IMMUNOLOGICAL ASPECTS OF MUSCLE INJURY AND
REGENERATION IN YOUNG AND OLD RATS.

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IMMUNOLOGICAL ASPECTS OF MUSCLE INJURY AND REGENERATION IN
YOUNG AND OLD RATS.

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Immunological Aspects of Muscle Injury and Regeneration in Young and Old Rats.

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The efficiency of skeletal muscle regeneration following injury is decreased with age, and a deficient macrophage response may be part of the cause. Since several important growth factors and cytokines are produced by macrophages, a deficient macrophage response may alter these important cellular signals, further inhibiting the regenerative process. PURPOSE: The primary aim of the study was to observe the response of three macrophage subpopulations (ED1+, ED2+, and IA+) and to quantify the appearance of IL-1β, FGF-2, TGF-β1 and IGF-I following exercise induced muscle damage in both young (11 months) and old (22-23 months) rats. These subpopulations are thought to be involved in different phases of muscle regeneration. METHODS: Male Fischer 344 rats (n=35 old, n=42 young) were sacrificed as controls, or at 2 hours, 1, 2, 4, 8 or 12 days after 90 minutes of running on a -16° slope at 11 m/min, and their soleus muscles removed. After sectioning (8µm), macrophage subpopulations were visualized via light microscopy using immunohistochemical staining and quantified at each time point. Muscle damage was assessed by observing H&E treated sections. RESULTS: Following exercise, there were significant increases (p ≤ .05) in ED1+ macrophages at day 2 in young rats, in IA+ macrophages at day 1 in old rats, and in ED2+ and IA+ macrophages at days 8 and 12 in both age groups. Despite sustaining significantly more muscle damage than young rats, increases in soleus macrophage subpopulations in old rats seemed to be generally less robust than in young. This was indicated by 1) the absence of a significant...
increase in ED1\(^+\) macrophages in old animals, and 2) a somewhat slower increase in both IA\(^+\) and ED2\(^+\) macrophage populations in the elderly age group. Among growth factors/cytokines, only IL-1\(\beta\) (increased at 2 hours in elderly over control and young) and FGF-2 (decreased at 1 and 2 days in old rats) were changed with the exercise stimulus. CONCLUSION: These results suggest that a deficient macrophage response may play a role in the delayed regeneration of damaged skeletal muscles in elderly animals, but this deficiency does not seem to directly affect growth factor concentrations.

Approved

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List of Abbreviations

BSA – Bovine Serum Albumin

DOMS – Delayed Onset Muscle Soreness

EMG – Electromyograph

ELISA – Enzyme-linked Immunosorbent Assay

FGF – Fibroblast Growth Factor

IGF – Insulin-like Growth Factor

IL – Interleukin

%DaF – Percentage of Damaged Fibers

%DeF – Percentage of Degenerating Fibers

%ReF – Percentage of Regenerating Fibers

PBS – Phosphate-buffered Saline

TGF – Transforming Growth Factor

TNF – Tumor necrosis factor
Background

Muscle injury: mechanisms and morphology

Skeletal muscle injury can result from a number of different stimuli, including crush, freeze, blunt force, exposure to toxins, mechanical stretch and unaccustomed exercise. All of these can occur normally, but in humans, injury in response to unaccustomed exercise occurs quite frequently during the course of normal living.

Several of the consequences of exercise-induced muscle injury are readily apparent. Strength loss following muscle injury is immediate and has been reported to last as little as 48 hours in rats (69), or several days (100) to more than a week (59) in humans. The source of this weakness is likely to be disruption or degeneration of myofilaments and other force transmitting elements in the muscle, brought about by mechanical overload or later chemical events such as the activation of constitutive proteases or enzymatic digestion by infiltrating inflammatory cells. It has also been suggested that motor unit recruitment patterns are disrupted for a time after the injurious exercise (100), further contributing to muscle weakness. Muscle soreness is also observed beginning 24-48 hours after the damaging event and lasts several days or longer, depending on the severity of the injury (94,101). The cause of this delayed onset muscle soreness (DOMS) is unknown but may not be directly associated with structural damage to the muscle. In addition, neuromuscular efficiency (torque/EMG) is also decreased and may outlast other symptoms of muscle damage (35).

Studies of exercise-induced muscle injury have shown that more severe injury occurs following lengthening (eccentric) contractions than following shortening (concentric) contractions. Armstrong et al. (4) subjected rats to downhill (primarily eccentric
contractions), level (both eccentric and concentric contractions), or uphill (primarily concentric contractions) running and observed enzymatic activity and morphological changes in muscle over the next eight days. They found that significant increases in plasma creatine kinase and lactate dehydrogenase, purported indicators of muscle damage, occurred only after downhill running. Increases in glucose-6-phosphate dehydrogenase, an enzyme associated with inflammation and repair, and phagocytic mononuclear cells occurred only after downhill and uphill running, although to a greater extent following downhill running. Other researchers have shown that both muscle soreness (8,30,83,101) and strength loss (84,100) is greater following eccentric exercise than concentric exercise.

The initial event in eccentrically induced muscle damage is not yet clear. Given the uniqueness of eccentric muscle contractions and the ability of skeletal muscle to produce greater forces eccentrically than concentrically (102) it has often been proposed that muscle damage is mechanical in origin, and numerous studies have sought to characterize the precise nature of this process. Warren et al. (114) have presented evidence that muscle injury is the result of the cumulative effects of a number of eccentric contractions. They found that isolated rat muscles undergoing up to eight eccentric contractions did not differ from non-exercised muscles in twitch tension and peak tetanic tension, time to peak tension and relaxation time. After more than eight contractions, however, all of these values declined significantly. The investigators concluded that the cause of these contractile deficiencies is the cumulative effect of a number of high tensile stresses, resulting in fatigue and damage to one or more of the force transmitting proteins present in the muscle (114). Further research from this same laboratory indicates that the decline
of the functional properties of isolated muscle after maximal eccentric contractions is most closely related to the peak forces sustained by the muscle (115).

In contrast, Lieber and Fridén (74) have found that contractile properties are most affected by rate of strain during the contraction, rather than peak force. Briefly, rabbit tibialis anterior was subjected to different forces (very high and moderate or low) and two different rates of strain (25% and 12.5%) while maximally activated. The authors reported that differences in contractile properties following treatment were associated with different rates of strain but not with different forces.

Other studies have reported that more eccentric muscle damage occurs at long muscle lengths than short muscle lengths (27,84), lending support to the mechanism supported by Lieber and Fridén (74). A particularly interesting hypothesis has been developed by Morgan et al. (82). These investigators have proposed that structural damage to sarcomeres occurs when sarcomere length inhomogeneities develop, creating stronger and weaker sarcomeres. Weaker sarcomeres become overextended to the point that actin and myosin no longer overlap, and active tension is no longer possible. This allows sarcomeres to “pop” from a viable length to one where structural proteins support the entire load of the muscle (82). If structural proteins cannot support such a load, they will sustain mechanical damage. Cellular structures likely to be damaged as a result of either high peak tension or excessive strain during eccentric contractions include non-contractile support proteins such as desmin and titin (45), contractile elements such as actin and myosin, and the plasma membrane.

Following the initial, mechanically induced injury, a chemically induced injury is initiated. Although the mediator of this secondary injury is not completely clear, it has
been hypothesized that unregulated calcium entry into the muscle cell and subsequent activation of calcium activated proteases may play a central role (5,11). One likely player in this process is the non-lysosomal protease calpain, which can be found within the sarcomere localized at the I-band and the Z-line regions. *In vitro*, calpains are known to cleave a number of myofibrillar constituents, among which are desmin, α-actinin, myosin light chain kinase, troponin, and tropomyosin (98). Destruction of these proteins by calpains *in vivo* may cause a pattern of damage in the sarcomere similar to that seen after exercise (11).

Although there is no direct evidence of a role for calpain in exercise induced damage, experimentally induced increases in intracellular [Ca$^{2+}$] have been shown to cause immediate intracellular damage (39,40,51,62), and there is some evidence indicating the development of elevated [Ca$^{2+}$], following chronic low frequency stimulation of isolated extensor digitorum longus (50) and treadmill running in rats (38,108). Therefore, it is possible that calpains are important in secondary destruction of muscle tissue following eccentric exercise.

Another possible chemical mediator of muscle damage and proteolysis is the ubiquitin-proteasome pathway. In this system, abnormal or misfolded proteins are specifically targeted for destruction by labeling with ubiquitin molecules. Labeled proteins are then degraded by the 26S proteasome (28). This pathway has been implicated in muscle wasting diseases and muscle atrophy (reviewed in (63)), and is likely to play a role in the early events of muscle regeneration following injury.

Both mechanical and chemical damage to muscle cells results in widespread morphological changes that can be visualized by light or electron microscopy. Hikida et
al. (56) examined human muscle biopsies after a marathon run and noted disrupted sarcolemma, increased incidence of free mitochondria and red blood cells in the extracellular space, basal lamina tubes lacking myofibrils, disoriented myofibrils, and Z-line streaming (widening) and degeneration following exercise. Ogilvie and colleagues (86) performed a similarly thorough investigation of eccentrically damaged rat muscles finding a predominance of A-band disruptions, which encompassed several fibers. In these fibers, the Z-line appeared structurally sound. In contrast, other fibers exhibited total lack of Z-lines but normal A-bands. Z-line deficient areas were surrounded laterally by hypercontracted sarcomeres.

**Wound repair and the inflammatory response**

In order to regain optimal functional ability from skeletal muscle following injury, it is necessary to repair damaged tissue. The sequence of events following injury is essentially the same regardless of the mechanism of injury. Figure 1 (adapted from Bischoff) (15) shows an idealized model of this process and each step is described here.

First, injured fibers (step 1) are observed to hypercontract or pull away from the central site of the injury, creating a gap between previously contiguous sarcomeres within the myofiber (step 2). This ‘retraction zone’ is usually given structure by the surviving basal lamina of the injured fiber. In the first few minutes after injury, intrinsic proteolytic systems are activated and degradation of damaged cell structures is initiated (5).

Within 45 min to 2 hours, inflammatory cells exit the vasculature (neutrophils are the earliest), invade the necrotic area of the retraction zone and begin to phagocyte
cellular debris (step 3). This is accomplished by the engulfment of debris particles and
destruction via lysosomal hydrolases and reactive oxygen species within the neutrophil.
Neutrophils die within 1-2 days and are replaced by their more powerful counterparts,
macrophages, in the wound. Macrophages not only complete the process of debridement,
but also produce and secrete numerous growth factors, which can attract and/or activate
other cells involved in the healing process, such as fibroblasts and satellite cells (93).

Under the direction of macrophage-derived growth factors and in response to various
other locally released chemical signals, satellite cells detach from their position between
the sarcolemma and basal lamina, migrate toward the wound site, proliferate and then
differentiate into myoblasts (step 4). Myoblasts arrange themselves into rows (step 5)
and fuse into myotubes, which eventually span the retraction zone and reconnect retracted myofibrils (step 6) to form a continuous functional contractile unit (step 7).

Clearly, macrophages are a central player in the wound healing process, having important roles in both wound debridement and healing. Of course, macrophages are themselves subject to influence by chemical signals present in the wound area. Cytokines produced by the injured muscle are chemoattractants for macrophages and may be largely responsible for the buildup of invading inflammatory cells (93). It is likely that macrophage activity at the wound site is dictated by continued production of cytokines and the presence of cellular constituents (such as extracellular matrix components) associated with an unstable cellular environment. Sources of these factors are numerous and may include (92), activated fibroblasts or platelets (36), disturbed extracellular matrix particles (33) and even non-phagocytic resident macrophages (109). As muscle regeneration proceeds, macrophages respond to the changing intracellular milieu, and phagocytic activity is phased out in favor of regenerative functions. As the wound resolves, production of cytokines and growth factors is down regulated. This combined with apoptosis of macrophages (110) results in the resolution of regenerative process.

**Macrophage derived growth factors**

Although there are innumerable growth factors involved in muscle regeneration, the scope of the current study includes only four. We believe these four growth factors are the most crucial for successful regeneration following muscle injury. A description of each growth factor and its role in regeneration follows.
1. Transforming growth factor

The transforming growth factor-beta (TGF-β) family is well known for its ability to inhibit growth in numerous cell types (14) and is represented by the closely related TGF-β1, 2 and 3 isoforms (all approximately 25 kDa) in mammals. In skeletal muscle, TGF-β is stored in the extracellular matrix bound to decorin and thus serves as a ready supply of the growth factor following structural damage. Additionally, degranulating platelets and activated macrophages present during inflammation produce and/or release TGF-β upon their entry into the wound site.

TGF-β has an important role in the maintenance of the extracellular matrix (ECM) following injury. It induces the synthesis of extracellular matrix components, such as proteoglycans and collagen types I, III, IV and V (61,95) by fibroblasts. It also stimulates the synthesis of protease inhibitors while inhibiting the production of ECM related proteases (105), thus facilitating the regeneration of the ECM and basement membrane that lend structure to invested myofibers.

TGF-β also plays a role in regulating myofiber regeneration following injury. In vitro it has been shown that while TGF-β is chemoattractant for satellite cells (15), it mildly inhibits their proliferation, and is a potent inhibitor of satellite cell differentiation (1,87). In addition, TGF-β can cause deactivation of macrophages, as measured by the production of cytotoxic H$_2$O$_2$, helping to end the phagocytic phase of regeneration and prepare the wound environment for the appearance of developing myofibers (111). Interestingly, TGF-β has also been shown to be chemoattractant to macrophages and to stimulate production of interleukin-1 (IL-1), a potent pro-inflammatory cytokine, by macrophages and other cell types (93).
**In vivo** studies further illuminate the role of TGF-β in the wound. A study by Lefaucheur and Sébille (71) found that following injection of anti TGF-β antibodies into regenerating rat muscles, the number of regenerating fibers at 11 days post injury had increased over controls. This suggests that TGF-β acted to decrease the proliferation of satellite cells in control rats.

Taken together, the functions of TGF-β are probably to stabilize the wound environment by first encouraging early inflammation, and then inhibiting the activities of proteases, macrophages and satellite cells and the consequent construction of myotubes and myofibers. This insures efficient construction of basement membrane and ECM that will serve as structural support for new maturing muscle fibers.

**2. Fibroblast growth factor**

Of the seven known forms of FGF, only FGF-1 and 2 (also called acidic and basic FGF, respectively) are thought to play significant roles in muscle regeneration, and only FGF-2 is known to be synthesized by macrophages (93). Both forms, however, are stored in the ECM bound to proteoheparin sulfates or in the muscle cytoplasm (29) and are released in response to muscle damage. FGF may also be produced by satellite cells during muscle inflammation (65).

Upon release into the wound site, FGF induces chemotaxis of satellite cells, in which it initiates proliferation and inhibits differentiation (44), although *in vitro*, differentiation may still occur if insulin-like growth factor I is present (1). In addition, FGF appears to promote angiogenesis (53,93), which proves critical to damaged regions of the muscle.

*In vivo* inhibition of FGF has been shown to cause a decrease in the number of regenerating myotubes in denervated, devascularized muscle (71). These results seem to
show that despite removal of the inhibition of differentiation by FGF, the absence of the pro-proliferative effects of FGF on myogenic precursor cells seriously impairs the regenerative process. Although the signaling pathway for FGF effects on muscle precursor cells is currently unknown, it seems likely that FGF regulates proliferation and differentiation via interaction with the myogenic determination genes MyoD1 and myogenin (44).

3. Insulin-like growth factor

The insulin-like growth factor (IGF) family includes IGF-I and IGF-II, similar peptides which both promote general anabolic actions, such as protein and glucose uptake by cells, protein synthesis, and inhibition of protein degradation (44). IGF is secreted by a number of cell types including muscle tissue, satellite cells (64) and macrophages (IGF-I only) (93), and is one of the few peptide growth factors found in the plasma at consistently significant levels, due mainly to synthesis in the liver (14).

Besides general anabolic effects, IGF has important effects on myoblasts with implications for muscle regeneration. IGF-I and II promote satellite cell proliferation (37) and, unlike TGF-β and FGF, differentiation (1). IGF is thus vital to the completion of muscle regeneration following injury. It seems likely that the stimulation of satellite cell differentiation is mediated through the myogenic determination gene myogenin, although the increase in myogenin mRNA is a somewhat slow process in vitro, taking 30-40 hrs (44).
The effects of both IGF-I and II are mediated by the IGF-I receptor (44), although the IGF-II receptor is abundant on cultured cells. Both IGF-I and II bind to the IGF-I receptor: IGF-I with high affinity, IGF-II with low affinity. Given these binding affinities, it would seem that IGF-I plays a greater role in satellite cell proliferation and differentiation, if both IGF-I and II are present.

In vivo, IGF interacts with other growth factors as well as numerous IGF binding proteins, produced by numerous tissues including skeletal muscle. IGF binding proteins can increase or decrease IGF activity, or even act on IGF receptors independently and thus play an important role in the regulation of IGF function. This complex interplay means that the measurement of IGF levels in regenerating muscle tissue, while a worthwhile first step, may not provide a complete picture of IGF’s role in muscle regeneration.

Because of the importance of IGF to muscle regeneration, therapeutic applications are beginning to be explored. For example, experimentally induced IGF-I overexpression in mouse hindlimb musculature induces increased muscle mass and strength in young mice by 15% and 14%, respectively. In aged mice, muscular strength was increased by 27%, while the age related decrease in muscle mass was prevented (10). Further experimentation on aged rats recovering from hindlimb immobilization shows that exogenously administered IGF stimulates both a recovery of muscle mass and an increase in satellite cell proliferation potential in hindlimb musculature (26). The results of these studies have important implications and may eventually lead to effective prevention of age-induced muscle atrophy in humans.
4. Interleukin-1

Interleukin-1 (IL-1) is perhaps the most widely functional growth factor in inflammation and early muscle regeneration. IL-1α and IL-1β (27 and 33 kDa, respectively), although chemically distinct, appear to have similar actions, despite the existence of specific receptors for each (93). IL-1 is produced by a wide range of cells, including endothelial cells, fibroblasts, B cells, natural killer cells, and macrophages. IL-1 production is regulated by an equally impressive array of stimulatory agents such as IL-1 itself, TGF-β, tumor necrosis factor-α (TNF-α), interferons, lipopolysaccharide and complement factor C5a. Most or all of these factors are present during inflammation.

The functions of IL-1 are largely pro-inflammatory. It is chemoattractant for several cell types, including fibroblasts, neutrophils and macrophages, in which it induces adhesion, increased respiratory burst (neutrophils and macrophages), and production of other pro-inflammatory cytokines and molecules such as IL-6, IL-8, TNF-α, prostaglandins, and interferons. IL-1 increases proteolysis of muscle cells, inhibits their differentiation, and can increase collagenase production by several cell types. IL-1 production is increased following exercise and plays a central role in initiating muscle inflammation, as indicated by its numerous effects above (22,23,41,43).

Since the effects of IL-1 are largely pro-inflammatory, prolonged action may be detrimental to wound healing. IL-1 can be regulated by the production of receptor antagonists or by other factors such as prostaglandin E2, which inhibits IL-1 production. In addition, as regeneration progresses and cellular debris is cleared by macrophages, the inflammatory environment produced by IL-1 may increasingly be dominated by other growth factors such as TGF-β and FGF. Macrophages may then be programmed for
destruction via apoptosis (110), resolving the inflammatory/phagocytic phase of muscle injury.

Effects of aging on skeletal muscle morphology, injury and repair

The aging of the human body produces many changes, and skeletal muscle is not unaffected. One prominent aging effect is the decrease in muscular strength seen after the age of 50. Between the ages of 50 and 70, maximal isometric strength may decline by 25-30% (70) (fig. 2). Strength loss is accelerated thereafter and, by the age of 80, an additional 30% of muscular strength may be lost (34). Increasing muscle weakness hinders the ability of the elderly to accomplish daily tasks and may increase the likelihood of falls and the resulting injuries (42).
Sarcopenia, the accelerated loss of muscle mass with age, is the primary, but not sole, contributor to strength loss, and has been well documented on both the whole muscle (47,52,73) and single fiber (47,70) levels. Muscle fiber atrophy seems to preferentially occur in type II fibers (70,73), and there is evidence of a gradual transition from fast to slow muscle fiber types, probably resulting from selective denervation of fast muscle fibers and subsequent reinnervation with a slow motor neuron (3,54). Together these adaptations lead to the existence of large, slow-type motor units (3) with decreased force production and slower contraction times. The process of denervation may be involved in the continuous drop in the number of muscle fibers with age, as reported in studies of cadaver muscles (reviewed in (52)).

There is also evidence that contractile abilities of muscle fibers decrease with age independent of changes in fiber size, although reports are conflicting. Several groups have reported a decline in the force per muscle cross sectional area, or specific force, with age in both whole muscles (17,91) and single fibers (47), while other evidence suggests that specific force is maintained during the aging process (46,117) (figs. 3 and 4). The difference in the findings of these studies may arise from methodology used to estimate muscle mass. Frontera et al. (47) also considered the effect of fiber type on specific force and found that after adjustment for size differences, fiber type-matched single fibers from younger subjects were stronger than fibers from older subjects, eliminating fiber type as a contributor to specific force changes with age.

Despite the myriad factors involved in weakness during old age, muscle atrophy is one that is somewhat reversible. Therefore significant research has focused on physical activity as a means to increase muscle mass and strength. Regular physical activity may
attenuate the decline of muscular strength, thus improving quality of life by allowing the elderly to safely perform tasks of daily living. Resistance training in the elderly seems to be a particularly promising intervention, since elderly subjects have been shown to be capable of achieving large strength gains (49,55) significant increases in muscle fiber cross sectional area, and favorable fiber type transitions (57) with high intensity resistance training.

Fig. 3(top). The decline in specific force with age for several different subject groups. Bars represent SEM. From Phillips et al. (91).

Fig. 4(bottom). The relationship between force and muscle mass is shown to be linear. Isometric strength in old women is lower due to aging atrophy. From Young et al. (117).
However, research using both mice (18) and rats (77) has shown that muscle from aged animals sustaining contraction-induced injury does not regain maximal isometric force as quickly as muscle from young animals, suggesting a reduced ability of muscle to regenerate after injury in the aged.

The inflammatory response initiated after muscle injury plays a vital role in the ultimate success of muscle regeneration and, therefore, recovery of muscle function. Age-induced macrophage dysfunction, or decreased macrophage accumulation following muscle injury could lead to both decreased debris clearance or inadequate or delayed production of critical growth factors such as TGF-β, FGF and IGF, and thus result in impaired or delayed muscle regeneration.

In vivo, the temporal sequence of growth factor release appears to largely control the progression of events of muscle regeneration, and alterations in this sequence could have severe consequences on the successful recovery of muscle function. Reports of macrophage dysfunction with age are not uncommon, and a number of studies have revealed inflammatory deficiencies that may contribute to altered tissue repair with aging. These deficiencies include decreased respiratory burst (90) and chemotactic activity (89) in neutrophils, and delayed accumulation of macrophages (21).

There is direct evidence that host specific factors such as macrophage function may hinder muscle regeneration with aging. A study by Zacks and Sheff (118) showed that autotransplants of minced muscle do not regenerate as well in old mice as in young mice, as indicated by decreased formation of myofibers, and decreased appearance of presumptive myoblasts and myotubes in regenerating old muscle observed by transmission electron microscope. When young minced muscle was transplanted into old
mice, the researchers observed decreased proliferation of satellite cells and a reduced clearance of damaged tissue. Old minced muscle transplanted into young mice, however, exhibited nearly normal regeneration, indicating that host specific factors may be more vital to the success of muscle regeneration than the properties of the muscle itself (118).

Carlson and Faulkner (25) performed whole muscle grafts of extensor digitorum longus (EDL) muscle from aged to young rats and vice versa. Following a 60 day recovery period, autografted young muscles had nearly two times greater mass than those that had been grafted into old rats and these muscles could generate more that 2.5 times greater maximum isometric force than their counterparts. Similarly, old muscle grafted into young rats maintained their mass just as well as young autografts and had significantly greater mass and maximum isometric tension generation than old muscle autografts (Figs. 5,6).

Fig. 5 and 6. Changes in muscle mass and maximum force following muscle transplantation. Comparison of mass of control (empty bars) and transplanted (shaded bars) EDL. Connected arrows with asterisks indicates that these two values are significantly different (p ≤ .05). From Carlson and Faulkner (25).
Additionally, both old autografts and young to old grafts were observed to have smaller fibers, wider interfiber space with more connective tissue and greater incidence of lipid deposits and central nuclei. The authors mentioned several host-related factors, which may have affected muscle regeneration, including success of reinnervation and revascularization, extent of physical activity during transplantation recovery, hormonal and growth factor effects, and phagocytic activity (25). None of these factors were investigated in that study.

Although important work on altered cutaneous wound healing with aging has been completed (6, 7, 21), research into the effects of aging on specific aspects of muscle regeneration, such as the response of macrophage populations, is limited. The role of macrophages in tissue repair is an important one as indicated by their dual roles in phagocytosis and wound healing. It has been shown that at least three distinct types of macrophages are present in injured rat skeletal muscle: those expressing either the ED1, ED2 or class II major histocompatibility type IA surface antigens (IA) (58). These three subpopulations are thought to carry out separate functions during inflammation and subsequent repair of damaged muscle tissue.

St. Pierre and Tidball (107) observed rat soleus muscle for 7 days following 10 days of hindlimb unloading. Reloading after hindlimb unloading causes injury due to muscle atrophy that occurs during the unloaded period. Using immunohistochemical staining of macrophage subpopulations, these researchers reported that ED1+ macrophage numbers were elevated above 0 day recovery values on days 2 and 4 of recovery (fig. 7A) and were seen both in the interstitium and inside necrotic muscle fibers, which were most abundant during this time.
Fig. 7. Changes in macrophage subpopulations following during recovery from hindlimb unweighting. A) ED1⁺ B) ED2⁺ and C) IA⁺ macrophage counts at 0, 2, 4 and 7 days recovery. Bars represent SE. * Significantly different from 0 days recovery (p<0.05). # Significantly different from control values (p<0.05). From St. Pierre and Tidball (107).
ED1$^+$ macrophage numbers returned to control values by 7 days of recovery. Ia$^+$ macrophage numbers were elevated above control and 0 day values at both 2 and 7 days recovery (fig. 7B) and were also observed to invade muscle fibers. In later stages of muscle regeneration (4 and 7 days recovery) ED2$^+$ macrophages increased in number. They were significantly more numerous at 4 days recovery than 0 day values. (fig. 7C), but did not invade muscle fibers.

It was hypothesized that because of their early appearance, ED1$^+$ macrophages are involved primarily in the phagocytosis of damaged muscle that occurs at the onset of muscle injury. The ED2$^+$ subpopulations appear to be more important in the muscle regeneration following the completion of phagocytosis at approximately 4 days recovery. The biphasic response of Ia$^+$ macrophages and their invasion of necrotic fibers indicate that they may be important in both phagocytosis and wound healing.

The results of this study are in agreement with other research. The absence of phagocytic activity in ED2$^+$ macrophages confirms earlier work by McLennan (79). In addition, the pro-regenerative functions of ED2$^+$ macrophages in cell culture have been demonstrated by Massimino et al. (76), who showed that purified macrophages expressing the ED2 receptor increased the proliferation of cultured myoblasts. This proliferation was accompanied by an increase in MyoD-positive nuclei. However, the isolated ED2$^+$ macrophages co-expressed the ED1 receptor, and the authors speculated that ED1$^+$ macrophages might possess the ability to differentiate into ED2$^+$ macrophages. This possibility has also been indicated following freeze damage, where some macrophages co-expressed either Ia and ED1 receptors or Ia and ED2 receptors (80). In summary, while it appears likely that discrete macrophage subpopulations exhibit
specialized functions during muscle regeneration, it is possible that some macrophages respond to changes in the wound environment by developing new capabilities, which serve to sustain the regenerative process.
Statement of problem and hypotheses

There have been no investigations into the activity of macrophage subpopulations in regenerating elderly muscle. It was therefore the primary aim of the research to compare the magnitude of the response of macrophage subpopulations as well as the time course of the appearance of selected growth factors/cytokines in soleus muscles of elderly Fischer 344 rats (22-23 months) to the same response in young (11 months) rats following contraction-induced muscle injury initiated by a 90-minute downhill running bout at a moderate speed. Since macrophages appear to be important not only for the removal of cellular debris, but also for initiation of the muscle regeneration, research into their activity in older populations following muscle damage is a key to understanding impaired recovery from muscle injury in the elderly.

In order to confirm the expected functions of the macrophage subpopulations, and to characterize changes in the intracellular environment throughout injury and regeneration, linear regression analysis was performed to discover associations between the presence of macrophages and the appearance of cytokines and growth factors associated with the phagocytosis of debris (IL-1β), fibrogenesis, angiogenesis, and satellite cell proliferation (FGF-2, TGF-β1) and differentiation (IGF-I).

Muscle morphology was used to quantify muscle injury. Since the macrophage response corresponds somewhat to the magnitude of injury, it was possible to gauge whether the observed macrophage response in the elderly population is comparable to the ‘normal response’ in the young population.

It was hypothesized that while the magnitude of injury (as assessed by histological observation) in response to an identical exercise bout may be higher in the elderly
population, the macrophage response would be less robust than expected in comparison to the same response in the younger population. It was further hypothesized that an altered macrophage response, if present would lead to a similarly altered appearance of one or more of the macrophage-produced growth factors. It is these deficiencies that would be partly responsible for the known delay in muscle regeneration seen in elderly animals.
Methods

Animals and exercise protocol

The study was approved by the Institutional Animal Care and Use Committee at Ohio University. Male Fischer 344 rats [42 young (11 months) and 35 elderly (22-23 months)] were obtained from the National Institutes on Aging aged rodent colony (Harlan Labs, Indianapolis, IN), housed individually, and maintained on a 12:12h light/dark cycle at the Ohio University Animal Facility. Animals were allowed to acclimate to the new holding facility for 3 weeks, during which time they had ad libitum access to water and commercial rat chow.

Eleven rats (five old and six young) were sacrificed as controls to make baseline measurements of immunological parameters. The remaining animals ran on a motorized treadmill at a speed of 11 m/min on a -16° slope for a total of 90 minutes, alternating 5 min of exercise and 2 min of rest. Although most required encouragement via gentle prodding and mild electrical shock, all animals were able to complete the exercise protocol. Six young and five old animals were sacrificed at each of six time points: 2 hrs, 1, 2, 4, 8 and 12 days post-exercise. At that time, the animals were deeply anesthetized using an intraperitoneal injection of sodium pentobarbitol equal to 70 mg/kg body weight. The soleus muscles were removed from both legs of each animal. Immediately following removal of the muscles, the animals were killed by cervical dislocation.

Tissue preparation

Soleus muscles from one hindlimb were cleared of excess tendinous and connective tissue, quickly weighed, snap frozen in liquid nitrogen, and stored at -80°C. In preparation for growth factor analysis, the frozen muscles were powdered in a
“biopulverizer” (Biospec Products, Oklahoma City, OK) and then homogenized in 7 volumes of ice cold PBS containing 5 µg/ml antipain, 10 µg/ml aprotinin, 10 µg/ml leupeptin (Sigma), and 4 mM Pefabloc (Roche Diagnostics) to prevent protein breakdown by endogenous proteases. Following homogenization, the samples were aliquoted, frozen in liquid nitrogen, and stored at -80°C. The contralateral hindlimb muscles were divided and oriented in tragacanth gum on a tongue depressor, snap-frozen in isopentane cooled by liquid nitrogen to -159°C and stored at -80°C to await immunohistological examination.

**Quantification of growth factors**

The levels of FGF-2, IL-1β and TGF-β1 were indirectly measured using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN). The assays for both FGF-2 and TGF-β1 were developed for measurement of human growth factors but cross-reacted with rat growth factors, while the IL-1β kit was specific for rats. All samples were thawed to room temperature and centrifuged at 13,500g for 5 min. The supernatant was withdrawn, the pellet discarded, and the sample supernatant was then pretreated, if necessary, as follows. For analysis of TGF-β1, 0.1 ml of 1N HCl was added to 0.5 ml of sample, which was incubated for 10 minutes and then neutralized with 0.1 ml of 1.2 N NaOH/ 0.5 M HEPES. Following pretreatment, 50-200 µl of sample or a standard of known concentration were added to each well and incubated at room temperature for 2-3 hours. The samples were then decanted and the wells washed several times with a provided washing buffer. A growth factor/biotin conjugate containing a polyclonal antibody against the specified growth factor was added to each well and incubated for 2 hours at room temperature, after which the wells were washed again.
Following development of color by addition of a chromogenic substrate solution to each well, the chromogenic reaction was stopped and the optical density of each well was measured using a microplate reader set to 450 nm with a wavelength correction at 540 nm. The growth factor concentration (in pg/ml) was then calculated by plotting the optical density of each sample on the best-fit line generated by the relationship between the concentrations and optical densities of the known standards. The growth factor concentration was then converted to pg/g of muscle by dividing by an experimentally produced factor to account for loss of connective tissue during both the homogenization and centrifugation procedures.

The level of IGF-I was determined using a competitive binding enzyme immunoassay (EIA) (Diagnostic Systems Labs, Webster, TX), specific for rat IGF-I. Sample preparation was necessary to separate IGF molecules from binding proteins, and this was accomplished via a pretreatment with provided buffers. Following pretreatment, the samples, were incubated with 100 µl of rat IGF-I/ biotin conjugate and 100 µl rat IGF-I antiserum for 1 hour at room temperature. Following a wash, 200 µl of streptavidin-enzyme conjugate was added to each well and incubated for 30 minutes. After another wash, a chromogen was added for a 30-minute development period; this reaction was stopped and the optical density measured at 450 nm with a wavelength correction at 600 nm. The growth factor concentration for each sample was measured as described above.
Immunohistochemistry and quantification of macrophage subpopulations

Muscle samples were thawed to -20°C and serially sectioned (8 µm) on a cryostat, placed on a coverslip pre-treated with Poly-L-Lysine (an adhesive) and air-dried overnight. Dried sections were fixed in cold acetone for 10 minutes, blocked with a 2% horse serum solution and then incubated with the primary antibody [ED1 (1:50), ED2 (1:500), IA (1:500)] in a diluting buffer, (1% bovine serum albumin (BSA), 0.1% Tween-20, 1% cold fish gelatin and 2% NaCl in 0.01 M PBS) overnight (ED1) or for three hours (ED2, IA) at room temperature. After a wash, (1% milk powder, 0.5% Tween-20 and 2% NaCl in 0.01 M PBS), sections were incubated in biotinylated anti-mouse IgG (Vector Labs, Burlingame, CA) with 2% rat serum (to block non-specific staining) and 2% horse serum in PBS for 1 hour at room temperature on a shaker. Sections were again washed twice for a total of 30 minutes and then treated with ABC reagent (Vector Labs) for 45 minutes. Endogenous peroxidase activity was quenched by treating sections in 0.3% H₂O₂ in absolute methanol for 10 minutes at room temperature. Antibody reactivity was developed by incubation with nickel-enhanced diaminobenzidine (Pierce, Rockford, IL) for 15 minutes. Sections were then lightly counterstained with eosin, dehydrated with an alcohol series and mounted on glass slides with Permount.

Image analysis of serial sections was performed on a Macintosh G3 computer (Apple Computer, Cupertino, CA) using the public domain NIH Image 1.62 program (http://rsb.info.nih.gov/nih-image/). Direct counts of macrophage subpopulations were performed on 3-5 random digitized frames from each section which, at 100x magnification with a light microscope (Carl Zeiss, New York, NY), were approximately
0.567 mm$^2$ per frame. In order to avoid error in the density counts that could have been introduced by the inclusion of large areas of connective tissue, these parts of the section were avoided. After all stained macrophages in the frames were counted, the average number of macrophages per frame was calculated, and the macrophage density expressed as macrophages per mm$^2$ (mac/mm$^2$). The density values in old rats were multiplied by a correction factor to account for the 15.4% smaller muscle fiber cross sectional area in these animals.

**Histological determination of muscle damage**

Cover slip mounted sections were fixed in acetone for ten minutes, washed in distilled water, and then stained with hematoxylin and eosin. Four to six frames of each section were observed at 100x via light microscope, and the following standards used to classify fibers as degenerating or regenerating (103).

Degenerating fibers: a) smaller than surrounding fibers, with an angular appearance, b) disrupted cytoplasm, poorly stained compared to surrounding fibers, c) large rounded appearance, with fissures in cytoplasm or d) filled with infiltrating cells.

Regenerating fibers: a) round and very small size, located on the periphery of the fascicle, or b) or several smaller ‘split’ fibers developing in the shape of a larger, healthy fiber.

This method of quantification has been used successfully by previous researchers (103) and has been reported to have a high intra-observer reliability. The number of each type of fiber in a section was counted and divided by the total number of fibers per section to be expressed as percentage of degenerating fibers (%DeF), percentage of
regenerating fibers (%ReF), and the overall percentage of damaged (degenerating or regenerating) fibers (%DaF).

**Statistical Procedures**

Data were analyzed using a 2x7 factorial ANOVA for age and time point interaction with the level of significance set at $p \leq .05$, and Duncan’s test used post-hoc to determine the source of differences revealed by ANOVA. In addition, linear regression analysis was used to determine interactions between growth factors and macrophages, with the dependent variable being either phagocytic or non-phagocytic macrophages and the independent variables being measured levels of growth factors. The level of significance for regression analysis was also set at $p \leq .05$. 
Results

Histological observations

There were no differences in body weight among young and old animals pre-exercise (control) or at any other time points. Among control rats, the soleus wet weight as a percentage of body weight (S%BW) of old rats was significantly lower than that of young rats (Table 1). This was also true at 2 hours, and 4, 8 and 12 days post-exercise. The S%BW in old rats was elevated above control values at day 2, and returned to control values thereafter. There was no increase in S%BW in the young rats following exercise.

| TABLE 1. Soleus wet weight and soleus weight as a percentage of body weight for young and old animals |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| | Soleus weight (mg) | Soleus weight as % of b.w. |
| | Young | Old | Young | Old |
| Control | 133.5±7.2 (n=6) | 116.6±8.7 (n=5) | .0323±0.001† | .0276±0.001 |
| 2 hours | 132.1±8.4 (n=6) | 119.6±4.7 (n=5) | .0327±0.001† | .0287±0.001 |
| 1 Day | 131.1±7.5 (n=6) | 126.3±2.5 (n=5) | .0324±0.001 | .0313±0.001 |
| 2 Days | 138.2±5.7 (n=6) | 135.9±7.7 (n=5) | .0336±0.001 | .0315±0.001† |
| 4 Days | 139.5±6.8†(n=6) | 110.4±7.5 (n=5) | .0335±0.001† | .0272±0.002 |
| 8 Days | 129.7±4.5 (n=6) | 115.1±6.8 (n=5) | .0328±0.001† | .0281±0.001 |
| 12 Days | 130.7±3.4 (n=6) | 115.2±3.8 (n=5) | .0323±0.001† | .0279±0.001 |

Values are ± SEM. † Significant difference from age-matched control values p≤ 0.05. ‡ Significant difference from old rat at same time point, p≤ 0.05.

Observation of H&E stained sections revealed the presence of degenerating and regenerating fibers in both control and post-exercise muscles (figs. 8-11). Degenerating fibers mostly appeared in the form of small, angular fibers (most frequent) (fig. 8B). Two subjects had many large, rounded, fissured fibers with unidentified nuclei in the cytoplasm (fig. 10A and B). However, no other animals were observed to have any of these types of muscle fibers.
Fig. 8. Healthy and damaged fibers, H&E treated. A) Healthy fibers (~75x mag), B) Small, angulated fibers, indicated by arrows, were the most common type of degenerating fibers observed. This is an old animal at day 2 post exercise. (100x mag).
Fig. 9. Two types of damaged fibers. A) This damaged fiber displays infiltrating inflammatory cells and a disrupted, poorly stained cytoplasm B) Arrow shows a smaller fiber growing in the outline of a larger one (arrowhead), giving the appearance of a ‘split’ fiber (100x mag).
Fig. 10. Appearance of some damaged fibers. A) Three of the observed muscles displayed numerous large rounded fibers, which dominated the field similar to what is seen above. This type of damage was rarely seen in the muscle fibers of most subjects. (~20x mag.) B) High magnification of fiber. Unidentified nuclei, indicated by arrow, were typical in this type of abnormality. (400x mag.)
Fig. 11. Serial cross-sections of a necrotic fiber. Two days after exercise stained with A) anti-ED1 (1:50), B) anti IA (1:500), and C) (following page) anti ED2 (1:500) antibodies. Note that while both ED1⁺ and IA⁺ macrophages (indicated by arrows) infiltrate the dead cell, few ED2⁺ macrophages are present and do not appear to enter the area the cell formerly occupied (400x mag).
Necrotic fibers with large numbers of inflammatory cells (figs. 9A, 11) were generally only seen in the first 2 days after exercise or occasionally even in control muscles, but were only seen in one day 4 and one day 12 muscle.

The results of the quantification of damaged fibers can be seen in figs. 12-14. Among control rats, there was no statistical difference between the percentage of degenerating fibers (%DeF) in the young (0.62%) and the old population (1.62%). This was also true for the percentage of regenerating fibers (%ReF) (0.27% young, 0.69% old) as well as for the total percentage of affected fibers (%DaF) (0.89% young and 2.31% old). Following the exercise bout, both the %DeF and %DaF was significantly greater in old rats at both 2 hours and 2 days. There were no observed differences in %ReF between age groups at any time during the post-exercise period. Recent evidence using immunohistochemical staining with developmental myosin heavy chain (dMHC) corroborates the low numbers
of regenerating fibers as well as the presence of dMHC in very small, round fibers (personal communication, L. Hilton-Taylor). When comparing results within the age groups, it was shown that %DeF in the elderly group was elevated above control values at 2 hours and 2 days post-exercise, but was not different at any other time point, while the %DaF was elevated only at 2 hours. %ReF did not change following exercise in the old rats. Young rats did not show an increase in %DeF, %DaF, or %ReF during the post-exercise period.

**Macrophage subpopulations**

The results of counts of macrophage subpopulations can be seen in figs. 15-17. ED1+ macrophages were found in very low concentrations in control muscles and were not different in density between young and old rats. When present, necrotic fibers were filled with ED1+ and IA+ macrophages (fig. 11A), the former of which seemed to be in higher concentrations in the area surrounding the necrotic fibers. Necrotic fibers were largely observed in the early stages of injury/regeneration, and were only seen in two muscles after day 2.

In young control rats, the ED1+ density was 9.8 ± 2.2 macrophages per mm² (mac/mm²). By day 2 this value had increased more than six-fold to 61.9 ± 19.4 mac/mm², a significant difference from the control value. The macrophage density returned to control values thereafter. In old control rats, the ED1+ density was 11.8 ± 5.1 mac/mm². Following exercise, this value was highest at day 2 (42.3 ± 20.7 mac/mm² – a 3.5-fold increase); however, at no time was this ED1+ density in old rats significantly different from control values.
Fig. 12. Damaged fibers observed in control and exercised soleus muscles of young and old rats. Values are ± SEM. * Significant difference from age-matched control values, $p \leq .05$.
† Significant difference from young or old rat at same time point, $p \leq .05$. 
Fig. 13. Degenerating fibers observed in control and exercised soleus muscles of young and old rats. Values are ± SEM. * Significant difference from age-matched control values, p ≤ .05. † Significant difference from young or old rat at same time point, p ≤ .05.
Fig. 14. Regenerating fibers observed in control and exercised soleus muscles of young and old rats. Values are ± SEM. * Significant difference from age-matched control values, p ≤ .05.
† Significant difference from young or old rat at same time point, p ≤ .05.
Fig. 15. ED1⁺ Macrophages in control and exercised soleus muscles of young and old rats. Values are ± SEM. *Significant difference from age-matched control values, $p \leq .05$.
† Significant difference from young or old rat at same time point, $p \leq .05$. 
Fig. 16. ED2+ Macrophages in control and exercised soleus muscles of young and old rats. Values are ± SEM. *Significant difference from age-matched control values, p ≤ .05. † Significant difference from young or old rat at same time point, p ≤ .05.
Fig. 17. IA+ Macrophages in control and exercised soleus muscles of young and old rats. Values are ± SEM. *Significant difference from age-matched control values, p ≤ .05. † Significant difference from young or old rat at same time point, p ≤ .05.
Compared to ED1+ macrophages, ED2+ macrophages were found in relatively high concentrations in control muscles of both age groups, but the density was significantly higher in old rats compared to young rats (168.1 ± 5.1 to 128.9 ± 16.2 mac/mm²). In contrast to ED1+ macrophages, ED2+ macrophages were never seen inside necrotic fibers (fig. 11C), and were evenly distributed in all areas of the muscle.

Following the exercise bout, ED2+ density was significantly higher in old than in young animals at 4 days post-exercise. At 8 days post-exercise, the macrophage density had increased above control values in both age groups, but there was no difference in absolute density between old and young, due to a 68% increase in the young population between days 4 and 8 compared to a 12% increase in the elderly rats during the same time period. At day 12 post-exercise, the ED2+ macrophage density remained significantly elevated above control values in both age groups.

The density of IA+ macrophages was less than that of ED2+, but greater than the density of ED1+ macrophages and, similar to ED1+ macrophages, IA+ cells were seen to invade necrotic fibers (fig 11B). There was no difference in IA+ density between the two age groups either pre exercise (67.0 ± 9.8 mac/mm² young, 64.9 ± 10.5 mac/mm² old), or at any time during the post-exercise period. Among old rats, there was a significant increase at 8 and 12 days post-exercise, with the highest value at day 12 (151.9 ± 9.9 mac/mm²). The day 12 value also represented a significant increase over the day 8 measurement. Among young rats, there was a significant increase over controls in IA+ density at 8 and 12 days post-exercise, with the highest mean at day 8 (166.6 ± 19.3 mac/mm²), remaining the same on day 12 (165.9 ± 6.8 mac/mm²).
Growth Factor concentrations

The results of the measurement of growth factors can be seen in figs. 18-20.

**Interleukin-1β**

The measurement of soleus muscle concentration of IL-1β by ELISA revealed no differences in pre-exercise values between young and old rats. There was a difference, however, at 2 hours post-exercise, when the IL-1β concentration was significantly higher in elderly rats (480.5 ± 109.2 pg/g of muscle (pg/g)) than in younger rats (311.8 ± 23.6 pg/g). The IL-1β level at 2 hours also represented a significant elevation over old rat control values. This was the only time point in which the IL-1β values were different from control. It is interesting to note, however, that the mean IL-1β levels at days 4, 8 and 12 were lower than the control value, and that the values at both 2 hours and 2 days were significantly higher than at these other time points. The possible significance of this finding will be discussed. The IL-1β concentration in young animals was unchanged from control throughout the post-exercise period.

**Fibroblast Growth Factor-2**

There were no differences between young and old control values for FGF-2, nor were there any differences between the young and old population in the level of this growth factor during the post-exercise period. When considering the pattern in the old population, a significant decrease was seen at both day 1 and 2 in the post-exercise period, but FGF-2 values returned to control values thereafter. The level of FGF-2 did not change significantly with exercise in the young rats.
Fig. 18. IL-1β concentrations in control and exercised soleus muscles of young and old rats. Values are ± SEM and expressed as pg/g of muscle. * Significant difference from age-matched control values, $p \leq .05$. † Significant difference from young or old rat at same time point. For young rats, n=6 except for IL-β at 2 hrs, and FGF-2 at 2 hrs and 12 d (n=5). For old rats, n=5 except at 2 hrs for all growth factors (n=4).
Fig. 19. FGF-2 concentrations in control and exercised soleus muscles of young and old rats. Values are ± SEM and expressed as pg/g of muscle. * Significant difference from age-matched control values, p ≤ .05. † Significant difference from young or old rat at same time point. For young rats, n=6 except for IL-β at 2 hrs, and FGF-2 at 2 hrs and 12 d (n=5). For old rats, n=5 except at 2 hrs for all growth factors (n=4).
Fig. 20. TGF-β1 concentrations in control and exercised soleus muscles of young and old rats. Values are ± SEM and expressed as pg/g of muscle. *Significant difference from age-matched control values, $p \leq .05$. † Significant difference from young or old rat at same time point. For young rats, n=6 except for IL-β at 2 hrs, and FGF-2 at 2 hrs and 12 d (n=5). For old rats, n=5 except at 2 hrs for all growth factors (n=4).
**Transforming Growth Factor-β1**

There were no differences in the concentration of TGF-β1 between young and old control rats, nor were there differences at any time point between the young and old animals measured in the post-exercise period. There was no distinguishable pattern of change in TGF-β1 in either young or old animals following the exercise bout.

**Insulin-like Growth Factor-1**

There was no detectable IGF-I in any of the animals, as tested by EIA. Since other studies have shown IGF-I to be present in rat soleus muscle, it is suspected that a technical error is responsible for these observations.
**Discussion**

A number of investigators have found that regeneration of skeletal muscle following injury is delayed, incomplete, or in some way deficient in older animals (18), but the mechanism for this phenomenon remains unclear (25,77,78,96,97,118). Macrophages are likely to play a central role in recovery from injury, not only clearing damaged cellular components via phagocytosis and promoting the inflammatory process by releasing pro-inflammatory cytokines, but also aiding in the process of regeneration through the release of important growth factors (reviewed in (93)). Because of these important functions, it was suspected that a decrease in macrophage response to injury or a change in the levels of cytokines and growth factors known to be released by macrophages and other sources may be at the heart of the incomplete recovery of skeletal muscle function seen in older animals.

This study was initiated to compare the response of three functionally distinct macrophage subpopulations, and of selected growth factors and cytokines to a 90-minute bout of downhill running in young and old rats. It was hypothesized that changes in the numbers of macrophages and/or the levels of growth factors at different time points after injury would be different between young and old rats.

**Macrophage subpopulations**

This study is the first to report on the magnitude of the ED1+, ED2+, and IA+ macrophage subpopulations in healthy or damaged elderly muscles. The major finding of the study is that despite the lack of a quantifiable amount of muscle damage, the response of ED1+ and late stage (days 2-12 post exercise) IA+ and ED2+ macrophages to a downhill running bout in young soleus muscle appears to be equal to or slightly more
vigorous than in elderly muscle. While this may not be apparent in terms of directly compared values at each time point, the overall change over time is suggestive of a blunted response in elderly animals. For example, in young rats, IA\(^+\) populations increased by 56% between days 2 and 4, and 107% between days 4 and 8, and remained high until day 12. This represented an overall increase of 246% from day 2 to 12 in IA\(^+\) macrophages in young rat muscles. In contrast, IA\(^+\) populations in elderly rats exhibited a somewhat more measured rise increasing by 38% between days 2 and 4, 8.5% from day 4 to 8, and 37% between days 8 and 12, for a total of a 106% increase from day 2.

A somewhat similar result is found in the response of ED2\(^+\) populations. The overall increase in these cells between days 2 and 12 was 78% in the young rats, and 52% in old rats. ED2\(^+\) macrophages in young rats show the sharpest increase (68%) between days 4 and 8, at a time when ED2\(^+\) cells are increasing more slowly in elderly animals (12% rise), although at the earlier time points, ED2\(^+\) macrophages are increasing more rapidly in elderly rats than in young (38% to 12%, respectively).

Likewise, ED1\(^+\) macrophages in elderly rats, while exhibiting an increased mean value, did not increase significantly. This same subpopulation did show a significant rise in the young rats.

Collectively, these data suggest that while ED2\(^+\) and IA\(^+\) macrophages found in elderly rats may begin to respond to muscle injury or exercise within the same time frame as in young rats, and may reach equal levels eventually, they might not possess the ability to increase their numbers as rapidly as they do in young rats. In addition, ED1\(^+\) macrophages do not seem to respond to the same level in elderly rats compared to young rats. These differences could influence regenerative capacity in elderly rats.
Several groups have reported deficiencies in phagocyte function in aged animals, including decreased respiratory burst (90), decreased responsiveness to chemotactic signals by neutrophils (89) and an attenuated neutrophil mobilization (24). Ashcroft et al. (21) have reported on changes in macrophage populations in young (20-39 years) middle aged (40-59 years) and elderly (60-90 years) humans up to 84 days after a punch biopsy of the skin of the upper arm. These investigators showed that the number of cells labeled with LP9 (a non-specific macrophage marker (88)) was higher in the young than in the old subjects 1, 3 and 7 days post-injury, but higher in the elderly subjects than any other subjects at day 84. This was cited as a delay in the accumulation of macrophages, although no statistical analysis was performed for within age-group macrophage numbers, and no control values of macrophage populations were noted.

The same study documented changes in cells labeled with 25F9 (a marker for mature, tissue macrophages in late-stage inflammation (120)) following cutaneous injury. It was shown that elderly subjects had increased numbers of these cells at all days when compared to young and middle-aged subjects.

Although these previous studies of immune function in the elderly were carried out in a different injury model or in vitro, it is still interesting to compare these results to our own. For instance, there are compelling similarities between 25F9\(^+\) cells and ED2\(^+\) macrophages, since both are described as ‘tissue fixed’ or resident macrophages, and since findings of elevated numbers of these cells in elderly humans at early stages of injury are similar to our own findings in elderly rats. This comparison is complicated, however, and since the possible relationship between the two antigens used to identify
these cells is unclear, it is unwise to view these two separate results as complementary at this time.

In contrast to the seemingly curtailed response to damage, the number of ED2\(^+\) macrophages in non-exercised soleus muscles was significantly higher in elderly animals. There is no clear reason for such a difference, but it may be related to a terminal differentiation of ED1\(^+\) macrophages into ED2\(^+\) cells. The ED2 antigen is a marker of macrophage differentiation (9), and Honda et al. (58) have suggested that one possible source of ED2\(^+\) macrophages in the muscle is differentiated ED1\(^+\) macrophages from the blood, where ED1\(^+\) macrophages are predominant. McLennan (79) has also explored possible macrophage differentiation and stated that ED2\(^+\) macrophages are unlikely to redevelop the ED1\(^+\) phenotype once they have been fixed in the tissue. The author cites observations that necrotic fibers containing large numbers of ED1\(^+\) macrophages are not surrounded by incoming ED1\(^+\) macrophages unless there is a nearby arteriole. If the source of the ED1\(^+\) cells were nearby differentiating ED2\(^+\) macrophages, then necrotic fibers would be surrounded by ED1\(^+\) cells regardless of their proximity to an arteriole.

Collectively, the above evidence leaves open the possibility that the conclusion of inflammation in damaged muscle is marked by the differentiation of a substantial number of ED1\(^+\) macrophages into ED2\(^+\) macrophages. If, as suggested by McLennan (79), these cells are then unable to return to their former phenotype, repeated episodes of damage and regeneration, even as a result of everyday activity, could conceivably lead to a gradual increase in the number of ED2\(^+\) macrophages found in otherwise healthy muscles. This could explain the elevated ED2\(^+\) levels seen in aged muscle, which presumably has undergone a greater number of damage cycles through its lifetime.
compared to muscle found in young animals. Other questions, specifically pertaining to the lifespan of ED2+ macrophages and the control of resting macrophage population numbers will have to be answered to fully explain this phenomenon. It has been reported, however, that ED2+ populations in injured rat solei are not fully returned to control values via apoptosis (110). In fact, no study, including the present one, has documented the return of ED2+ populations to baseline values after injury. Thus, current evidence does not rule out the possibility of longer lived populations of ED2+ macrophages.

The changes in macrophage subpopulations following exercise observed in the current study are similar to a previous study of macrophage response to injury by St. Pierre et al. (107), and our results seem to agree with their interpretation of the functions of these subpopulations in injured muscles. In general, ED1+ macrophages were early responders to damage, and appeared to serve a phagocytic function during the first 2-4 days of the damage cycle. ED2+ macrophages responded later (8 days), and did not invade necrotic fibers, thus seeming to be more involved in late stage regenerative events. IA+ cells appeared to have the ability to serve in both capacities, participating in early phagocytosis, and then rapidly increasing in number in a manner similar to ED2+ macrophages beginning at 8 days post-exercise. The absolute densities of macrophage subpopulations in both healthy and damaged muscles were lower than those reported by St. Pierre et al. (107), but this may be due to species differences.

In contrast to previous results, which showed a sustained ED1+ response from 2 days to 7 days post injury, we have observed a relatively quickly resolved response, wherein the density of ED1+ macrophages returned to control values by day 4. The response of
both ED2\(^+\) and late-stage IA\(^+\) macrophages to downhill running, however, is somewhat later compared to the response to hindlimb unweighting, increasing only after 8 days instead of four.

The most obvious explanation for a disparate macrophage response would be differing amounts of muscle damage, but the above results are not easily interpreted in this context. The shorter phagocytic phase seen after downhill running seems indicative of a less extensive injury; however, the delayed ED2\(^+\) and IA\(^+\) macrophage response induced by this intervention seems more likely the result of a more damaging protocol. A direct comparison of damage might resolve the question, and St. Pierre et al. (107) have estimated damage by counting the number of ‘invaded’, or necrotic fibers. In our case, however, necrotic fibers were very rare: only 15 out of 77 muscles examined contained even one, with only one soleus muscle containing more than one. Thus our preferred method of assessing muscle damage was to count all degenerating and regenerating fibers. This makes a direct comparison of damage impossible and interpretation of the macrophage differences difficult.

**Muscle damage**

Muscle damage was less than expected for the exercise protocol. However, there were indications that the method used to quantify muscle damage may not be sensitive enough to fully detect the extent of muscle damage. First, there was a lack of quantifiable damage at day 1 post exercise in the elderly animals, even though there was significant damage at both 2 hours and 2 days. Second, there was a vigorous macrophage response in the young animals, despite the fact that there was no measureable increase in muscle damage following exercise. A more sensitive method of detecting muscle
damage might have allowed a better comparison of the macrophage/growth factor response to the exercise bout, however, we do not doubt the presence of muscle damage in the elderly animals.

The finding that a similar exercise bout results in a greater amount of damage in elderly muscles was somewhat expected based on the results of previous studies, but may be partly explained by our measurements of muscle wet weight. Our data revealed that soleus muscle weights of old control rats were a smaller percentage of their total body weight compared to young control rats (table 1). This means that soleus muscles of aged rats were subjected to a greater percentage of their maximal force during downhill running, and were therefore more likely to be damaged. Based on previous studies, however, this does not seem to be the sole reason for the difference in muscle damage between young and old populations.

Brooks et al. (19) measured maximal force in situ one minute after single stretch of maximally activated extensor digitorum longus muscles and found that the resulting force deficit was greater in old mice (25-28 months) than in young mice (9-12 months). Reportedly, both old and adult muscles performed the same amount of total work during the stretch. This study confirmed earlier research from the same lab, which showed old mice to be more susceptible to eccentrically induced injury as indicated by force measurements made 3 days post-injury (119). These data would seem to indicate that inherent properties of aged muscle, rather than differences in relative load during exercise, are responsible for differences in muscle damage between old and young muscle.
Growth factors and cytokines

Although several studies have reported on plasma levels of growth factors and cytokines following exercise, investigations of these substances in tissue are uncommon. This study is the first to report the tissue concentrations of several growth factor proteins for as many as 12 days after contraction-induced muscle injury. The known sources of these growth factors in muscle are listed in table 2.

**TABLE 2: Sources of growth factors and cytokines in muscle.**

<table>
<thead>
<tr>
<th>Growth Factor/cytokine</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Macrophages, fibroblasts, neutrophils</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Macrophages, ECM, fibroblasts, muscle cells, satellite cells</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Macrophages, ECM, neutrophils, platelets</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Macrophages, satellite cells</td>
</tr>
</tbody>
</table>

IL-1β is a well-known pro-inflammatory cytokine and its presence during inflammatory situations such as exercise is expected. A number of studies have investigated the presence of IL-1β in plasma and skeletal muscle following exercise with conflicting results. Increases in IL-1β activity have been reported in human plasma samples as early as 3 hours after concentric exercise (21) and following 45 minutes of downhill running (23,41). In contrast, serum samples from mice after 3 hours of downhill running were negative for IL-1β (32), whereas two studies in humans after long distance running (85,106) and one following high intensity eccentric exercise (104) also failed to show any increases in plasma IL-1β. IL-1β may be more easily detectable in the muscle than in the plasma. Two studies have reported that IL-1β in muscle tissue as quantified by immunohistochemistry is elevated immediately and sustained for 5 days following downhill running in humans (22,43) but quantification of IL-1β mRNA
expression by reverse transcriptase-rapid polymerase chain reaction did not reveal significant levels in muscles of exercised mice (32). The negative finding in the latter study may be the result of a methodological issue, as another study of IL-1β in normal and diseased muscles revealed that the detection of IL-1β mRNA in muscle is dependent on the use of ‘optimized’ RNA extraction and quantification procedures (12).

Our results confirm the presence of elevated IL-1β within 2 hours of downhill exercise and show ELISA to be a reliable method of quantification in this exercise model. Since our measurements continued through 12 days post-exercise, both the immediate IL-1β response and the return of this cytokine to control values in elderly animals was observed, unlike previous studies. We report that, in old rats, IL-1β exhibits an immediate response to injury, being significantly elevated at 2 hours post-exercise, and returns to control values by one day. There is also a slight increase of IL-1β at day 2, which, although nonsignificant, appears to mirror the increase in the percent of degenerating and damaged fibers also observed in old rats at 2 hours and 2 days. This similarity is confirmed statistically, as there was a significant correlation between both %DeF and IL-1β (r=.251, p≤ .05) and %DaF and IL-1β (r=.239, p≤ .05). This finding would seem to indicate that muscle damage itself is a fairly potent stimulus for an increase in IL-1β in the muscle, but our data do not shed light on potential sources of IL-1β in the muscle, and it is possible that infiltrating neutrophils, resident macrophages, damaged muscle cells, and even activated satellite cells are responsible for the IL-1β response. It has been shown, however, that stimulated serum cells collected after exercise show an increased ability to secrete IL-1β (23), and further studies on the secretory abilities of intramuscular inflammatory cells such as ED2+ and ED1+ would
provide further clues as to the sources of IL-1β after exercise. The early presence of IL-1β in damaged muscle and its relationship to muscle damage seems to further validate the argument that IL-1β plays a role in the initiation of muscle inflammation following strenuous or injurious exercise (reviewed in (109)).

In agreement with several other studies (103,113), we have shown that mild damage is present even in non-exercised control muscles, as indicated by a small number of degenerating, regenerating and even necrotic fibers, suggesting that everyday activity can cause quantifiable muscle damage. Our results further indicate that the percent of damaged fibers and the level of IL-1β, after a significant increase in old animals at two hours and two days, return to values below (although non-significantly) the measured control values, and remain there for days 4, 8 and 12 post-exercise. Thus, following a bout of eccentric exercise, everyday activity seems to be less injurious to soleus muscles than before the exercise. Besides further illustrating the tight relationship between muscle damage and the muscle concentration of IL-1β, these results seem to provide histological and chemical evidence of the well-described protective effect of eccentric exercise (20,31).

As described earlier, the 16 kDa growth factor FGF-2 has various, sometimes highly potent in vitro effects on satellite cells, endothelial cells, fibroblasts and macrophages, among others. In vivo studies have shown that FGF-2 plays an important role in muscle regeneration, most likely through its effects on these cell types. LeFaucheur et al. (71) have demonstrated that immune neutralization of FGF-2 in vivo results in reduced capillary number, reduced phagocytic macrophage invasion, delayed myofiber necrosis, and delayed proliferation of myofibers (72). This indicates the extent of involvement of
FGF-2 in the normal function of numerous cell types and several important steps in the regenerative process.

While the physiologic functions of FGF-2 have been thoroughly investigated both *in vivo* and *in vitro*, few investigators have measured the time course and level of FGF-2 appearance in damaged muscle. To our knowledge, this study is the first to use ELISA in an attempt to map the pattern of FGF-2 protein expression in muscles exposed to damaging contractions.

The few studies that have measured FGF-2 in skeletal muscle do not lead to a consensus on the FGF-2 response to exercise or muscle damage. Gavin et al. (48) have reported no change in FGF-2 mRNA in gastrocnemius muscles of Wistar rats 1 hour after completion of an hour-long bout of uphill running. In agreement with this finding, Sakuma and colleagues (99), measuring FGF-2 protein using immunohistochemistry, have reported similar findings in rat plantaris muscles over a three-week period of functional overload induced by synergist ablation.

Other groups have seen increases in FGF-2 following tissue damage, and have presented evidence of its importance during muscle regeneration. Anderson et al. (2) measured FGF-2 for 11 days following crush injury to the tibialis anterior in SJL/J and BALB/c mice. In these animals, an increase in localized FGF-2 immunofluorescence for 2 days in SJL/J muscles and for 1 day in BALB/c muscles was reported. In addition, SJL/J muscles exhibited a greater mononuclear cell infiltration, which peaked earlier than the response in BALB/c mice. Many of these cells stained positively for FGF-2, but more so in SJL/J mice. Since BALB/c mice are known to have a decreased ability to regenerate following injury, the authors concluded that FGF-2 expression might be
related to this deficiency. Ashcroft et al. (6) have measured FGF-2 immunostaining intensity in skin wounds of mice and found a significant increase only in old mice after 14 days of healing.

Clarke et al. (29) have presented evidence that FGF-2 might be released from the cytoplasm of muscle fibers following injury, rather than from the ECM. In this scenario, FGF-2 released from muscle fibers would migrate to the outside of the cell and bind to proteoheparin sulfates in the extracellular matrix, in position to act locally to aid in the regeneration of damaged fibers.

Ours is the first group to provide evidence of a decline in FGF-2 protein following injury. It is possible that the lack of a consensus in both the previously cited and current studies is due to the use of different injury models. Only the current study utilized a physiologic model of muscle damage, and thus comparisons between this and other studies, especially those using non-damaging exercise (48) or skin wounding (7) as a model are unlikely to be enlightening. Another possibility for the discrepant results is the use of a different quantitative method. Since the primary source and release mechanism of FGF-2 is unclear, the use of ELISA to quantify FGF-2 levels may yield differing results than immunohistochemistry.

Given the speculative role of FGF-2 as a ‘wound hormone’, which might be an initiator of the inflammatory response following muscle damage (109), an early increase in FGF-2 levels (within 1 day) was expected. It may be that the early response is not of great enough magnitude to be detected by the current method. The primary mechanism of an early increase of FGF-2 levels is expected to be the release of matrix-bound FGF, as described earlier in this paper, but the quantity of FGF stored in this manner and the
mechanism leading to its release following exercise are as yet unknown, making conclusions regarding the lack of an early FGF-2 response obscure.

A suitable explanation for the reported decrease in muscle FGF-2 may be related to the overall (young and old rats combined) negative relationship ($r= -.337$) between FGF-2 and the number of phagocytic macrophages. It is possible that this relationship is indicative of a ‘permissive effect’ by FGF-2 on the phagocytic phase of muscle regeneration, although FGF-2 does not have any known inhibitory effects on macrophages. The decrease in FGF-2 at days 1 and 2 post-injury does correspond to the elevation of ED1$^+$ macrophages seen at this time, and as FGF-2 is generally expected to be more important in late-phase inflammation, given its chemoattractive and proliferative effects on satellite cells as well as its known angiogenic properties (53), a drop in FGF-2 during the phagocytic phase of regeneration is not necessarily inconsistent with previous research. In addition, while FGF-2 is known to be secreted by macrophages (93), it is unknown whether this is true of all macrophage subpopulations, and therefore the return of FGF-2 to control levels during days 4-12 may be the result of FGF-2 secretion by ED2$^+$ or IA$^+$ macrophage populations that are increasing during this time. It is interesting, however, that the depression in FGF-2 levels is significant only in elderly rats, yet only young rats showed a significant increase in ED1$^+$ macrophages. The importance of this finding is unclear.

The physiologic functions of TGF-β have been thoroughly studied, and much is known about the role of this growth factor in the wound environment, as described earlier. To our knowledge, however, there have been no studies into the time course of the expression of this growth factor during muscle regeneration. Our study confirms the
presence of TGF-\(\beta\)1 in normal soleus muscle, but does not reveal any changes with damage.

McLennan and Koishi (81) have provided evidence that TGF-\(\beta\)2 and 3 are both present following non-physiologic muscle injury. Following localized freeze damage, affected muscle fibers were shown to have high amounts of TGF-\(\beta\)2, as measured by quantitative immunohistochemistry. In addition, both satellite cells and immature myotubes were shown to express this growth factor. TGF-\(\beta\)3 was expressed by invading inflammatory cells. Although TGF-\(\beta\)1, 2 and 3 are thought to have somewhat similar functions, the evidence presented by McLennan and Koishi indicates that they are preferentially expressed by different cells and in different time courses, and thus must be considered separately. TGF-\(\beta\)1 is known to be expressed in pathologic conditions such as myositis (112) and muscular dystrophy (116) and is purported to be elevated in the early stages of muscle damage (60), although no published research is available on this matter. \textit{In vivo} results demonstrate the importance of TGF-\(\beta\)1 in the regulation of muscle regeneration, as immune neutralization of this factor results in an increased number of regenerating myofibers in devascularized, denervated mouse extensor digitorum longus. This finding confirmed that TGF-\(\beta\)1 is inhibitory to myofiber proliferation during muscle regeneration, although the mechanism remains unclear.

Since there is no clear evidence from either previous research or the current study that TGF-\(\beta\)1 is measurably elevated following physiologic muscle damage, it seems possible that the effects of this growth factor are regulated by other factors present in the wound environment. For example, TGF-\(\beta\)1 has been shown \textit{in vitro} to deactivate macrophages (111), an event which is of course necessary for effective regeneration of muscle cells.
When, however, macrophages are co-incubated with TGF-β1 and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), TNF-β, or interferon-γ, macrophages still exhibit elevated activation levels as measured by H₂O₂ production (111). Thus, regulation of phagocytosis in the wound environment may come about through a decrease in the concentration of macrophage activation factors, rather than through an increase in the level of TGF-β1. Thus, knowledge of TGF-β1 levels may provide only part of the picture of the importance of TGF-β1 during muscle regeneration.

Our attempts to measure IGF protein levels in damaged muscle using ELISA were not successful; however, several studies have provided evidence of increased IGF following damaging or strenuous exercise. Keller et al. (67) have reported an increased level of both IGF-I and II at 4, 7 and 10 days after eccentrically-induced muscle damage in mice. Developmental myosin heavy chain (a marker of myofiber regeneration) was often co-expressed with IGF at days 7 and 10. Interestingly, inflammatory cells in this study did not stain positively for IGF at any time, indicating that these cells were not a significant source of this growth factor. Another study (64) showed similar expression of IGF-I in regenerating, but not damaged muscle cells. IGF-I expression by muscle cells was noted at approximately 3 days after ischemic injury to rat extensor digitorum longus muscles. Earlier expression of IGF-I was seen in Schwann cells, endothelial cells and satellite cells.

IGF mRNA response to injury has been studied in old rats (75). Following injection of bupivicaine (a myotoxin), IGF-II mRNA was expressed to a lesser extent in adult (12 mo.) and old (24 mo.) rats than in young (2 mo.) rats during early regeneration. IGF-I mRNA expression, however, was similar in all age groups, but remained elevated over a
15 day period in adult and old rats while returning to normal in young rats. The authors speculated that these differences might play a role in the slowed regeneration seen in old rats. It can be argued, however, that these differences may be related to continuing growth and development in 2-month-old rats compared to mature rats.

Several studies have shown that exogenous supplementation of IGF can counteract age-related deficiencies in skeletal muscle function. A study by Chakravarthy et al. (26) showed that the proliferative potential of satellite cells in old rats (25-30 mo.) is decreased after several immobilization-induced atrophy/regeneration cycles. These cycles also resulted in an 11% loss of muscle mass. Exogenous delivery of IGF-I to the atrophied muscles, however, both restored the proliferative potential of satellite cells and resulted in the recovery of almost half of the lost muscle mass. Barton-Davis (10) and colleagues, explored a virally mediated over expression of IGF-I in elderly rats and found a preservation of muscle mass and strength over a 9 month period of observation.

There seems to be little question that both mRNA and protein IGF expression are increased at some time during muscle regeneration. It is also possible that the presence of this growth factor changes with aging and maturation. In addition, experimentally induced increases in IGF-I in old rat muscles has been shown to increase muscle mass and strength. Thus, it seems the level of IGF expression may be related to the inability of older muscle tissue to regenerate efficiently.
Macrophage/growth factor interactions

Based on past studies of these macrophage subpopulations (68,79,107), as well as the evidence presented in this study, it was possible to categorize macrophages as phagocytic or non-phagocytic. This grouping enabled a meaningful investigation of the interaction between growth factors and macrophages during muscle injury.

ED1⁺ macrophages were considered phagocytic at all time points, while ED2⁺ macrophages were considered non-phagocytic at all time points. IA⁺ macrophages, while expected to be non-phagocytic in control muscles, have been shown in this study and previous studies to invade necrotic myofibers (13,107) in the first several days after muscle damage and are thus expected to be phagocytic in the presence of acute muscle damage. In both our study, and previous studies, however, they were very rarely seen to invade muscle cells later than 2 days post-injury. Thus, following the resolution of the early phases of muscle regeneration, they probably serve a similar function as ED2⁺ macrophages, which are non-phagocytic. In addition, our data show a clear separation between the early and late response of IA⁺ macrophages. Therefore, IA⁺ macrophages were characterized as phagocytic at the 2 hour, 1 day and 2 day time points, and non-phagocytic in control muscles, and at the 4, 8 and 12-day time points.

Macrophages did have some relationships with growth factor levels. The only factor significantly related to the number of non-phagocytic macrophages was the level of FGF-2, with a correlation of $r=.393$. Two factors were shown to bear a significant relationship to the number of phagocytic macrophages. IL-1β showed a strong positive correlation ($r=.422$), while the level of FGF-2 was negatively related ($r= -.337$). These data suggest that the increased numbers of a particular macrophage subpopulation may not directly
lead to increases in growth factors, although the opposite may be true, as in the case of IL-1. This is likely due to the existence of multiple sources of growth factors in damaged muscle.
Conclusion

The results of this study seem to confirm our hypothesis that old rats exhibit a somewhat blunted macrophage response to physiologic muscle damage, as indicated by the absence of a significant increase in ED1\(^+\) macrophages in old animals, and a seemingly more sluggish increase in ED2\(^+\) and IA\(^+\) subpopulations between days 2 and 12. The altered macrophage response did not seem to directly affect growth factor levels as expected, and this is probably due to the contribution of other sources of growth factors to the post-injury milieu. IL-1\(\beta\) exhibited a response that mirrored muscular damage, whereas FGF-2 was significantly decreased following muscle injury. The finding that old rat muscles have a higher baseline ED2\(^+\) population was unexpected and is yet another example of the changes occurring in skeletal muscle with age. It is possible that the elevated ED2\(^+\) macrophage density in elderly rats is simply the result of a number of damage/repair cycles where infiltrating ED1\(^+\) macrophages are differentiating into ED2\(^+\) macrophages at the conclusion of the phagocytic response. Further research is needed test this hypothesis.

We had originally expected to see a prominent IGF-I, TGF-\(\beta\)1, and FGF-2 response later in the regenerative process, given their expected functions during that time, but individual growth factors did not appear to be restricted to discrete phases of regeneration. Based on this finding, it seems likely that the interaction of all of the growth factors present in regenerating muscle is more important than the response of any individual growth factor. Given the number of growth factors known to be present in this situation, a more productive approach might involve the use of microarray and similar
technology to obtain a more complete picture of the chemical control of muscle regeneration.

Future directions of research should include further investigations of interventions and therapies to improve muscle healing. Injection of FGF-2, IGF-I and nerve growth factor have been shown to improve recovery of tetanic strength in mice (66), and studies into factors which might improve macrophage proliferation and/or migration would also prove interesting. What is the cause of the deficient macrophage response in aged animals? Does chronic exercise training with aging facilitate or further degrade the macrophage response? The answers to these and other questions may be related to the causes of age-induced sarcopenia, a phenomenon that has long baffled researchers. It is hoped that research into these questions will lead to interventions to improve muscle regeneration in elderly humans and contribute to improved quality of life.
Cited Literature


