DOES AN ALTERED INFLAMMATORY RESPONSE HAVE A ROLE IN DELAYED MASSETER MUSCLE REPAIR?

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To my husband, Adam, who supported me tirelessly with love and understanding throughout the adventure of receiving my post-graduate education

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOI	Area of interest
EIMD	Exercise induced muscle damage
FGF	Fibroblastic growth factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HGF	Hepatocyte growth factor
IFN	Interferon
IL	Interleukin
IP	Interferon gamma induced protein
KC	Keratinocyte chemoattractant
LIF	Leukemia inhibitory factor
LSD	Least significant difference
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PEMS	Post-exercise muscle soreness
RANTES	Regulated upon activation, normal T cell expressed and secreted
SCF	Stem cell factor

TA	Tibialis anterior muscle
TGF	Transforming growth factor
TMD	Temporomandibular disorders
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Abstract of Thesis Presented to the Graduate School
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Background - Temporomandibular disorders (TMD) are a prevalent orofacial pain condition and consist predominately of muscle pain that can have a profound negative impact on those affected. Parafunctional habits, a behavior that is commonly associated with TMD, may cause muscle pain and this pain may be due to exercise induced muscle damage in the masticatory muscles. Inflammation plays a crucial role in promoting tissue repair following injury, but a balance between inflammatory cell populations and the inflammatory mediators released is required for the normal healing process to occur. The differences in the healing process observed between masseter and tibialis anterior muscles could be the result of a varied inflammatory process within masseter. Mast cells appear to play a major role in this variation, causing delayed healing and increasing fibrotic tissue through the release of various mediators. *Purpose* – (1) To quantify the differences in inflammatory mediators present in the masseter muscle and the tibialis anterior muscle following standardized muscle freeze injury over the first seven days of healing; and (2) To determine whether healing is improved in the masseter muscle with daily injections of a mast cell-stabilizer following muscle injury. *Methods* - Fifty-two female CD-1 mice, 6 weeks of age, were allocated to the following

groups: masseter muscle injury plus saline injections, masseter muscle injury plus mast cell-stabilizer injections, tibialis anterior muscle injury plus saline, and unmanipulated control. A cryoprobe was applied to the surface of the muscle for 5 seconds causing a standardized 5 mm freeze injury in posterior superficial masseter or tibialis anterior and the region of injury was harvested after 1, 4 and 7 days of repair. The Milliplex ™ Mouse Cytokine/Chemokine Kit, 96 well plate assay was used to detect 32 different cytokines in the injured muscles and controls at each time point. Histological analysis of repair in the three groups 7 days post injury determined how well the muscle healed by measuring areas of inflammatory exudate. Differences between groups and across time were analyzed using parametric statistics (ANOVA, LSD test). *Results* – The area of inflammatory exudate in the injured tibialis anterior muscle at 7 days post injury more closely approximated that of masseter muscle with cromolyn injections. Significant changes in cytokine/chemokine expression were most pronounced during repair of tibialis anterior, followed by expression in the masseter+cromolyn group. The masseter+PBS group exhibited no statistically significant changes in expression of any cytokine/chemokine examined at any time point. The majority of the cytokines/chemokines that varied across time were related to macrophage activation, suggesting the important role of macrophages in the inflammatory process leading to repair. *Conclusion* - When compared to tibialis anterior muscle, the masseter muscle exhibits a blunted cytokine/chemokine response that is enhanced with the administration of cromolyn. The expression of cytokines/chemokines that affect the migration and activation of macrophages are increased during muscle repair in tibialis anterior but not in repair of masseter.

CHAPTER 1
INTRODUCTION

Pain associated with temporomandibular disorders (TMD) is the most common chronic orofacial pain condition, and it affects between eight and fifteen percent of women and three to ten percent of men.[1] Muscle pain is a frequent characteristic of temporomandibular disorders, and the masseter muscle has been implicated in nearly sixty percent of cases.[2] The basis for muscle pain associated with temporomandibular disorders could be linked to exercise induced muscle damage (EIMD) which follows increased muscle contraction, as in bruxism or clenching. Masticatory muscle pain affects women three times more than men.[3] The explanation for a gender difference in the prevalence of muscle pain conditions in women appears to have a hormonal basis.[4,5,6] In addition to hormonal differences in pain perception, the role of inflammation in the healing of damaged muscle is crucial to understand. The masseter muscle has been found to have a significant delay in healing compared to limb muscle such as the tibialis anterior (TA).[7] Morris-Wiman and Widmer have identified an increased number of mast cells in the masseter muscle after injury and mast cells could influence the decreased healing response exhibited by the masseter.[8] This study has been designed to determine whether or not an altered inflammatory response has a role in delayed masseter muscle repair.

Temporomandibular Disorders, Bruxism, and EIMD

Temporomandibular disorders is a term used to describe musculoskeletal conditions in the temporomandibular region involving pain in the muscles of mastication (myofascial pain), in the joint itself (arthralgia), or in both.[1] Bruxism is considered a risk factor, with an odds ratio of up to 4.8, for TMD myofascial pain with or without

arthralgia.[9,10] The high level of concentric and eccentric muscle contractions that occur during parafunctional habits and bruxism could cause exercise induced muscle damage (EIMD). During nocturnal bruxism there is a sustained, rhythmic masticatory muscle activity, and at times both jaw openers and jaw closers are co-contracting.[11]. The increased intensity of the jaw closers (concentric contraction) and the co-activation of the jaw openers (eccentric contraction) could theoretically cause muscle damage. Muscle pain following exercise is referred to as delayed onset or postexercise muscle soreness (PEMS) and has been associated with EIMD. Abnormal muscle activity, such as nocturnal bruxism, probably causes some form of PEMS in certain individuals. PEMS is presumably caused by the inflammatory events that accompany the mechanical breakdown of myofibrils and connective tissue in the muscle.[12] Initial injury to the muscle and the accompanying inflammation and pain may form a basis for muscle pain associated with TMD.

Gender Differences in Muscle Pain

Females exhibit muscle pain conditions more frequently than males, and the basis for this increased prevalence in females is not completely understood. In a study examining jaw pain induced by clenching, females exhibited a different response than males to exertional pain by having increased pain twenty-four hours later.[13] One study found chewing increased masticatory muscle pain not only in patients diagnosed with temporomandibular disorders but also in female controls.[14] This shows the increased susceptibility of females to muscle pain. Gender differences in muscle pain conditions are not fully understood, but it appears sex hormones could play a significant role.[4,5,6] It has been suggested that estrogen plays a significant role in the healing of damaged muscle by protecting against further damage, but the exact mechanisms by which this

occurs have yet to be determined.[5] It is also possible that the increased prevalence for muscle pain in women could be due to the influence of estrogen or progesterone on pain perception.[15] The literature is inconclusive on the exact role of estrogen or progesterone, but the studies have shown that these hormones impact pain perception. This could potentially explain the increased prevalence of muscle pain in females versus males.

Inflammatory Response to Muscle Damage

Tissue injury induces inflammation in order to protect the body, and the inflammatory response involves a cascade of events. After tissue injury macrophages, mast cells, fibroblasts, and injured muscle fibers release cytokines, chemokines, and growth factors. Tumor necrosis factor (TNF-α), various interleukins, and monocyte chemoattractant protein (MCP-1) are important inflammatory mediators released following muscle injury.[16,17] TNF-α is involved in the acute phase reaction of inflammation, and it serves to regulate immune cells by promoting the accumulation of neutrophils and macrophages in skeletal muscle.[18] Recovery of function in injured muscle has also been shown to be impacted by TNF-α, which could be controlling expression of muscle regulatory genes, including MyoD.[19] In addition, TNF-α also upregulates class I major histocompatibility complex (MHC) in myoblasts and regenerating myofibers. This could cause the myoblasts and myofibers to become a target for inflammatory cells, which would delay healing in the muscle.[20] Interleukin 1β (IL-1β) and Interleukin 6 (IL-6) are key proinflammatory cytokines that are also involved in the acute phase reaction. IL-6 has been shown to increase significantly following eccentric exercise.[21] Circulating IL-6 has even been shown to exert an anti-inflammatory effect by inducing the production of anti-inflammatory cytokines and

reducing circulating TNF-α.[22] MCP-1 is a chemokine that guides the migration and activation of macrophages in damaged tissue. Impaired muscle repair in MCP-1 deficient mice suggests macrophages and MCP-1 are required for a normal healing response in muscle.[23] The ability of damaged myofibers to express a variety of cytokines including TNF-α, IL-1β, and IL-6, as well as MCP-1 could prolong the inflammatory response and cause further injury.[24] The inflammatory mediators that are released, which is dependent on the type of muscle injury, serve to control the healing process of the tissue.[17]

One of the main objectives of inflammation is to clear cellular debris and prepare the tissue for regeneration.[25] Following the initial injury the tissue is infiltrated by various inflammatory cells, including neutrophils, macrophages, and mast cells. Neutrophils have been shown to quickly respond to muscle damage and arrive in the tissue within one hour of increased muscle use. The neutrophil population can remain elevated within the muscle tissue for up to five days and can either increase tissue injury or promote tissue repair depending on the inflammatory mediators released.[17] It has been shown that following muscle injury neutrophils seem to contribute to muscle injury and may impede the healing process.[26] Macrophage populations also increase following injury, with the initial population promoting tissue injury and the subsequent population promoting tissue repair.[27] Following injury, degranulation of resident mast cells provides an instantaneous source of preformed mediators.[28] Mast cells accumulate in injured tissue even if the injury is minor and could prolong an acute inflammation leading to chronic inflammation.[29] Mast cells serve as a source of cytokines and inflammatory mediators that serve to promote inflammation in the

damaged tissue. The presence of mast cells has been shown to be imperative for neutrophil recruitment and normal tissue repair.[30] Mast cells have also been shown to be a factor in tissue necrosis. The delicate balance and timing of neutrophils, macrophages, and mast cells determines whether or not these cells cause increased damage or serve to promote healing. The difference in the proportion and timing of these cells in masseter and limb muscle could provide the cellular foundation for the differences in healing.

Healing and the Influence of Inflammation

The process by which healing and regeneration occurs in muscle involves activation of satellite cells. Satellite cells are dormant muscle precursor cells that reside between the basal lamina of muscle fibers and the sarcolemma. Satellite cells are formed late in fetal development by the resident progenitor population. Satellite cells are characterized by the expression of Pax 7, a gene important for satellite cell specification.[31,32] Upon injury to the muscle, satellite cells are activated by several factors, the most prominent of which is hepatocyte growth factor (HGF).[33] Satellite cells differentiate to myoblasts and fuse to form new myofibers, or they fuse to damaged myofibers. An important characteristic of satellite cells is self-renewal through asymmetric division, thus the healing potential of muscle is not decreased with additional injury. The inflammatory mediator Interleukin 4, IL-4, is a potential myoblast recruitment factor that contributes to the formation of myotubes.[34] Muscle precursor cells can also differentiate into myofibroblasts during regeneration leading to the formation of scar tissue, which delays rehabilitation of the muscle. Fibroblasts are activated by factors released due to muscle damage, which could also cause increased

fibrous repair. Preventing fibrosis in healing muscle would therefore involve the prevention of the activation of fibroblasts that cause scar formation.

Delayed Healing in Masseter Muscle

The masseter muscle exhibits delayed healing when compared to tibialis anterior muscle.[7] The basis for this difference in healing is not completely understood. It has been shown that in order for effective muscle regeneration to take place necrotic tissue must be removed.[35] If phagocytosis is incomplete, muscle regeneration is unsuccessful.[36] Masticatory muscles have a different embryonic origin than limb muscles, which develop from somites. Masticatory muscles develop from somitomeres, presomitic mesoderm located adjacent to the embryonic brain. Differences in the number and proliferative rate of myoblasts in masseter compared to tibialis anterior may be responsible for differences in regeneration.[7]

It is possible that differences in the inflammatory process are also responsible for delayed healing in masseter, and preliminary data has shown one of the most significant differences between masseter and tibialis anterior following injury is the increased number of mast cells in masseter.[8] Mast cells exhibit different phenotypes depending on the local environment, and this affects cytokine expression and functional differences.[37] Chronic inflammatory conditions (e.g. psoriasis, rheumatoid arthritis, allergy, parasite infection, and inflammatory bowel disease) and fibrotic disorders (e.g. pulmonary fibrosis, liver cirrhosis, and Crohn's disease) are characterized by mast cell hyperplasia.

Mast cells are a source of a variety of cytokines, chemokines, growth factors, and matrix metalloproteinases (MMPs). Mast cells also control extracellular matrix regeneration and potentially fibrosis through the release of pro-fibrotic cytokines,

including fibroblastic growth factor (FGF-2) and IL-4.[38] Disodium cromoglycate, cromolyn, stabilizes mast cells by impeding calcium mobilization and degranulation.[39] It has been shown that blocking degranulation of mast cells through cromolyn administration decreases the number of neutrophils in injured muscle.[30] This highlights the important role mast cells play in the muscular inflammatory response. It has also been shown that preventing mast cell degranulation through daily cromolyn injections can decrease muscle fiber necrosis.[40]

Summary

Temporomandibular disorders are a prevalent orofacial pain condition, and the muscle pain associated with TMD can have a profound negative impact on the quality of life for those affected. Women have a higher tendency to develop myofascial pain with TMD, but the explanation for this observation is not completely understood. Estrogen appears to play a role in both inflammatory events within muscle and pain perception, which could explain the gender differences detected in myofascial pain. Inflammation plays a crucial role in promoting tissue repair following injury, but a balance between inflammatory cell populations and the inflammatory mediators released is required for the normal healing process to occur. Shifting the balance can cause increased injury, prolonged healing, or fibrous repair. The differences in the healing process observed between masseter and tibialis anterior muscles could be the result of a varied inflammatory process within masseter. Mast cells appear to play a major role in this variation, causing delayed healing and increasing fibrotic tissue through the release of various mediators. Analysis of the inflammatory mediators present following a standardized injury to the masseter muscle and the tibialis anterior muscle will lead to a better understanding of the inflammatory process occurring in masseter versus tibialis

anterior. Quantifying the differences in cytokines present following muscle injury between the masseter muscle and the tibialis anterior muscle would help explain the reasons for different proportions of mast cells and diverse healing in the two muscles.

Significance

The biological basis and the etiology of myalgia associated with temporomandibular disorders are inadequately explained by the present information available. Muscle pain in the jaw closer muscles is frequently associated with TMD, with the masseter muscle more highly affected. At this time, treatment for muscle pain conditions is palliative, and it does not address the underlying muscle damage associated with muscle injury. Models of muscle injury to help determine the molecular basis of inflammation would be beneficial in explaining the biological basis of myalgia. The masseter muscle has been shown to have a varied healing response following muscle injury. An increased number of mast cells has been found in the masseter following injury, potentially explaining the delayed healing response. Defining the inflammatory mediators present following muscle injury could account for the presence of an increased number of mast cells in the masseter muscle. With a better understanding of the inflammatory process, mast cells could serve as a potential target for treatment of myalgia in jaw closing muscles. The effect cromolyn has on muscle inflammation following injury will help determine if it is a prospective therapeutic for muscle pain conditions.

Hypotheses

1. The masseter muscle will exhibit an altered cytokine/chemokine expression compared to tibialis anterior, which will help explain the different inflammatory cell

profile exhibited by the masseter, particularly the increased number of mast cells, and delayed muscle repair following injury.

2. Daily systemic injections of cromolyn (a mast cell stabilizer) will improve the healing of the masseter muscle. A difference will be evident in the cytokine/chemokine profile between masseter muscle injected with cromolyn and masseter muscle injected with saline.

Specific Aims

1. Evaluate the expression of inflammatory mediators following muscle injury to determine if a varied inflammatory profile, temporally and/or quantitatively, exists between the masseter muscle and the tibialis anterior muscle that could help to explain the differences in healing exhibited by these two muscles.

2. Improve the understanding of the role of mast cells in muscle inflammation following injury. Determine if cromolyn has an effect on inflammatory mediators in injured muscle and determine its potential as a therapeutic agent for myalgia associated with TMD.

CHAPTER 2
MATERIALS AND METHODS

Animals

Fifty-two female CD-1 mice, six weeks of age, were used in this study. The mice were allocated to three groups (Table 2-1). Sample size was calculated based on data from previous studies examining immunofluorescent-labeling of inflammatory cells in masseter and tibialis anterior muscles after injury. In those studies, an effect size of 1.5 or greater was observed for mast cell numbers. Therefore, using the parameters of an effect size of 1.5, a power of 0.80, and an alpha level of 0.05, we required 4 subjects per group. The cromolyn and physiological saline (PBS) injections were given systemically at the time of the muscle injury and each subsequent day until sacrifice. The dose of cromolyn was 100 mg/kg in 0.1 ml IP. PBS (0.1 ml) was injected IP in masseter control animals and in the tibialis anterior animal group. Mice were anesthetized using ketamine (10-14 mg/kg body weight) and xylazine (70-80 mg/kg body weight). A small incision approximately 3 mm in length was made in the tissue overlying the posterior portion of the superficial layer of the masseter muscle or the central portion of the tibialis anterior muscle. A Keeler Ophthalmic cryoprobe (Broomall, PA) was applied to the surface of the muscle for 5 seconds causing a standardized 5 mm freeze injury in posterior superficial masseter or tibialis anterior. The probe was placed midway on the posterior portion of the superficial layer of the masseter muscle, between the tendon of the superficial masseter and the posterior attachment of the masseter to the mandible. The probe was placed in the center of the tibialis anterior muscle. Skin incisions were closed using surgical glue, cyanoacrylate. The mice were sacrificed at 1, 4, or 7 days post-injury (Figure 2-1) and the area of muscle injury was

harvested using a standardized 3 mm punch biopsy. Biopsies were placed in 1.5 ml tubes and snap-frozen in acetone-cooled isopentane for analysis. For histological analyses on day seven (T7), whole masseter and tibialis anterior muscles were harvested, orientated on cardboard, embedded in Fisher TissuePrep and snap-frozen in acetone-cooled isopentane.

Cytokine/Chemokine Analysis

The Milliplex ™ MAP Mouse Cytokine/Chemokine 32-plex kit was used for the simultaneous detection and quantification of 32 cytokines and chemokines (Table 2-2) in injured muscle and control samples. Protein was extracted from 3 mm biopsy muscle samples using Invitrogen™ Tissue Extraction Reagent I according to manufacturer's instructions. Briefly, the reagent was warmed to room temperature and mixed well. Protease inhibitor cocktail (10 µl, Halt Protease Inhibitor, 100x, Thermo Scientific) was added to 1 ml of Tissue Extraction Reagent I just prior to use. Tissue Extraction Reagent I (200 µl) was added to each biopsy specimen. This amount was found to allow adequate extraction of protein from the 3 mm biopsy samples which ranged from 7 to 10 mg in weight. The samples were homogenized in the reagent using a battery-powered pestle and then centrifuged at 10,000 RPM for 5 minutes to pellet the tissue debris. The supernatant was collected and frozen. A 10 µl aliquot of each sample was used to determine protein concentration using the Pierce® BCA Protein Assay Kit according to manufacturer's instructions. A 35 µl aliquot of each sample was sent to Millipore Assay services for analysis utilizing 96 well multiplex Luminex xMAP technology. In the multiplex Luminex® assay, multiplex capture antibodies are attached to 5.6 micron polystyrene beads internally dyed with red and infrared fluorophores of differing intensities. After an analyte from a test sample is captured by the bead or

microsphere, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. With the Luminex® 200™ detection system, the microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter. Millipore Assay services provided an excel spreadsheet of cytokine/chemokine concentrations for each sample as well as the precision values, MFI (mean fluorescent values) and standard curves from which these concentrations were derived. The mean amount of each cytokine was calculated for the T0 samples. In order to normalize the data, concentration values for each individual cytokine sample were divided by the respective mean T0 value. The raw data and the normalized data were statistically analyzed. *Statistical Analyses*: Differences between groups and across time were analyzed with a 2 way ANOVA followed by post-hoc testing using the LSD test when appropriate. A probability level of 0.05 was used for all statistical tests.

Histological Evaluations

Frontal cryosections (14 μm thick) of muscle samples from the three groups: TA+PBS; masseter+PBS; masseter+cromolyn, at 7 days post-injury were stained with Mayers Hematoxylin. Sections were viewed on a Nikon FXA microscope under brightfield and images acquired through the region of greatest muscle damage using an Mrc5 Zeiss digital camera and AxioVision (Zeiss) software. Collages of the damaged regions were constructed using Photoshop CS2. Using Image Pro software the areas of damage on each collage were outlined, specifying the area of interest (AOI). A

standardized square AOI (10,000μm^2) was placed in the uninjured area of the section. A segmentation procedure was then performed in order to determine the cellularity within each AOI. The histogram based segmentation procedure involved separating the inflammatory exudate pixels from the background pixels in each image based on color range. The background pixels were then set as black and the range of color intensities to be emphasized were set as white. Once the segmentation procedure was performed, a mask was applied and a 2 pass median filter, 5x5 kernel was completed. A median filter is used to remove random high impulse noise (spots or points that vary significantly from the background) by replacing the center pixel with the median value in its neighborhood. *Statistical Analyses*: Differences between groups were analyzed with an ANOVA followed by post-hoc testing using the LSD test when appropriate. A probability level of 0.05 was used for all statistical tests.

Table 2-1. Number of mice examined for each group/timepoint.

	Cytokine/Chemokine Analyses				Histology
Injury Groups	*T0 (Uninjured)*	*T1*	*T4*	*T7*	*T7*
Masseter plus PBS	4	4	4	4	4
Masseter plus cromolyn		4	4	4	4
Tibialis Anterior plus PBS	0 (4 TA samples from same mice as above)	4	4	4	4

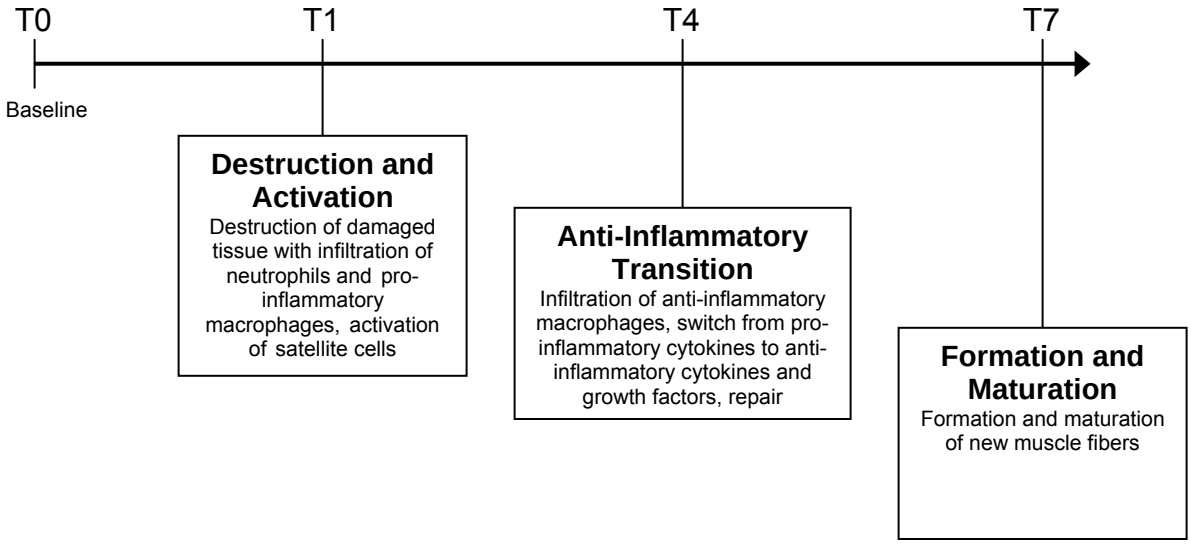


Figure 2-1. Timeline of major events.

Table 2-2. Cytokines/chemokines examined.

Hematopoietic Agents
G-CSF
GM-CSF
M-CSF
IL-3
Pro-inflammatory Cytokines
IFN-γ
IL-1α
IL-1β
IL-2
IL-5
IL-6
IL-9
IL-12(p70)
IL-15
IL-17
LIF
TNF-α
Anti-inflammatory Cytokines
IL-4
IL-10
IL-12(p40)
IL-13
Chemokines
IP-10
MIP-2
KC
Eotaxin
LIX
MCP-1
MIP-1α
MIP-1β
MIG
RANTES
Growth Factors
VEGF
IL-7

CHAPTER 3
RESULTS

Histological Comparison of Muscle Damage

Examination of histological sections revealed that TA was more advanced in the healing process than masseter+PBS; muscle fiber sizes were more uniform and larger and the original muscle architecture had been restored. Healing in masseter+cromolyn muscle also appeared to be more advanced than in masseter+PBS and the histology of the repairing region resembled that observed in TA (Figure 3-1). The analysis of the area of muscle damage seven days post-injury in histological sections revealed statistically significant differences between the three groups examined: TA, masseter+PBS and masseter+cromolyn (ANOVA, $F = 5.0$, $p < 0.05$). Masseter+PBS exhibited a greater area of damage than masseter+cromolyn ($p = 0.04$, LSD test) and TA ($p < 0.02$, LSD test) (Figure 3-2A). The percentage of the damaged area occupied by an inflammatory exudate at seven days post-injury was also significantly different among the three groups (ANOVA, $F = 18.0$, $p < 0.001$) and was found to be significantly increased in masseter+PBS compared to masseter+cromolyn ($p < 0.01$, LSD test) and TA ($p < 0.002$, LSD test) (Figure 3-2B). The percentage of the area occupied by non-muscle cells in the uninjured standardized area was not statistically different across any of the groups (ANOVA, $F = 2.1$, $p = 0.18$) (Figure 3-2C).

Cytokines/Chemokines in Injured Muscle Compared to Control

Six cytokines/chemokines were not detected at any time point or were detected at levels below the standard range of the detection system at all times points (Table 3-1). Seven cytokines/chemokines were detected at levels below standards at T0 but had an

increased expression as a response to damage and repair. Nineteen cytokines/chemokines were detected at baseline and at all time points during repair.

Significant differences in baseline expression between uninjured masseter and TA were found for four cytokines/chemokines (ANOVA, $F=103$, $p < 0.01$; t-test, $p < 0.05$). IL-12(p70), IL-17 and Eotaxin all were observed to have a significantly increased expression in the masseter muscle when compared to the TA while VEGF had a significantly increased expression in TA compared to masseter. The four cytokines/chemokines were also found to significantly change over time (Table 3-1).

Ten cytokines/chemokines were determined to significantly change in expression over baseline levels during repair (Table 3-1).

One cytokine, VEGF, decreased significantly in expression at both time points, one and four days post-injury, from baseline (T0) in TA (Figure 3-3). VEGF expression did not vary between baseline (T0) and any time point during repair for either masseter group (masseter+PBS, masseter+cromolyn).

At twenty four hours post-injury (T1), the expression of G-CSF, KC and MCP-1 was significantly increased in TA but returned to baseline levels at day 4 (Figure 3-3). Expression of IL-12(p70) also was increased significantly in TA at T1 and its expression was further increased at T4 before returning to baseline at T7 (Figure 3-3). No significant differences in the expression of these cytokines were observed for either masseter group at any time point examined.

Four days post-injury (T4),the expression of cytokines/chemokines Eotaxin and IP-10 was increased in the masseter+cromolyn group but an increase was not observed for the TA or masseter+PBS groups (Figure 3-4). MIG was also significantly increased

at this time in both the TA and masseter+cromolyn groups (Figure 3-4). The two cytokines IL-4 and M-CSF were observed to be differentially expressed between the three groups at different time points.

IL-4 expression was significantly decreased in both TA and masseter+cromolyn at T1. Expression levels rebounded to baseline levels at T4 in masseter+cromolyn, but not TA. Expression of IL-4 in TA remained depressed until T7 where expression was increased over baseline levels. Expression of IL-4 showed no significant differences at any time point for masseter+PBS (Figure 3-4). M-CSF was determined to have a greater level of expression at baseline (T0) in TA than in either masseter group. Whereas, expression levels did not significantly change in either masseter group during repair, M-CSF expression decreased in TA at T1 and again at T4 until expression was increased to exceed baseline levels at T7 (Figure 3-4).

Cytokine/Chemokine Main Effects and Interactions

Values for cytokine/chemokine expression were normalized to baseline for each group (TA or masseter) to allow the comparison of cytokine/chemokine expression between the three groups (TA, masseter+PBS, and masseter+cromolyn) over time. Seven cytokines/chemokines were determined to have significant main effects in expression by day, by muscle group or by muscle group and day (Table 3-2).

Two cytokines/chemokines were shown to differ significantly by day with no significant interactions. Both KC and MCP-1 had an increased expression at T1 for all muscle groups (Fig 3-5).

Five cytokines/chemokines: G-CSF; M-CSF; IL-4; MIG and IL-12(p70), showed significant interactions of muscle by day. Expression of G-CSF by TA at T1 was significantly increased over that observed for either masseter group at T1 or for any

group at later repair time points (Fig 3-6). Interestingly, M-CSF expression by TA was significantly increased at T7 over that observed for either masseter group or TA at other time points (Fig 3-6). Differences were also observed at T4 between TA and both masseter groups for M-CSF. The expression of M-CSF remained relatively unchanged during repair in both masseter+PBS and masseter+cromolyn. In contrast to G-CSF and M-CSF, a decrease in expression at T4 was observed for IL-4 in TA that was not apparent in either masseter group or TA at other times points (Fig 3-6). MIG expression increased in both TA and masseter+cromolyn between T1 and T4 and, in both groups, expression levels returned to baseline at T7 (Fig 3-6). Significant differences in MIG levels were not observed between the TA and masseter+cromolyn groups at T4 but were present between both groups and masseter+PBS. No significant differences in MIG expression were detected in the masseter+PBS group during repair. Finally, IL-12(p70) expression was observed to be significantly increased at both T1 and T4 in TA compared to expression in either masseter group at all time points and TA at T7 (Fig 3-6).

Figure 3-1. Representative frontal cryosections (14 μm thick) of muscle samples stained with Mayers Hematoxylin from the three groups at 7 days post-injury. The area of damage is delineated by a dashed line. A) TA+PBS. B) Masseter+Cromolyn. C) Masseter+PBS. D) Higher magnification of TA+PBS. E) Higher magnification of Masseter+Cromolyn. F) Higher magnification of Masseter+PBS. In D and E, new muscle fibers (arrows), identified by their central nuclei, are uniform in size and that there is little cellular exudate within the damaged region. In F, new muscle fiber size is variable and regions between areas of new fibers are occupied by inflammatory exudate (arrow heads).

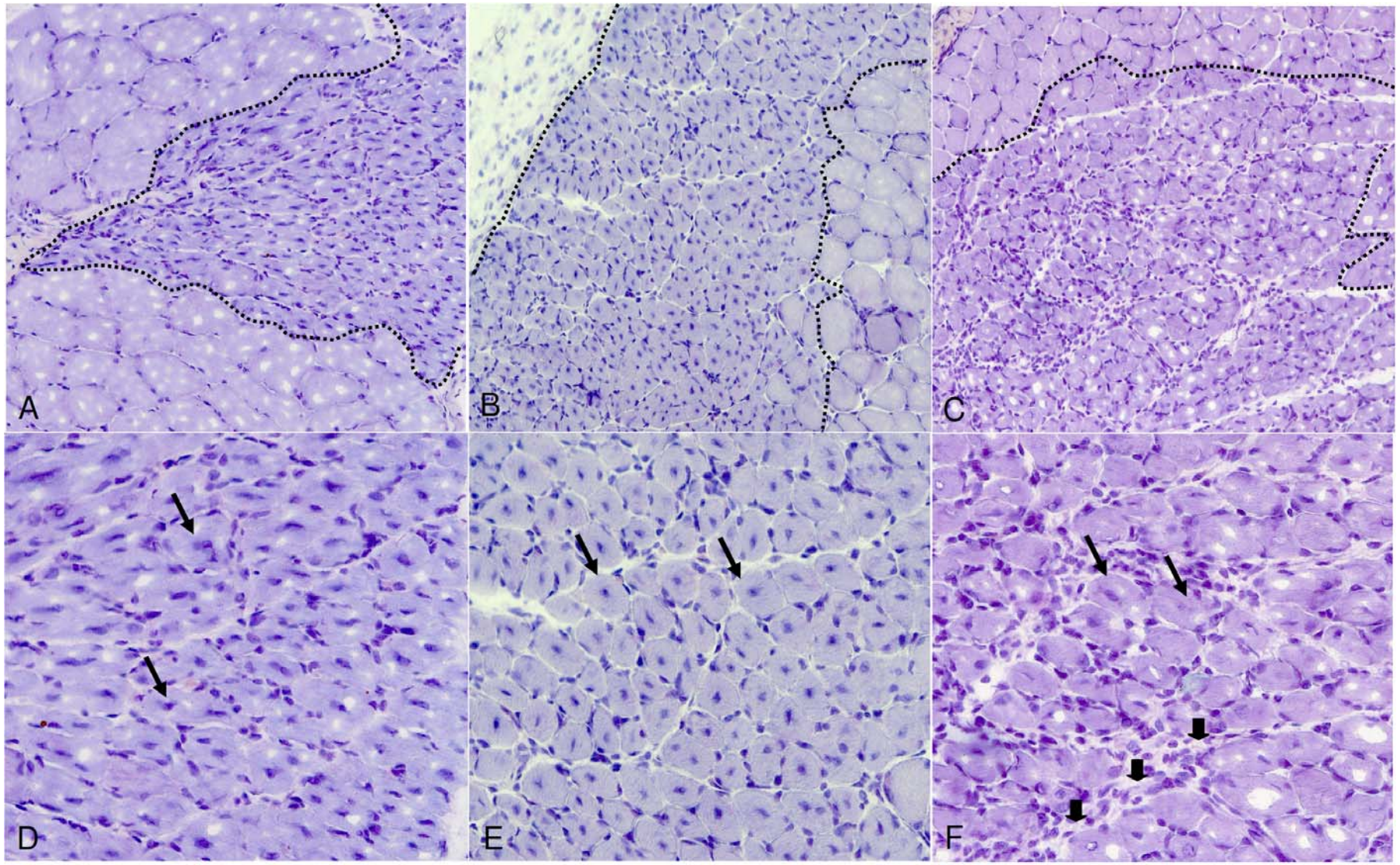
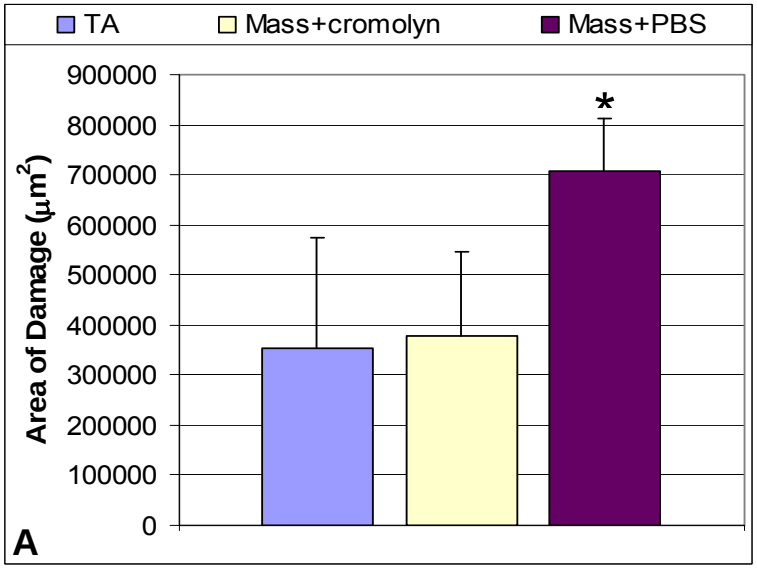
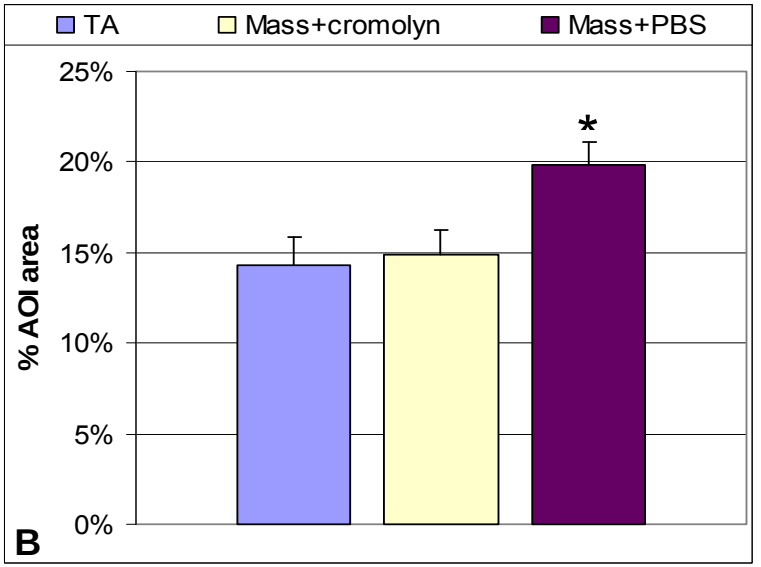

A
B
C
D
E
F

Figure 3-2. Comparison of the area of damage and the cellularity between collages of TA+PBS, Masseter+cromolyn, and Masseter+PBS frontal cryosections (14 μm thick). A) Significant differences were found in the area of damage between Mass+PBS and TA and Mass+cromolyn groups ($p<0.05$, ANOVA, post-hoc LSD test). B) A segmentation procedure was used to determine the percent of the damaged area containing inflammatory exudate in each image or cellularity in a standardized square area in the uninjured area of the section. Significant differences were observed between Mass+PBS and the TA and Mass+cromolyn groups ($p<0.05$, ANOVA, post-hoc LSD test). C) No differences were found in the cellularity of control areas between groups.


TA
Mass+cromolyn
Mass+PBS
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Area of Damage (μm²)
*
A


TA
Mass+cromolyn
Mass+PBS
25%
20%
15%
10%
5%
0%
% AOI area
*
B

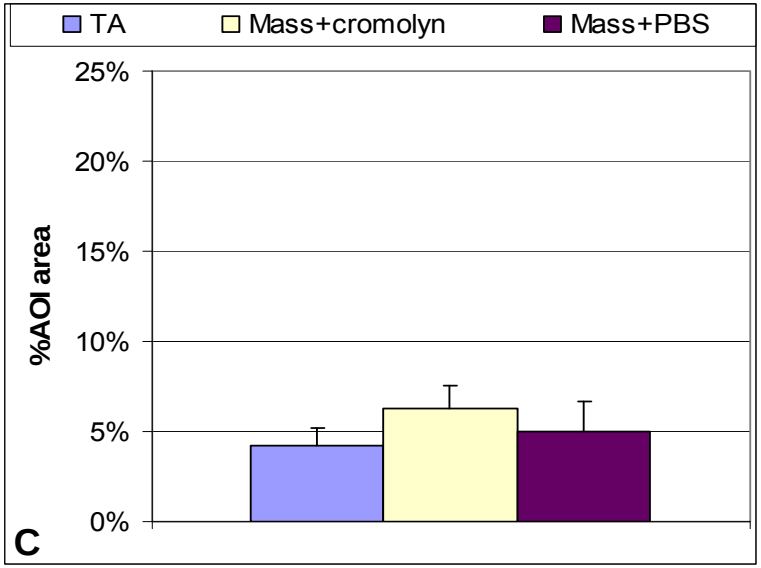

TA
Mass+cromolyn
Mass+PBS
25%
20%
15%
10%
5%
0%
%AOI area
C

Table 3-1. Characterization of cytokines/chemokines.

Cytokines/Chemokines Below Detectable Levels	Cytokines/Chemokines Not Detected at Baseline	Cytokines/Chemokines Detected Over Time
IL-3	G-CSF	GM-CSF
IL-7	IL-2	M-CSF
MIP-2	IL-13	IL-1α
LIF	MCP-1	IL-1β
MIP-1β	LIX	IL-5
TNF-α	IFN-γ	IL-6
	RANTES	IL-9
		IL-12p70
		IL-15
		IL-17
		IL-4
		IL-10
		IL-12p40
		IP-10
		KC
		Eotaxin
		MIP-1α
		MIG
		VEGF

Table 3-2. Cytokines and chemokines exhibiting main effects and interactions.

Cytokine/chemokine	F value	p value
KC	7.0	0.004
MCP-1	5.3	0.01
G-CSF	3.7	0.02
M-CSF	7.4	0.001
IL-4	2.7	0.05
MIG	3.0	0.04
IL-12(p70)	18.7	0.000001

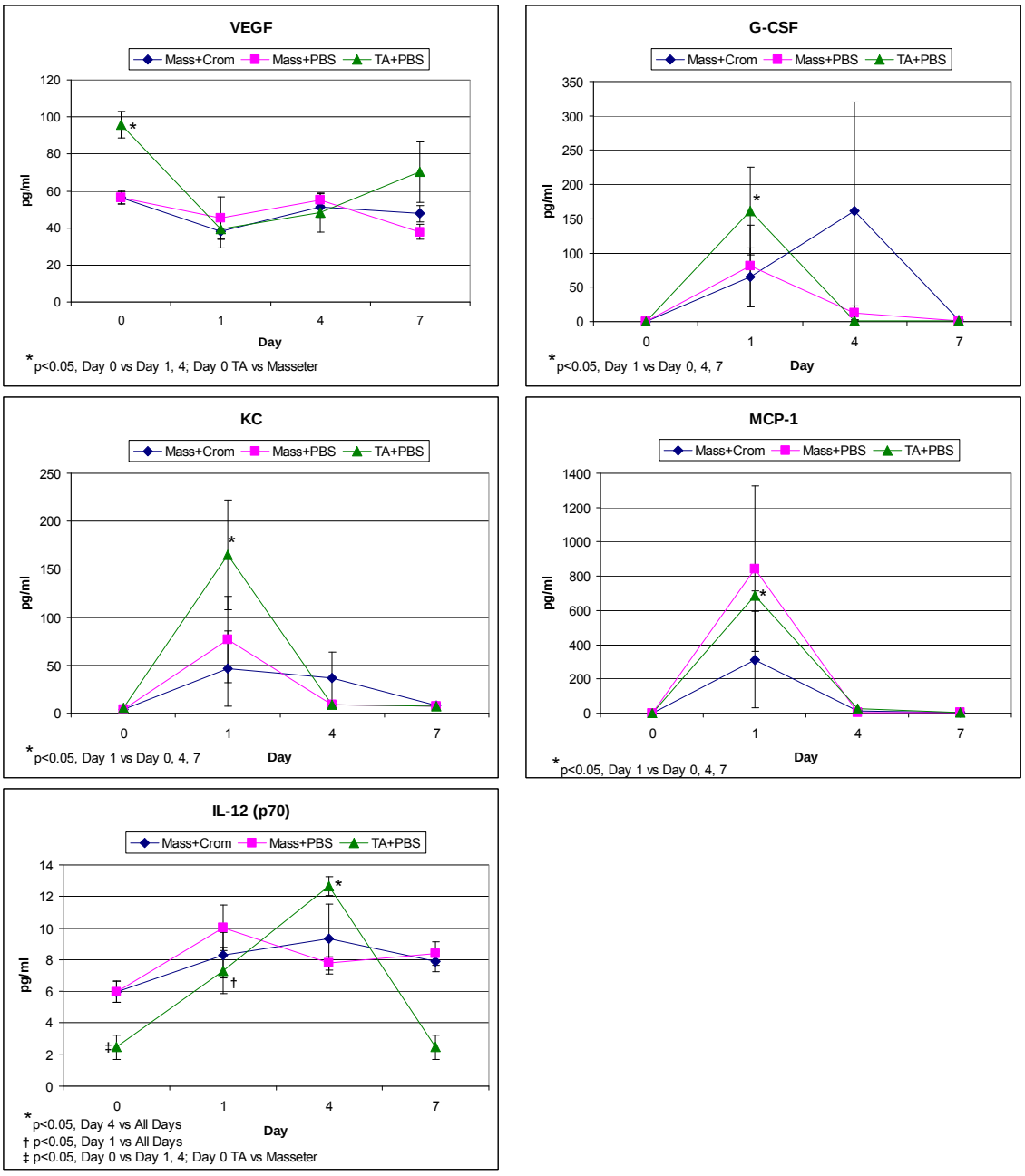


Figure 3-3. Significant differences in cytokine/chemokine expression detected within groups at baseline (T0) and/or during early stages of repair (T1).

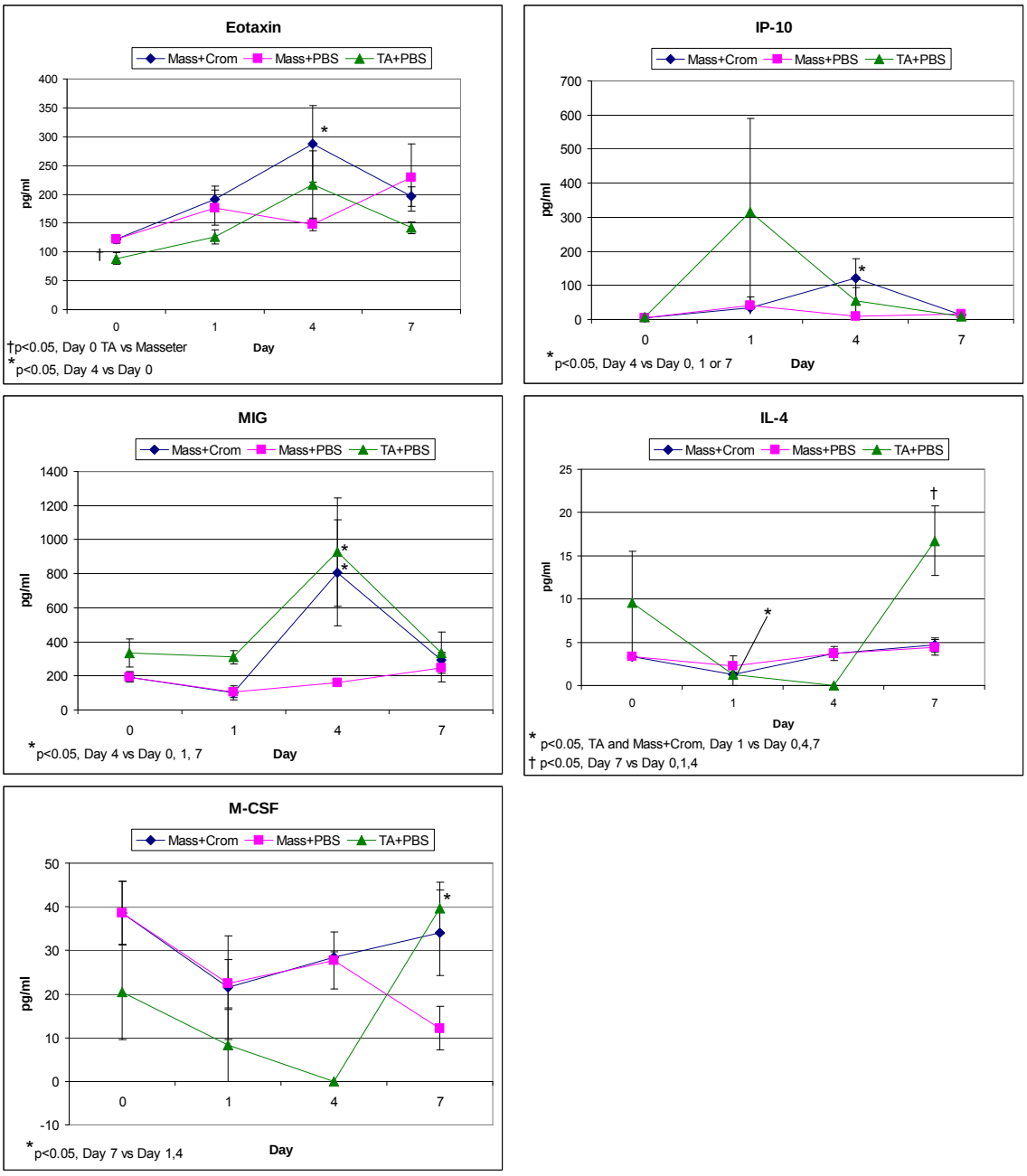
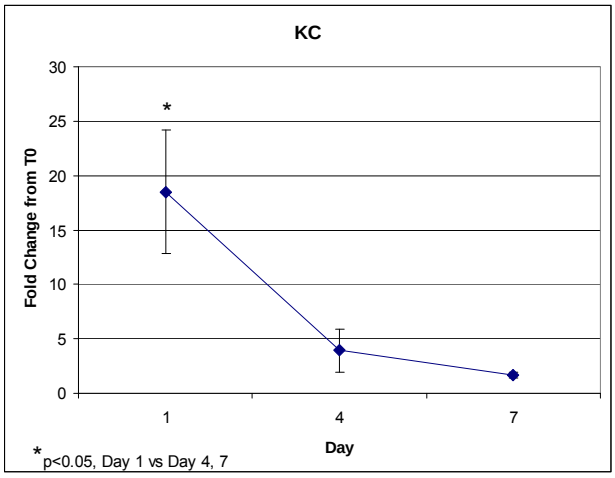


Figure 3-4. Significant differences in cytokine/chemokine expression detected within groups at later stages of repair (T4 and T7).



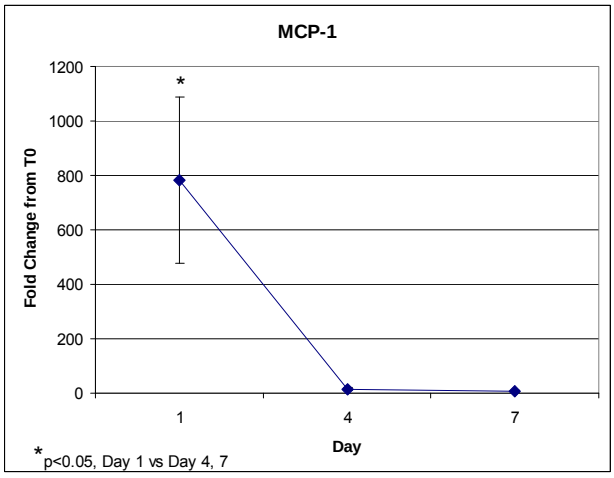


Figure 3-5. Cytokine/chemokine main effects by day.

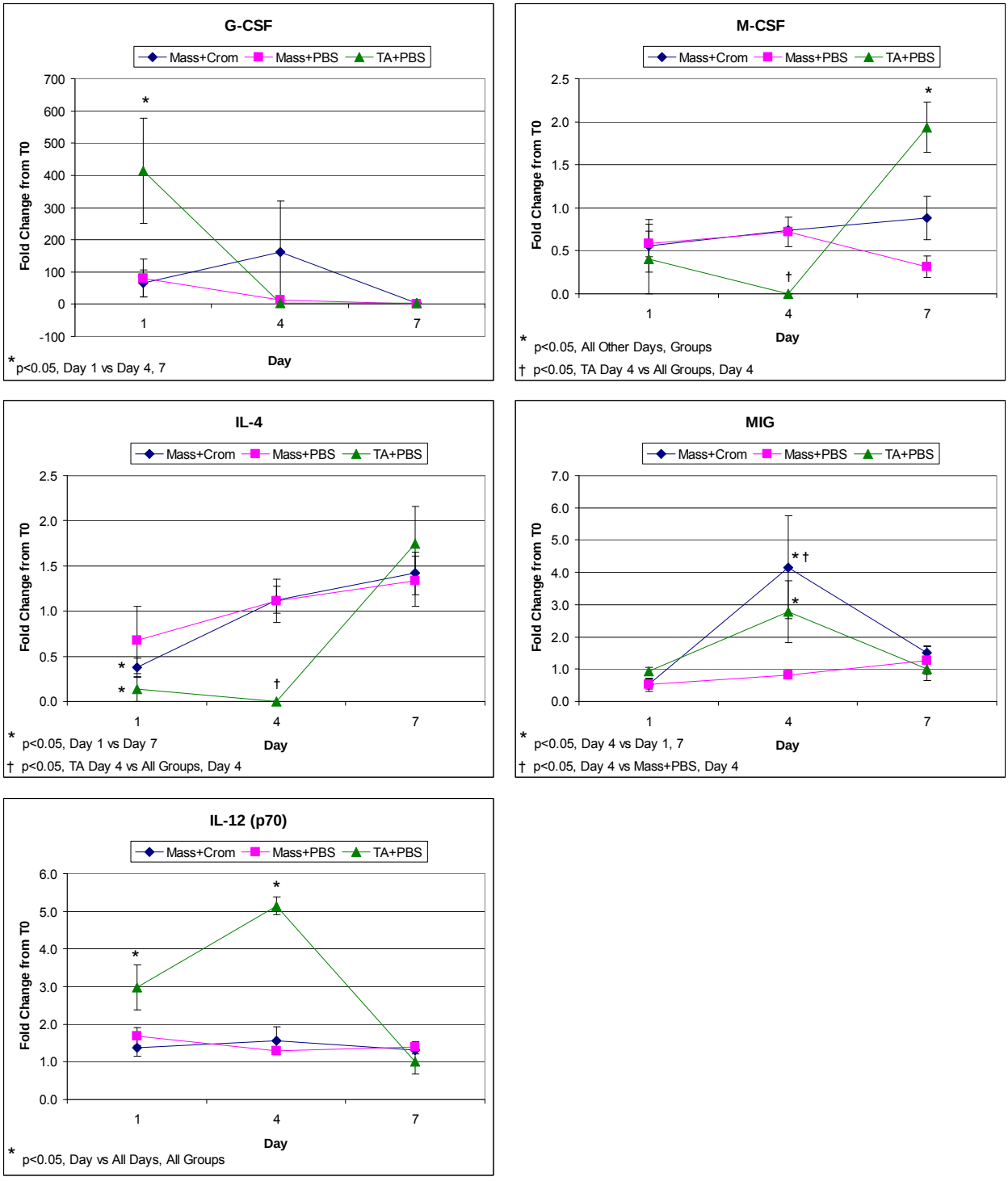


Figure 3-6. Cytokine/chemokine interactions of muscle by day.

CHAPTER 4
DISCUSSION

Tibialis Anterior Reflects Normal Muscle Healing

Inflammation following injury to tibialis anterior muscle and subsequent healing was compared in this study to injury of masseter muscle and its inflammatory and healing process. Histological evaluation of the tibialis anterior muscle showed more advanced healing, a smaller area of damage with less inflammatory exudate, and larger, more uniform muscle fibers by day 7. Previous studies have also shown the healing of masseter muscle is delayed and exhibits more fibrosis than tibialis anterior.[7,8] The histological evaluation confirmed a difference between masseter+cromolyn and masseter+PBS, with masseter+cromolyn more closely approximating the healing of TA. This is suggestive of a therapeutic effect of disodium cromoglycate, cromolyn, and its effect on various cytokines/chemokines in the masseter muscle.

Baseline Cytokine/Chemokine Differences between TA and Masseter

The expression of cytokines/chemokines in the uninjured masseter muscle was similar to that observed in the uninjured tibialis anterior muscle except for four cytokines: Eotaxin, IL-17, IL-12(p70) and VEGF. Eotaxin and IL-17 were both present in increased concentrations in the masseter control compared to tibialis anterior. IL-17 has been shown to induce the expression of eotaxin, a chemoattractant for mast cells.[41,42] The fact that mast cells have been observed in increased numbers in masseter muscle when compared to TA may be explained by the inherent increased concentration of cytokines/chemokines specific for mast cell recruitment in masseter muscle.

VEGF had higher basal levels in the TA control when compared to the masseter control. VEGF decreased significantly in TA following injury, especially during the destruction and activation phase and the anti-inflammatory transition, when compared to baseline controls. It has been shown that VEGF affects dystrophin-positive regenerating fibers, endogenous muscle regeneration, microvascularization, and level of fibrosis.[43] It has also been shown that VEGF expressing muscle-derived stem cells improve muscle repair in the animal model of muscular dystrophy.[43] Macrophage recruitment, an event dependent on chemokine receptor 2, is associated with the restoration of tissue VEGF levels after an initial decrease.[44] The masseter muscle does not have the initial decrease of VEGF typical of injured muscle, which could be evidence of an altered inflammatory response. An inverse relationship has also been shown between regenerated muscle fiber size and capillary density.[44] Visual examination of the masseter muscle histological sections revealed masseter to be highly vascularized, and this could be associated with the fact the muscle fibers are smaller in healing masseter muscle. The temporal expression of VEGF, particularly an increased concentration at baseline, a significant decrease following injury, and a later return to baseline concentration, appears to be important in the healing of skeletal muscle and this pattern was not observed for the masseter muscle.

Normal Cytokine/Chemokine Response

Overall, it was found that TA exhibited changes in the expression of eight different cytokines/chemokines during the inflammatory process, while the masseter+PBS group did not exhibit changes in the expression of any of the cytokines/chemokines. With the administration of cromolyn, the number of cytokines/chemokines that changed during the inflammatory process in the masseter muscle injury group was increased to four.

This is evidence that the masseter muscle has a blunted cytokine/chemokine response when compared to tibialis anterior muscle and that cromolyn selectively had an effect on the expression of a subgroup of cytokines/chemokines in the masseter.

When the cytokines/chemokines were normalized to baseline values, MCP-1 and KC were shown to increase during the destruction and activation phase in all muscle groups. MCP-1 is produced by monocytes and endothelium, and it guides chemotaxis of macrophages and also causes degranulation of mast cells. KC is produced by keratinocytes, monocytes, and macrophages and activates neutrophils. Since chemokines are important early in the inflammatory process, it is logical that chemokines MCP-1 and KC were increased initially to promote chemotaxis of various inflammatory cells, including neutrophils and macrophages.

IL-12(p70) was increased during the destruction and activation phase and peaked during the anti-inflammatory transition in TA. There was a significant difference in baseline levels between TA and masseter (TA > masseter) and TA had a significantly higher levels on days 1 and 4 compared to the other muscle groups. GM-CSF stimulates murine bone marrow precursors to form GM-BMM macrophages which preferentially secrete IL-12(p70), a pro-inflammatory cytokine important in neutrophil stimulation.[45] Other macrophage populations, e.g. BMM (stimulated by M-CSF), do not secrete IL-12(p70).[46] The fact that TA produced IL-12(p70) in significant quantities, and IL-12(p70) is only produced in certain populations of macrophages suggests the macrophage populations present in TA may be different than those present in masseter muscle. Histological evaluation in a previous study by Widmer and Morris-Wiman showed fewer macrophages in masseter muscle than TA.[8] Differences in the number

and type of macrophages may be crucial in explaining the varied healing process exhibited by masseter muscle.

Colony-Stimulating Factors

Colony-stimulating factors also appear to play a major role in the inflammatory process of tibialis anterior muscle compared to masseter muscle. G-CSF and M-CSF were both elevated in tibialis anterior but not masseter. G-CSF was increased during the destruction and activation phase in TA, and previous studies have shown that following injury G-CSF may increase muscle fiber diameter, inhibit inflammation, and augment muscle mass regeneration through increased proliferation of satellite cells.[47, 48] Early release of G-CSF in TA but not in masseter is suggestive of its importance in the healing process potentially by early activation of satellite cells.

M-CSF or CSF-1 decreased during the anti-inflammatory transition and increased during the formation and maturation phase in TA, and it may have played a crucial role in the remodeling process of the damaged muscle. M-CSF regulates the survival, proliferation, and differentiation of mononuclear phagocytes.[49] Previous studies suggest M-CSF is required for normal development in tissues undergoing rapid morphogenesis or tissue remodeling, and inflammatory conditions involve macrophages that are not dependent on M-CSF.[50] Since M-CSF was increased during formation and maturation of new muscle fibers, its primary role was probably related to tissue remodeling, and absence of an increase in M-CSF in injured masseter muscle may help explain the aberrant architecture of the muscle fibers.

Overlapping Effects between Masseter+Cromolyn and TA

Two cytokines, IL-4 and MIG, showed similar effects in masseter+cromolyn and TA. IL-4, which is released by mast cells, was decreased during the destruction and

activation phases in TA and masseter+cromolyn and also during the anti-inflammatory transition in TA when compared to their respective controls. The level of IL-4 in TA during the anti-inflammatory transition was lower than in all other muscle groups on each day. Following the initial decrease, IL-4 increased in TA on day 7 above baseline values. IL-4 is a critical factor in muscle growth and it may promote the fusion of myoblasts to myotubes.[34] A decrease initially in IL-4 following injury is potentially related to the importance of inducing a normal inflammatory process and not promoting myoblast differentiation and fusion muscle until the damaged area has been cleared. IL-4 was significantly lower in TA and masseter+cromolyn groups on day 1 compared to masseter+PBS, but this difference did not persist for the masseter+cromolyn group during the anti-inflammatory transition (day 4) where IL-4 levels increased to control levels. The varied response between TA, masseter+cromolyn and masseter+PBS groups, where a decreased IL-4 expression was maintained in TA, transiently decreased in masseter+cromolyn and did not change from baseline in masseter+PBS, could contribute to the varied healing response seen between the two groups, with masseter+cromolyn more closely approximating TA.

MIG is a chemokine that is increased in masseter+cromolyn and TA during the anti-inflammatory transition. MIG is involved in chemotaxis of monocytes and is produced by macrophages. This may be additional evidence for the importance of macrophages in the inflammatory process in order for normal healing to occur, with TA and masseter+cromolyn potentially exhibiting similar numbers of active macrophages. It has been shown that the presence of macrophages directly affects satellite cell

proliferation, skeletal muscle regeneration, and fibrosis, and their presence is crucial for a normal healing process to occur.[51]

Effects of Cromolyn on Masseter

IP-10, a chemokine that attracts monocytes and is produced by macrophages, fibroblasts, and endothelial cells, was increased in masseter+cromolyn during the anti-inflammatory transition when compared to baseline controls. The expression of IP-10 did not change in the masseter+PBS or TA groups. It was shown in this study that, histologically, healing in masseter+cromolyn was similar to TA. It was shown previously that macrophages are more prominent in TA than in masseter during repair.[8] Since IP-10 is released by macrophages, an increased amount of IP-10 in masseter+cromolyn may be evidence of an increased number of active macrophages present at the site of injury.

Major Inflammatory Cytokines Exhibiting No Difference

Pro-inflammatory cytokines like TNF-α and IL-1β that were expected to increase in damaged muscle did not show an appreciable change from the baseline values and were similar for all muscle groups. Tidball (2005) provided evidence that the role of TNF-α may vary in muscle injury and repair depending on the type, severity, location, and stage of injury.[17] It is possible TNF-α is not needed for the repair of muscles with mild damage such as after a freeze-injury. Another explanation for the lack of response could be that the response occurred outside of the time points examined. If TNF-α increased immediately post-injury and decreased back to baseline values within the first 24 hours, an effect would not have been observed. Including a time point that evaluates the cytokine/chemokine response within several hours of muscle damage may show if there is indeed an effect in these and other cytokines.

Directions for Future Research

It was beyond the scope of this project to evaluate the level of every cytokine/chemokine predicted to play a role in muscle repair. Several cytokines/chemokines that could be examined in future studies are SCF, TGFβ, and NGF. Stem cell factor (SCF) is a growth factor unique to mast cells, shown by the fact that mast cells do not exist in the absence of SCF. SCF causes local proliferation of mast cells and enhanced production of SCF by fibroblasts could lead to fibrotic processes. TGFβ and nerve growth factor (NGF) are also responsible for mediating chemotaxis of mast cells.[52,53] Mast cells have been shown to have a direct effect on inflammation and healing, but they are also involved in neuroimmune interactions. The association between mast cells and nerves could serve as a means of amplifying inflammation in muscle and causing increased pain. In addition to being activated by neuropeptides and possibly nerve stimulation, mast cells release nerve growth factor (NGF) and express trk-A, a high affinity receptor for NGF.[54] NGF is a neurotrophin responsible for causing sensitization of nociceptors, and in animal and human studies, inflammation has been shown to cause increased levels of NGF resulting in hyperalgesia.[55,56] NGF can cause degranulation of mast cells as well as increased cytokine expression. The relationship between mast cells and nerve associated factors, including neuropeptides and NGF, could be significant in the inflammation and healing process in muscle and the accompanying pain experienced by the individual.[57]

Macrophages and the cytokines/chemokines associated with them have been shown to be important in the inflammatory process. GM-BMM macrophages express an integrin, CD11c, that is not expressed by BMM macrophages.[58] Evaluating the specific populations of macrophages present in TA and masseter through antibody labeling of

the CD11c integrin may also help explain healing differences. It is possible masseter is deficient in certain factors, therefore macrophages do not migrate properly into the injured area. Evaluating the subtypes of macrophages present in the injured muscle would help determine if this is a reason for the delayed healing seen in masseter muscle.

Conclusions

Temporomandibular disorders commonly include myofascial pain, and the masseter muscle is one of the primary muscles affected. Following damage, the masseter muscle has been shown to exhibit delayed healing and a varied inflammatory response when compared to limb muscle. When compared to tibialis anterior muscle, the masseter muscle exhibits a blunted expression of cytokines/chemokines that is enhanced with the administration of cromolyn. The reasons for this may be explained by the fact:

1. Certain cytokines/chemokines are present in significantly different concentrations in the control TA and masseter muscles, e.g. Eotaxin and IL-17, and this may prime the condition of the muscle to lead to a varied inflammatory response.
2. Cytokines related to macrophage function and recruitment are increased in TA, emphasizing the importance of macrophages for normal healing to occur.
3. The temporal expression of VEGF is also different in TA and masseter and could explain differences in healing.

When the degranulation of mast cells is blocked through administration of cromolyn, the healing of masseter muscle appears to normalize to TA histologically and

through the release of IL-4 and MIG in similar concentrations to that of TA. IP-10 is also increased in masseter+cromolyn which suggests the normalized healing could be occurring due to an increased number of macrophages.

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