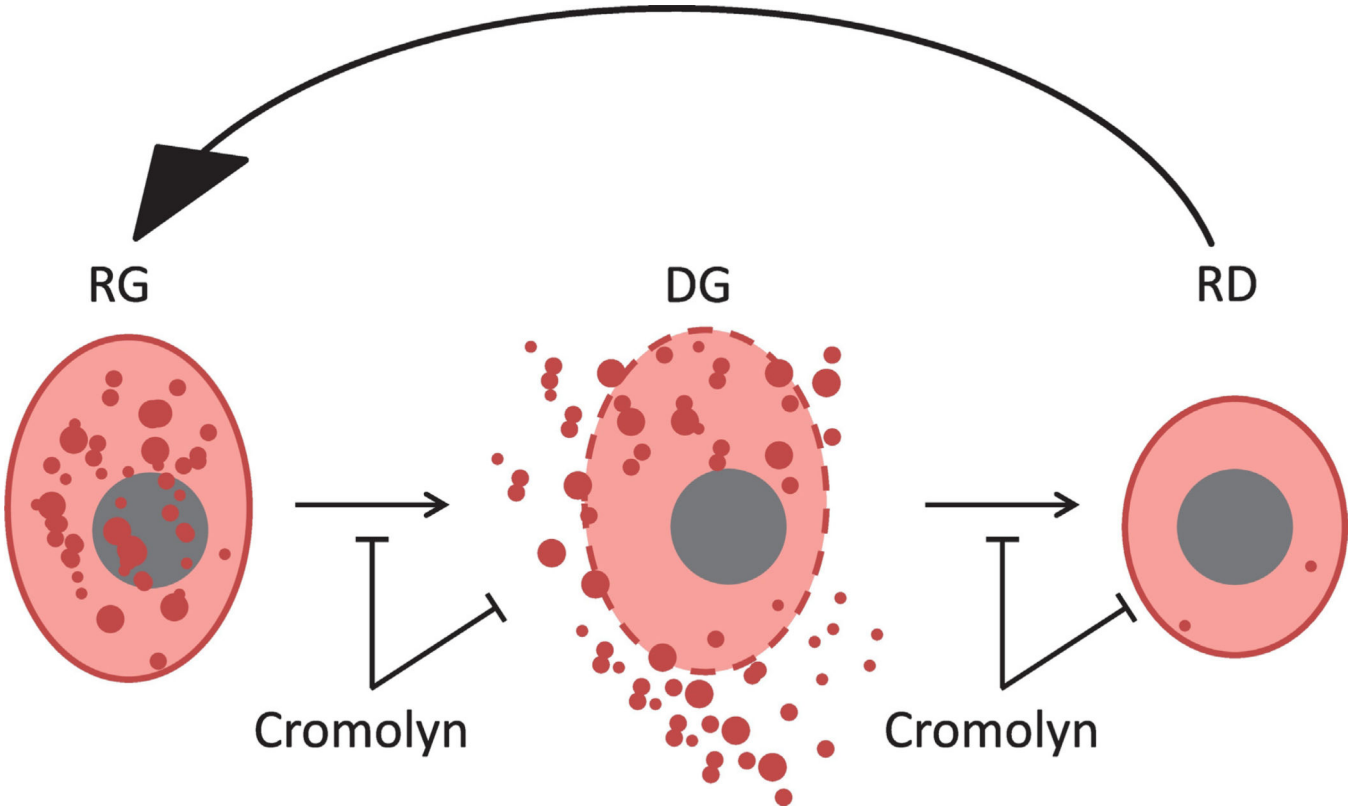

Fig. 7.

Schema showing cellular mechanisms of mast cell inhibition by cromolyn. Note that cromolyn antagonizes both the activation of mast cells (DG) from RG cells as well as the production of RD cells, preventing the regeneration of RG cells. Cromolyn may also act by directly inhibiting or removing DG and RG cells from the regeneration cycle. RG, resting granulated; DG, degranulating; RD, resting degranulated (RD).