Heparin-containing Hydrogels for Sustained Release of Cathepsin Inhibitors for Treatment of Tendon Degeneration

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Introduction: Overuse of the rotator cuff, particularly the supraspinatus tendon (SST), may lead to degenerative changes, caused by a host of cell-mediated responses. The design of injectable controlled drug release vehicles for protease inhibitors can complement current surgical reconstruction techniques of the rotator cuff, which fail to address the underlying pathological causes of tendon disorders. We investigated the activity of cysteine cathepsins, potent collagenases, in a rat SST overuse model and used this to inform the design of a heparin-containing, hydrolytically degradable hydrogel system for the release of a model protein and, finally, the cathepsin inhibitor cystatin C. To further evaluate the release system, the bioactivity of released cystatin C was evaluated for up to 12 days.

Methods: Dahl salt resistant rats (330±20g initial weight) were subjected to a daily downhill running regime[1] for 4 or 8 weeks (n=6) in order to develop an overuse injury in the SST. Age-matched rats allowed cage activity served as controls (n=6). For cathepsin zymography, SST samples were homogenized in lysis buffer and the supernatants were collected. Samples in non-reducing loading buffer were loaded into 0.2% gelatin SDS-polyacrylamide gels and resolved[2]. Gels were renatured and incubated for 17h in pH 4 activating buffer at 37°C. Thereafter, gels were stained with Coomassie Blue, less-dense bands analyzed by densitometry using ImageJ (NIH) and values were normalized to the cathepsin V band within the same gel. Data were analyzed by t-tests.

Heparin methacrylamide (hep MAm), PEG-diacrylate (PEG-DA, M_w ~ 3,400), and oligo[poly(ethylene glycol) fumarate] (OPF 10K, M_w ~ 10,000) were synthesized. Hep MAm was incorporated at 5 and 10wt% with PEG-DA and OPF to comprise a hydrogel of 10% initial gel weight. The mixture of macromers was dissolved in buffer, 10mM dithiothreitol (DTT), and 0.05 wt% D2959 (Irgacure). For model protein release, histone was mixed into each hydrogel solution. Solutions were dispensed in 30µL aliquots into molds and crosslinked (365nm, 15mW/cm^2). Each hydrogel, containing 41µg of histone, was placed in PBS and 200µL samples were removed and exchanged at 3h, daily for 4d and weekly for 4w. Samples were analyzed with a BCA kit.

A longer release formulation was chosen to encapsulate 54µg cystatin/hydrogel. Complete volume of supernatant was collected at 3h, 4d, 8d, and 12d. Supernatant was analyzed for cystatin C bioactivity by incubating samples with 50µg/mL DQ gelatin (Invitrogen) and 5pmol of cathepsin K. Fluorescent values (ex 488nm/em 525nm) were recorded at 3h. All values were corrected for a no enzyme, gelatin-only control.

Results: Histology demonstrated slight damage, evidenced by fiber disorganization, in 8-week overused tendons compared to controls (data not shown). Cathepsin zymography performed on 4-week samples revealed high molecular weight (50-100kDa) bands within the gels that represent cathepsin K bound to extracellular matrix. Analysis showed that the SST of the overuse rats exhibited greater than 400% increase in cathepsin K activity compared to the control (Fig. 1b). Furthermore, cathepsin L activity was ~180% higher in overused SSTs than controls (Fig. 1c).

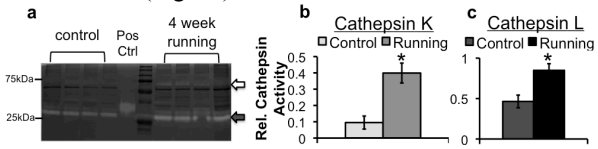

Fig. 1. (a) Zymography in control and overused tendon. Overused group had higher cathepsin activity than controls for cathepsins (b) K and (c) L. n=3-4; mean ± s.d. ; * p<0.05

OPF, with a lower crosslinking density than PEG-DA, can decrease the hydrogel degradation time when incorporated at greater amounts. Hydrogels of 45wt.% OPF hydrolytically degraded within 3d (5% hep MAm) or 4d (10% hep MAm). These hydrogels released 49-64% of the encapsulated histone. Hydrogels with 30wt.% OPF showed a prolonged release over 26d (5% hep MAm) and 23d (10% hep MAm), with 47-54% of histone released (Fig. 2). Hydrogels with 30wt.% OPF released less histone than the faster degrading formulations at 3h. At the time of degradation, the 10% hep MAm with 30% OPF released more cumulative histone than the faster degrading formulations (Fig. 2).

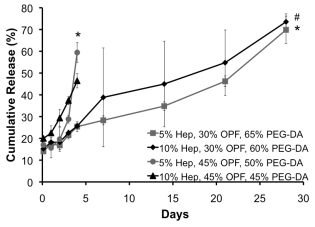

Fig. 2. Cumulative release of histone from degradable, hep MAm hydrogels. * significantly different than ▲; # significantly different cumulative release % than ▲ and ● at time of degradation; p<0.05

Heparin-containing hydrogels can electrostatically interact with cystatin C, and during degradation, release bioactive cystatin. Supernatant containing released cystatin collected over 12 days inhibited cathepsin K-mediated gelatin cleavage by more than 67% of the no inhibitor control (data not shown).

Conclusions: Our results show that cathepsin activity increases in the SST by 4 weeks of overuse. This finding provides a potential drug target that has been so far unexplored in tendon disorders. The *in vivo* studies also informed the development of a tunable hydrogel carrier that, depending on the original formulation, is hydrolytically degradable over a similar time scale as the development of tendon tissue degeneration. The sustained release of a bioactive cathepsin inhibitor from this injectable hydrogel suggests that this material is an exciting potential drug delivery system to treat degenerative tendon diseases.

Acknowledgements: NFL Charities, Regenerative Engineering and Medicine Center (GT/Emory) **References:** [1]Soslowsky et al. *J Shoulder Elbow Surg.* 2000; 9(2): 79-84, [2]Wilder et al. *Arch Biochem Biophys.* 2011; 516(1): 52-57