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Title: Age influences the early events of skeletal muscle regeneration: Studies of whole muscle grafts transplanted between young (8 weeks) and old (13-21 months) mice.
Year: 2008
Journal: Experimental Gerontology
Volume: 43
Issue: 6
Pages: 550-562
Date: June
Publisher: Elsevier
ISSN: 0531-5565
DOI: http://dx.doi.org/10.1016/j.exger.2008.02.005
Keywords: ageing, skeletal muscle, regeneration, inflammation,angiogenesis
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CSU ID: CSU310595

Age influences the early events of skeletal muscle regeneration: studies of whole muscle grafts transplanted between young (8 week) and old (13-21 month) mice

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Running title: Early regeneration events in aged muscle

Key words: ageing, skeletal muscle, regeneration, inflammation, angiogenesis

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Abstract

Injured skeletal muscle generally regenerates less efficiently with age, but little is understood about the effects of ageing on the very early inflammatory and neovascular events in the muscle repair process. This study used a total of 174 whole muscle grafts transplanted within and between young and old mice to analyse the effects of ageing on the early inflammatory response in two strains of mice (BALB/c and SJL/J). There was a very slight delay in the early inflammatory response, and in the appearance of myotubes at day 4 in BALB/c muscle grafted into an old host environment (implicating systemic events). In SJL/J mice, the initial speed of the inflammatory response was slightly delayed with old muscle grafts regardless of host age (implicating muscle-derived factors), while an old host environment transiently affected myogenesis (myotube formation). The slight delays in inflammatory and neovascular responses in old mice did not dramatically impact on the overall formation of new muscle. The neovascular response to injured young and old muscle tissue was further analysed using the corneal micropocket assay. This showed a very clear 1-2 day delay in angiogenesis induced by old versus young BALB/c muscle tissue implanted into the young rat cornea, indicating that new blood vessel formation is at least partly determined by muscle-derived factors. Taken together these results indicate that, while there are slight age-associated delays in inflammation and neovascularisation in response to injured muscle, there is no detrimental effect on myogenesis in the mouse model used in this study.

Introduction

Old age is associated with a progressive loss of muscle mass and a decrease in force and power. It is well recognised that this is due to atrophy of individual myofibres, although the reasons for this are still the subject of intense research (reviewed in Grounds, 2002; Shavlakadze and Grounds, 2003; Deschenes, 2004; Lynch et al., 2007). One contributing factor is denervation (Carlson 1995; Cartee 1995; Shavlakadze and Grounds, 2003; Ehrhardt and Morgan 2005; Lynch et al., 2007) and this is likely to be combined with a loss and/or change in contractile properties of muscle motor units (Faulkner et al., 1995; Kadhiresan et al., 1996; reviewed in Luff, 1998). There is strong evidence that the ability to re-innervate injured muscle declines with age (Shavlakadze and Grounds, 2003), and thus the long-term regeneration of old muscle is generally less efficient and less successful than young muscle with respect to both morphological (Sadeh, 1988) and functional properties (Gutmann and Hanzlikova, 1976; Grounds, 1987; Carlson and Faulkner, 1989; reviewed in Carlson, 1995; Brooks, 1996). In classical regeneration studies, whole muscle autografts in old rats had impaired return of muscle function when analysed at 60 days compared with almost normal function achieved by even very old (34 month) muscles cross-transplanted into young hosts (Carlson et al., 2001). More recently, a small population of muscle precursor cells that exist well into adulthood was identified, and, while these cells can efficiently form new muscle, their activation and full potential depends on both the muscle and the systemic environment (Collins et al., 2007). This confirms the excellent regenerative capacity of old muscles and emphasises the important role of the host environment in the age-related decline in muscle repair. However, many other factors contribute to the successful repair of injured skeletal muscle and the effects of age, particularly on the highly complex early *in vivo* events, are not well understood.

Myogenesis involves the activation, proliferation, and fusion (to form myotubes) of resident muscle precursor cells (myoblasts) that are normally quiescent on the surface of myofibres where they are termed satellite cells (reviewed in Grounds, 1999; Ehrhardt and Morgan, 2005; Zammit et al., 2006). This process is tightly regulated by a complex interplay between various growth factors, and signalling and extracellular matrix molecules (Grounds, 2008), and involves a range of cell types including leukocytes, macrophages, and endothelial cells. Infiltration of the damaged muscle by inflammatory cells is critical for the breakdown and removal of necrotic tissue (reviewed in Cannon, 1995; Cannon, 1998; Grounds, 1998) and when inflammation is prevented by irradiation (Robertson et al., 1992) or administration of anti-inflammatory agents (Grounds et al., 2005), new muscle formation is impaired. The ingrowth of new blood vessels (neovascularisation) is also required to support the process of new muscle formation (reviewed in Roberts and McGeachie, 1992; Smythe et al., 2002).

The inflammatory and neovascular responses in injured skeletal muscle are dependent on changes in many components including the extracellular matrix (Grounds, 2008)

and the release of growth factors/cytokines from both muscle and non-muscle cell types. Damaged muscle tissue produces many factors, and neutrophils, macrophages and other inflammatory cell types that invade necrotic muscle release further cytokines to amplify the inflammatory response (Robertson et al., 1993; Grounds and Davies, 1996). There is evidence that injured skeletal muscle also secretes neovascular growth factors, as do neutrophils, macrophages and other inflammatory cell types (reviewed in Charge and Rudnicki, 2003; Tidball, 2005; Wagers and Conboy, 2005). However, despite strong evidence that inflammatory (Ashcroft et al., 1998; Linton et al., 2005) and neovascular (reviewed in Edelberg and Reed, 2003) responses are impaired with age in many tissues, the impact of age-associated changes in these responses on muscle regeneration has not been closely investigated.

The present study analysed key early inflammatory, neovascular and myogenic events following skeletal muscle injury, using the whole muscle graft model of muscle regeneration (Roberts et al., 1989; Roberts and McGeachie, 1995; White et al., 2000; Shavlakadze et al., 2004; Grounds et al., 2005) in which mouse extensor digitorum longus (EDL) muscles are grafted onto the surface of the tibialis anterior (TA) muscle. This model causes widespread necrosis within the grafted muscle, followed by a highly reproducible series of events leading to complete new muscle formation. This study uses classical cross-transplantation studies between young and old mice to determine the effects of the age of the systemic environment on inflammation, neovascularisation, and myogenesis. The ability of young and old muscle to stimulate new blood vessel growth was tested specifically using the corneal micropocket assay (reviewed in Hasan et al., 2004).

The early events of muscle regeneration were also compared for two strains of mice, BALB/c and SJL/J, which have previously been shown to have differing capacities for efficient muscle regeneration (Mitchell et al., 1992; .Maley et al., 1994; McGeachie and Grounds, 1995; Roberts et al., 1997). The superior regeneration of SJL/J muscles is due to a faster and more extensive inflammatory response combined with a greater capacity for myotube formation (Mitchell et al., 1992); intrinsic muscle factors appear to play a central role rather than the genotype of the bone marrow-derived macrophages (Mitchell et al., 1995). The SJL/J mice are also dysferlin-deficient and have an autoimmune myopathy with many immunohistochemical changes in older muscles (Nemoto et al., 2007).

There was no marked effect on overall new muscle formation at 7 days between young and old muscles in hosts of the different ages. In both BALB/c and SJL/J mice, an old host environment adversely affected the appearance (timing and number) of new myotubes in muscle grafts. There was a very clear delay in the neovascular response elicited by old BALB/c muscle in the rat corneal micropocket assay associated with an extended phase of inflammation, indicating that the timing and/or profile of angiogenic factors released by muscle does decrease with age.

Materials and Methods

Transplantation of whole muscle grafts in mice

Mature inbred female mice were obtained from the Animal Resources Centre (Murdoch, Western Australia). All animal procedures were carried out in strict accordance with the guidelines of the National Health and Medical Research Council of Australia. Young BALB/c and SJL/J mice were all 2 months (8 weeks) of age, old BALB/c mice were aged 15-21 months, and old SJL/J mice were aged 13 months. All mice received 2 grafts of whole intact extensor digitorum longus (EDL) muscles, either from the same mouse (autograft), or a cross-transplant from a different aged mouse of the same strain (allograft). The transplantation procedure is described in detail elsewhere (Roberts et al., 1989; Roberts and McGeachie, 1995). Briefly, mice were anaesthetised, and the EDL muscles were removed from both hindlegs of donor mice and relocated over the tibialis anterior (TA) muscles of the host. Each EDL muscle was sutured proximally to the distal tendon of the quadriceps femoris muscle and distally to the distal tendon of the TA, and the skin closed with sutures (Roberts and McGeachie, 1995).

Muscle sampling and histological analysis

Surgery was taken as day 0. Between 2 and 7 days after grafting (see Tables 1-3) mice were euthanased by cervical dislocation. Whole EDL muscle grafts with underlying TA muscle were removed, immersed overnight in full-strength fixative (0.1 M phosphate buffered 10% formalin, pH 7.2) at 4°C and processed in a Lynx automatic tissue processor. Muscle grafts were dissected in the mid-region, embedded with both cut surfaces at the top of the paraffin block, and transverse sections (5 μm) collected on silanated glass slides. Sections were stained with haematoxylin and eosin (H&E) to analyse general structure and polyclonal rabbit anti-desmin (Biogenex) antibody conjugated to biotin-avidin was used to identify muscle cells (myoblasts and myotubes) in regenerating grafts (Roberts et al., 1997). The antibody for desmin was detected by colour development using the standard diaminobenzidine system with haematoxylin counterstaining.

All samples were coded, then analysed "blind" using a light microscope. Each section was examined at low power (10x) through to high power (under 100x oil immersion) and analysed histologically, specifically for the infiltration of the inflammatory cells and the presence of desmin-positive myogenic cells. In this model, infiltration of inflammatory cells and the formation of new myotubes occurs progressively from the outer perimeter (periphery) to the centre of the graft (Roberts et al., 1989; Roberts and McGeachie, 1995; White et al., 2000; Shavlakadze et al., 2004; Grounds et al., 2005, see also Fig. 1). Myotubes were distinguished from myoblasts as cells with a diameter greater than 15 μm with central nuclei and condensed cytoplasm: they were very clearly identified with the desmin immunostaining (see Fig. 1). Data for inflammatory cell infiltration and myotube

numbers are shown in Tables 2-4 in semi-quantitative format to enable representation of the large range of variability observed in these parameters.

Rat corneal micropocket assay

Since it was difficult to clearly distinguish newly formed blood vessels invading muscle grafts (because antibodies such as PECAM-1/CD31 that identify blood vessel endothelial cells do not distinguish between original persisting and newly formed vessels (Shavlakadze et al., 2004) the corneal micropocket assay was used to analyse the capacity for young and old injured muscle to elicit a neovascular response. On the day of implantation of mouse muscle tissue into rat corneas (that are avascular), young (2 months) and old (21 months) female BALB/c mice were sacrificed, the EDL muscles removed and maintained in PBS until required for implantation. The removal of the muscle from its neurovascular connections was considered sufficient to induce extensive muscle injury. A total of 20 male Wistar rats aged 6 weeks were used, 10 received young and 10 received old BALB/c EDL muscle tissue implants in both eyes as previously described (Smythe et al., 1997; Hasan et al., 2004). Briefly, the rats were anaesthetised (1.5% v/v halothane/oxygen) and one eye immobilised using clamps. Lignocaine (0.1 v/v in sterile saline) was dropped onto the surface of the eye as a topical anaesthetic. A small incision was made in the cornea (slightly eccentrically) and fine jewellers forceps used to burrow a micropocket within the corneal stroma, working towards the nearest edge of the corneoscleral junction. A small (1-2 mm^3) piece of old or young mouse EDL muscle was implanted into the micropocket, and the pocket pressed closed. Rats were examined under anaesthesia on days 3, 5, 7, 9, 12 and 14 after surgery (day of surgery was taken as day 0) for the presence of a neovascular response. This was identified as a localised bundle of vessels invading the cornea from the area of the sclera nearest the implanted mouse muscle tissue. The percentage of the total distance from the limbus to the nearest implant border that the vessel tips were located was estimated (this could not be quantified accurately due to the changing curvature of the cornea, and the lack of set points from which to measure blood vessel growth). On each observation day after implantation, rats were sacrificed, both eyes removed and immerse-fixed in paraformaldehyde (4% v/v in PBS) for 15 minutes, then stored in 70% ethanol. Two rats were sampled on each of days 3, 5, 7, and 9, while one rat was sampled on each of days 12 and 14. The cornea and adjacent limbal sclera were separated from the remaining orbital tissue and processed through paraffin as described above for muscle grafts. H&E-stained 5 μm sections of each cornea were examined for histological differences in the neovascular responses elicited by old and young muscle. Blood vessels were very easily identified in the corneal stroma early in development due to the presence of erythrocytes in the lumen, and later as obvious capillaries.

Results

Histological analysis of whole muscle grafts transplanted between young and old mice

A total of 174 grafts were analysed over a wide range of sampling times to provide a broad picture of the key regenerative events. In all grafts, a zonal pattern of regeneration was seen where the necrotic donor muscle was infiltrated by inflammatory cells and replaced by myoblasts that fused to form myotubes progressively from the periphery of the graft towards the centre, as has been described extensively elsewhere (Roberts et al., 1989, Roberts and McGeachie, 1990, Roberts et al., 1997). Staining with antibodies to desmin readily identified myoblasts and myotubes (see Fig. 1). As discussed in detail below, slight differences were seen in the timing of regeneration, as measured by the extent of leukocyte infiltration and the appearance of myotubes within the grafts, between the auto- and cross-transplants in young and old hosts, and also between the two strains (BALB/c and SJL/J) of mice.

BALB/c mice

Two experiments were carried out using BALB/c mice; a preliminary one with 15 month old mice (Experiment 1, see Table 1) and a later one using 21 month old mice sampled mainly between 4 and 7 days, with many grafts (n=5-8) analysed at day 5 (Experiment 2, see Table 1). Data from the study using 15 month old BALB/c mice (Experiment 1) showed only slight differences between any of the groups of grafts with respect to the speed and extent of leukocyte infiltration over the first 7 days, as observed in H&E sections (Table 2). In all cases, leukocytes were present within 2-3 days of grafting, and progressively infiltrated towards the centre of the graft. This appeared to be slightly slower at day 4 in old autografts and in young grafts in old (15 month) host mice (groups 2 and 4) where leukocytes had infiltrated about 200μm into the graft, compared with young autografts and old (15 month) grafts in young hosts (groups 1 and 3) where infiltration had progressed up to 300μm. However, this trend at day 4 was not seen for old autografts and young grafts in old 21 month host mice (groups 5 and 7) where leukocyte infiltration was the same or better than in young autografts and in old grafts in young hosts (groups 1 and 6). i.e all of the grafts in Experiment 2 had similar levels of inflammatory cell infiltration by day 4.

At days 6 and 7, leukocytes had completely penetrated to the centre of some young autografts (group 1), but overall the extent of inflammatory infiltration was slightly less in the four old (15 month) autografts (group 2) examined at day 7. Leukocyte infiltration was similarly slightly less in all grafts in old 21 month hosts (Table 2, groups 5 and 7) at days 6 and 7 compared with all grafts into young hosts (Table 2, groups 1 and 6), indicating that infiltration of inflammatory cells was overall slightly more effective in young host mice (Table 2).

Myotubes were readily observed in transverse sections of grafts stained with H&E and with desmin immunostaining, and were first seen at 4 days in most grafts (Table

with the exception of 3 of the 4 groups of grafts implanted into old host mice (Table 2, groups 4, 5 and 7). At day 5, the numbers of myotubes in grafts was generally low and variable. Notably, myotubes were not detected in any of the 6 autografts of young muscle (Table 2, group 1). This lack of myotubes at day 5 was later confirmed in an additional group of 6 young autografted BALB/c mice (12 grafts; data not shown). Desmin staining was used to confirm that myoblasts and myotubes were not present in any grafts at earlier time points. By days 6 and 7, many myotubes were conspicuous in all grafts.

At day 6 in Experiment 2 (where grafts were intensively sampled between 4 to 7 days and the old mice were aged a further 6 months), fewer myotubes were present in all grafts made into old hosts (Table 2, groups 5 and 7 compared with groups 1 and 6 respectively). This indicates an adverse effect of the old host environment, and the role of host factors on myotube formation is emphasised by the similarity of both young and old grafts (Table 2, groups 1 and 6) in the young host environment. However, this difference was transient and all groups of BALB/c mice had similar numbers of myotubes by day 7 (Table 2).

Taken together these data for the first 7 days after transplantation suggest that there is a very slight delay in the progression of inflammatory cell infiltration into whole EDL muscles grafted into an old host systemic environment (old autografts and young grafts in old host mice). What is more consistent is a transient delay (of about one day) in numbers of myotubes formed in old hosts (regardless of the age of the graft), implicating host factors in the efficiency of myotube formation with some delay in aged hosts. The slight delay in the progression of myotube formation in old hosts (that was evident in the 6 day grafts) correlated weakly with the small delay in the inflammatory response. Importantly, there was no overall difference in the appearance of any grafts at one week after transplantation, showing that the older host environment has no dramatic adverse effect on the overall muscle regeneration process in this mouse model of regeneration.

SJL/J mice

Since previous studies have reported differences in the efficiency of leukocyte infiltration and muscle regeneration between BALB/c and SJL/J mice, whereby the latter showed more efficient regeneration overall (Roberts et al., 1997), Experiment 3 investigated whether the mouse strain influenced the pattern of regeneration in old (13 month) SJL/J mice, with an emphasis on the earlier time points (comparable to Experiment 1 for BALB/c mice).

At 3 days, more leukocytes were seen in young autografts (group 8 in Table 3) compared with old autografts (group 9), suggesting that the initial leukocyte response was more adversely affected by age in this mouse strain (Fig. 1). However, an age-related host influence was not supported as the reverse trend was seen at 3 days in both sets of cross-transplanted grafts (Table 3, groups 10 and 11) (Fig. 1, A compared

with C, B compared with D), instead suggesting a correlation between reduced leukocyte infiltration and age of the muscle graft. No marked differences in leukocyte infiltration were apparent at 4 days or 5 days between the groups of grafts and, as for the BALB/c mice, young autografts were fully infiltrated by inflammatory cells by day 7 whereas the duplicate old autografts were not (Table 3).

Myotubes were first observed in young SJL/J autografts at 3 days, but they were not present in old autografts (group 9) until day 4 (Table 3). Some adverse influence of host age on myotube formation was further suggested by data at day 4 for groups 10 and 11, in which at least half of the grafts remained devoid of myotubes. A positive effect of the young systemic environment on myotube formation was further supported by the good myotube formation at day 5 in the one old graft implanted into a young host (group 10) (Fig. 1).

In summary, in SJL/J mice, inflammatory cell infiltration was slightly faster at day 3 in response to young muscle grafts regardless of host age, suggesting that there is a more potent chemoattractant stimulus for leukocytes produced by young muscle. Myotube formation was earlier and more extensive in young autografts compared with old autografts at days 3 and 5; while the numbers of grafts are relatively few, these observations combined with data for the cross-transplanted grafts support an adverse effect of host age on myotube formation.

Since a slight early difference in the pattern of inflammatory cell infiltration occurred in cross-transplanted muscles between BALB/c and SJL/J mice, the morphology of the underlying host TA muscle was examined closely in each strain and age group. While a small amount of damage was sustained at the surface of the host TA (underlying the graft) in most cases, we focussed on the structure of the deeper, undamaged muscle. Interestingly, with the exception of a slight hypertrophic change in the 21 month muscles (probably accounted for by normal growth that increases myofibre size), there were few obvious changes in the BALB/c host muscle morphology between 8 weeks, 15 months, and 21 months of age with respect to centrally nucleated myofibres (a sign of previous regeneration), or numbers of inflammatory cells (Fig. 2). However, all TA muscles from 13 month old SJL/J host mice were conspicuously hypertrophic (i,e increased in size of overall muscle and of individual myofibres), had numerous centrally nucleated myofibres, and contained many inflammatory cells throughout the tissue (Fig. 2), compared with the 8 week old SJL/J mice. The hypertrophy observed in old SJL/J muscles was also noted in previous studies at 12 months of age (Bergman and Grounds, unpublished data). These differences in the host muscle properties are likely to contribute to the observed strain-specific differences, particularly the early inflammatory events.

Corneal micropocket assay in rats

The neovascular responses elicited by young and old muscle were assessed using the corneal micropocket assay. This assay was used to measure *in situ* blood vessel

growth in response to neovascular stimuli produced by muscle tissue. Small pieces of young (3 month) or old (21 month) BALB/c EDL muscles were implanted into the corneas of young (2 month) Wistar rats, and the growth of new blood vessels from the sclera into the normally avascular cornea examined at days 3, 5, 7, 9, 11 and 14 after implantation (day 0). Corneas were examined *in situ* in anaesthetised rats with the aid of an operating microscope and the distance (as a percentage) from the edge of the sclera to the cornea estimated and recorded (Fig. 3). At each time point, corneas were also sampled for histological analysis (Fig. 4).

On day 3, few blood vessels were present within the corneas and there were no obvious differences in blood vessel growth induced by tissue of either age group. By day 5, blood vessels had grown about 75% of the distance between the sclera and young muscle implants, but only about 60% of the distance towards old muscle implants (Fig. 3). The differences between implant types were most striking at 7 days, when vessels had reached and invaded the young implants, but had not grown any further towards the old implants (Fig. 3). By 9 days, vessels had reached and invaded the old implants. Vascularisation was sustained for both implant types up to 14 days (i.e. there was no apparent regression of the vessels).

Histological analysis of H&E sections of the corneas showed that the implants first induced an inflammatory response, followed by a neovascular response. Many polymorphonuclear leukocytes were present in the corneas by day 3 and there were no differences in the intensity of the initial inflammatory response induced by young and old muscle implants (Fig. 4). There were very few leukocytes remaining in corneas implanted with young muscle at day 5, and this was concomitant with the appearance of corneal blood vessels (Fig. 4). In contrast, corneas implanted with old muscle still exhibited a strong inflammatory response and remained devoid of blood vessels at 5 days (Fig. 4). By 9 days many blood vessels were easily observed throughout the stroma of all corneas, although the strong inflammatory response in corneas containing old muscle implants persisted (Fig. 4). By 14 days the stroma of all corneas was mostly devoid of inflammatory cells, and only the old muscle implants themselves retained a relatively high concentration of leukocytes.

These results demonstrate that when implanted into the corneas of young rats, old BALB/c muscle induces a more prolonged inflammatory response and a delayed neovascular response compared with young BALB/c muscle, and suggests that there are age-associated changes in the profile of both inflammatory and neovascular factors released by injured muscle.

Discussion

The combined results of the present study are in accordance with the hypothesis that the inflammatory and neovascular responses are delayed with age, although the effects were very small. While these differences are subtle and barely significant in this small mouse model, such age-related differences might be critical in the regeneration of large muscle masses after injury or transplantation in bigger animals. There was no evidence of an impaired capacity for myogenesis (i.e myoblast activation, proliferation, differentiation and fusion to form myotubes) between old and young mice. The data are discussed below with respect to the age of the host and graft, and strain-specific differences.

Inflammatory events

It is well established that an efficient inflammatory response is important for successful muscle repair to take place (Robertson et al., 1992; Grounds et al., 2005), and it has been reported that inflammatory processes in general are impaired with ageing (Ashcroft et al., 1998; Linton et al., 2005). With ageing, both deficiencies in cytokine release as well as production of a number of inflammatory mediators are reported (reviewed in Krabbe et al 2004), and it seems likely that there is an overall imbalance in the ability to mount an efficient inflammatory response to injury. To determine the effects of ageing of the systemic and/or local muscle environments on the pattern of inflammatory cell infiltration into injured muscle, the present study analysed whole muscle autografts in young and old mice, and muscles allografted (cross-transplanted) between young and old mice at 2-7 days after grafting. This was also compared between two strains of mice with varying capacities for muscle repair to determine if there is a correlation between inflammation and muscle regeneration. It was noted that when young or old muscles were transplanted into a very old (21 month) systemic environment, the overall progression of leukocyte infiltration was inconsistent, with the initial response being rapid and aggressive, but with slow overall progression towards the centre of the graft. At later time points (day 7) old BALB/c autografts were not fully infiltrated by inflammatory cells compared with young autografts. While old muscles may have a larger cross-sectional diameter than young muscles, this is not expected to affect the distance from the edge of the graft that leukocytes have infiltrated. It should also be noted that only one 21 month old host mouse (with 2 grafts) was available for the day 7 data for both the autograft and allograft (groups 5 and 7), and unfortunately greater numbers of old mice were not available in this study for cross-transplantations between old and young mice to address the influence of host versus graft age at late time points. Therefore, further studies may be required to clearly dissect the specific events and the contribution of systemic versus local muscle factors at late time points during the regenerative process.

A transient difference in inflammatory cell infiltration was indicated in the first few days between young and old autografts in SJL/J mice, whereby the progression of leukocytic infiltration was slightly delayed in old muscles in old hosts. Interestingly

young muscles grafted into old host mice more closely represented the young autografts, while the old muscles grafted into young hosts resembled the old autografts, indicating an association with the age of the muscle itself. This is contradictory to other studies reporting that the systemic environment exerts the most influence over the process of muscle regeneration (Carlson and Faulkner, 1989; Conboy et al., 2005). However, given that many of the growth factors released by injured muscle are chemotactic for inflammatory cells (Grounds and Davies, 1996; Charge and Rudnicki, 2003; Tidball, 2005; Wagers and Conboy, 2005), an age-associated effect driven by the muscle itself is not unexpected. It is likely that both systemic and local muscle factors work in close concert to co-ordinate inflammatory, neovascular and myogenic events in the regeneration process. A possible explanation for the fact that this effect was observed only in SJL/J mice is that ageing may have a greater impact on the production of inflammatory mediators by the muscle itself, and a less dramatic impact on the systemic environment and this may well represent a strain-specific difference between SJL/J and BALB/c mice.

The underlying host TA muscles in 13 month old SJL/J mice contained many inflammatory cells, and the ready availability of these cells to respond to cytokines secreted by young muscle grafts may contribute to the more rapid inflammatory response that was observed. It is well documented that the SJL/J mice have a strong autoimmune response and inflammatory muscle disease that increases by 8 months of age and is pronounced by 12 months (Weller et al., 1997). There are many altered immune parameters in SJL/J muscles related to inflammation including more mast cells and pronounced immunohistological changes in old mice (Nemoto et al., 2007). It is now recognised that SJL/J mice are dysferlin deficient and are a model for the late onset dysferlinopathies that have many immunological aspects (Wenzel et al., 2005). Previous studies indicated that it is the SJL/J muscle that is responsible for the enhanced inflammatory response rather than the systemic environment per se (Mitchell et al., 1995), and this is endorsed by the present cross-transplantation experiments whereby the age of the transplanted muscle was a stronger determinant of the inflammatory response than the age of the host environment.

Neovascular response elicited by old versus young muscle

The results from the corneal micropocket assays carried out in this study clearly show a delayed neovascular response induced by old versus young BALB/c skeletal muscle, suggesting that the profile of muscle-derived neovascular growth factors changes with age. Furthermore, old muscle induced a stronger and more prolonged inflammatory response in the rat cornea. This suggests that young and old injured muscles secrete different types or levels of soluble growth factors that are chemotactic and/or mitogenic for leukocytes. Leukocytes are generally associated with corneal neovascularisation (Fromer and Klintworth, 1975; McCracken et al., 1979; Strieter et al., 1992), although studies in x-irradiated animals demonstrated that neovascularisation still occurs in the absence of leukocytic invasion (Eliason, 1978). There is evidence that leukocytes mediate new blood vessel growth by releasing

proteases or endothelial cell chemoattractants (Fromer and Klintworth, 1976; Montesano et al., 1984; Brannstrom and Norman, 1993), and this is also likely to be the case in regenerating muscle where leukocyte infiltration just precedes new blood vessel invasion (Roberts et al., 1989; Roberts and McGeachie, 1990). A prerequisite for new blood vessel formation is localised breakdown of the basement membrane and the interstitial extracellular matrix (ECM). This precedes the proliferation and migration of capillary endothelial cells into the surrounding tissue and formation of new vessels (reviewed in Hallmann et al., 2005). Since the complex molecular composition and amount of ECM changes with age around the myofibres of the graft and in the underlying host tissue (reviewed in Grounds, 1998; Grounds, 2007), this is also likely to affect the speed of new blood vessel growth into the muscle grafts.

While experimental studies have clearly demonstrated that revascularisation of injured muscles is essential to the repair process (Roberts and McGeachie, 1992), the effects of ageing on neovascularisation were relatively mild and did not impact on the process of muscle regeneration in the small grafts of mice aged up to 21 months. Regeneration of larger skeletal muscle masses may reveal more amplified effects of delayed neovascularisation especially in very old animals.

New muscle formation (myogenesis)

The formation of new muscle following injury is clearly dependent on the presence of inflammatory cells and associated revascularisation (in situations where blood vessels are damaged) (Roberts and McGeachie, 1992; Bondesen et al., 2004; Grounds et al., 2005). There were no apparent differences in the timing and extent of myotube formation between most graft types in the study using 15 month old BALB/c mice, and this is in accordance with autoradiographic studies where the onset of myoblast replication in muscle grafts was the same in young (2 month) and old (39 week) mice, although the time to peak myoblast replication was delayed by 26-36 hours in old compared with young muscles (McGeachie and Grounds, 1995). Again, the lack of any striking age-related effects in these early events may reflect differences that were too small to detect in such mouse grafts. However, a more marked 1-2 day delay in the appearance and numbers of myotubes occurred in muscles (young or old) grafted into a 21 month old host environment, being most evident in grafts sampled at days 4 to 6. A similar delay in myotube numbers related to an older host environment was observed in (young or old) muscles transplanted into the old SJL/J hosts. However, the number of myotubes increased rapidly and by one week after transplantation was similar in most graft types. These *in vivo* results emphasise that there is no dramatic adverse effect on these early events of new muscle formation in old animals, and that any slight differences do not impact significantly on the overall outcome of muscle regeneration by one week in this mouse model of regeneration.

While old muscle retains an excellent capacity to form new muscle, the majority of studies in this area indicate that functional muscle regeneration is generally less efficient and successful compared with young muscle (reviewed in Grounds, 1998).

This can be attributed to the effects of ageing on both local and systemic factors that interact to regulate the critical inflammatory and neovascular responses that precede new muscle formation as discussed above, combined with the well documented adverse effects of the old host environment on re-innervation that is essential for muscle contraction and function (Shavlakadze and Grounds, 2003). Recent studies have demonstrated that certain signalling pathways involved in satellite cell activation are affected by ageing (Conboy et al., 2003) and that these events are controlled predominantly by the systemic environment (Conboy et al., 2005). In particular, it seems that a serum factor associated with the Wnt pathway is involved in the delayed activation of satellite cells and increased fibrosis in aged muscle (Brack et al., 2007). The present study supports a role for the host environment in myotube formation. Other studies in young animals emphasise that the systemic environment has a strong influence over muscle regeneration, since whole muscle autografts in young SJL/J mice regenerated more rapidly and efficiently than young BALB/c autografts, but BALB/c muscle regeneration was improved by cross-transplantation of muscles into SJL/J host mice (Roberts et al., 1997).

It is also important to consider the age of the old mice used in the present study. In the conventional sense, "old" usually refers to post-maturation with a loss of homeostasis (e.g. mice aged more than about 12-18 months, or rats aged from about 24 months). Many studies in the literature instead compare young rodents with older (but not aged) adults that range from 2 month old mice (Pastoret and Sebille, 1995), to 10 month (Yablonka-Reuveni et al., 1999) and 18 month old rats (Marsh et al., 1997); yet such comparisons may barely reflect the changes seen in very old geriatric animals such as the 31, 32-34 and 35 month old rats used by Marsh *et al,* (1997), Carlson *et al.* (2001) and Kaminska *et al.* (1998) respectively. It seems reasonable to consider that the processes of normal growth and maturation may be distinct from the main events responsible for pathological ageing especially in the post-reproductive phase of life (Martin et al., 1996). In the present study, the oldest animals used were 21 month BALB/c mice. It was expected that differences would be apparent at this age since the life span of laboratory mice is generally about 2 years, however extreme age may be required to reveal age-related changes in rodent muscles (Kaminska et al., 1998) and this should carefully be considered in the design of future experiments.

In summary, the results reported here indicate that the inflammatory, neovascular and myogenic responses to skeletal muscle injury by whole muscle grafting are very slightly delayed in old muscle and/or an old muscle environment in BALB/c and SJL/J mice. These subtle delays are transient, and by 7 days after whole muscle transplantation no significant morphological differences were observed between any experimental groups. While these results demonstrate that ageing of the host and/or muscle environment does not strongly impact on overall muscle repair following injury in this small animal model, the slight differences observed here are likely to translate to stronger adverse effects on muscle regeneration in larger animals.

Acknowledgements

The authors thank Ms Hannah Radley for technical assistance. This research was supported by funding from the National Health and Medical Research Council of Australia (MDG).

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Table 1: Total numbers of whole muscle grafts analysed for each experiment.

Group		**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Total**
	Experiment 1 BALB/c (2 and 15 months)							
1	Yg/Yh*	1	1	3	6	4	4	19
2	Og/Oh	8	4	4	4	0	4	24
3	Og/Yh	2	2	2	2	0	0	8
4	Yg/Oh	4	2	4	2	0	0	12
								62
	Experiment 2 BALB/c (2 and 21 months)							
5	Og/Oh	0	2	3	8	4	2	19
6	Og/Yh	0	2	6	5	4	4	21
7	Yg/Oh	0	2	8	6	4	2	22
								62
	Experiment 3 SJL/J (2 and 13 months)							
8	Yg/Yh	2	2	4	2	0	2	12
9	Og/Oh	4	4	3	2	0	2	17
10	Og/Yh	2	4	4	2	0	0	12
11	Yg/Oh	2	4	3	0	0	0	9
								50

Yg/Yh = young autografts, Og/Oh = old autografts, Og/Yh = old graft in young host, Yg/Oh = young graft in old host,

* all Yg/Yh grafts performed in Experiment 1 and Experiment 2 were combined to increase sample sizes. A further 12 Yg/Yh autografts were sampled at 5 days for myotube analysis (data not shown - see Results section).

Table 2: Whole EDL muscles grafted in young (Y=2 month) and old (O=15 month or 21 month) BALB/c mice. The young autografts from both experiments are pooled as group 1 and used for comparison throughout. For groups 2-4, old grafts/hosts were 15 months (O=15 months), and for groups 5-7 old grafts/hosts were 21 months (O=21 months). Grafts were sampled at 2 to 7 days after transplantation, transverse sections stained with H&E or with desmin antibodies and analysed for the presence of leukocytes (L) and myotubes (M). The extent of leukocyte infiltration and myotube formation are indicated (with numbers in grafts having this appearance shown in brackets).

Group	Graft/host	Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
		L	M	L	M	L	M	L	M	L	M	L	M
1	Yg/Yh (auto)	-	- (1)	*	- (1)	***	- (1)	**/***	- (5)	****	++++ (2)	****	+++ (2)
	2 month					***	+ (2)	***	- (1)	comp	++++ (2)	comp	+++/++++ (2)
2	Og/Oh (auto)	*	- (6)	*	- (4)	*/**	- (2)	**/***	- (2)			**	++++ (1)
	15 month	**	- (2)			**	+ (2)	***	++ (2)			***	++++ (3)
3	Og/Yh (Xtrans)	-	- (1)	*	- (1)	***	+ (2)	**	- (1)				
	15 month	*	- (1)	**	- (1)			**	++ (1)				
4	Yg/Oh (Xtrans)	**	- (4)	**	- (2)	*	- (2)	***	- (1)				
	15 month					**	- (2)	***	+++ (1)				
5	Og/Oh (auto)			**	- (1)	*	- (1)	***	- (5)	**	- (2)	**/***	++++ (2)
	21 month			***	- (1)	***	- (2)	****	- (3)	***	+++ (2)		
6	Og/Yh (Xtrans)			*	- (2)	*/**	- (4)	**	- (1)	***	++++ (2)	****	+++ (2)
	21 month					**	+ (2)	***	+ (4)	comp	++++ (2)	****	+++ (2)
7	Yg/Oh (Xtrans)			*	- (1)	*/**	- (5)	**/***	- (3)	**	+/++ (2)	***	++++ (2)
	21 month			***	- (1)	***	- (3)	****	- (3)	**	+++ (2)		

Key:

Yg/Oh (auto) =Young autograft
Og/Oh (auto) =Old autograft
Og/Yh (Xtrans) =Old muscle graft in young host (cross-transplant)
Yg/Oh (Xtrans) =Young muscle graft in old host (cross-transplant)

L: extent of leukocyte infiltration into the transplant as measured from the peripheral edge of the graft (- = no cellular infiltration, * = up to 100μm, ** = up to 200μm, ***= up to 300μm, **** = up to 400μm, comp = complete cellular infiltration to the centre of the graft).
M: number of myotubes (- = no myotubes, + = 1-10 myotubes, ++ = 11-20 myotubes, +++ = 21-40 myotubes, ++++ = >40 myotubes, comp = myotubes packed throughout the graft).

Table 3: Whole EDL muscles grafted in young (Y=2 month) and old (O=13 month) SJL/J mice. Grafts were sampled at 2 to 7 days after transplantation, transverse sections stained with H&E or with desmin antibodies and analysed for the presence of leukocytes (L) and myotubes (M). All other details are the same as for Table 2. (# the second graft at this time was unsuccessful for technical reasons).

Group	Graft/host	**Day 2**		**Day 3**		**Day 4**		**Day 5**		**Day 6**		**Day 7**	
		L	M	L	M	L	M	L	M	L	M	L	M
8	Yg/Yh (auto)	*	- (2)	**	+ (2)	**	- (1)	***	++ (1)			comp	++++ (2)
	2 month					**/***	+ (3)	comp	++++ (1)				
9	Og/Oh (auto)	*	- (4)	*	- (4)	**	+ (3)	**	+ (1)			***	++++ (2)
	13 month							comp	++ (1)				
10	Og/Yh (Xtrans)	*/**	- (2)	*	-(3)	**	- (2)	***	++++ (1)#				
	13 month			* *	- (1)	**	+ (2)						
11	Yg/Oh (Xtrans)	*	- (2)	*	- (1)	**	- (2)						
	13 month			**	- (3)	**	+ (1)						

Figure Legends

Figure 1: Representative histology of H&E (A-D) and desmin (E, F) stained sections of whole EDL muscle grafts in SJL/J mice sampled at 3 (A-D) and 5 (E, F) days. (A) autografts in young (Yg/Yh) and (B) 13 month old (Og/Oh) mice, (C) young muscles cross-transplanted into old hosts (Yg/Oh) and (D) old muscles transplanted into young mice (Og/Yh). All grafts are orientated with the underlying TA at the bottom of the picture. Grafts at 3 days show a slightly stronger inflammatory response in the periphery (indicated by "p") in young muscle grafts regardless of host age (A, C) compared with old muscles grafted into either old or young hosts (B, D). Desmin staining at day 5 demonstrates fewer desmin-positive myotubes (arrows) in the periphery of old autografts (E) compared with old grafts in young hosts (F). Activated desmin-positive myoblasts (*) "cuffing" the scaffold of dying myofibres, probably satellite cells, were more abundant in old grafts in young hosts (F) compared with old autografts (E) at 5 days. Bar = 100 μm for A-D, 50 μm for E-F.

Figure 2 H&E stained sections of BALB/c (A-C) and SJL/J (D, E) host TA muscles underlying the EDL grafts. Between 8 weeks (A), 15 months (B) and 21 months (C) of age, the only obvious change in BALB/c muscle morphology was slight fibre hypertrophy, as shown by the increased diameter of most muscle fibres compared with younger muscles. In contrast, in SJL/J mice, between 8 weeks (D) and 13 months (E) of age, there was striking myofibre hypertrophy and also many centrally nucleated myofibres (arrows) and clusters of inflammatory cells (*). For all images the bar = 100 μm.

Figure 3 Quantitative analysis of blood vessel growth elicited in rat corneas by young (3 month) and old (21 month) BALB/c muscle implants. Mouse muscle was implanted on day 0, corneas were examined on days 3, 5, 7, 9, 11 and 14 to measure the percentage of the distance between the sclera and the implant that was vascularised. Data show the average distance (as a percentage of the total distance) covered by new blood vessel on each day for 4 different rats (n=8 corneas) ± S.D. There was an obvious delay in the rate of growth of new vessels induced by old compared with young muscle at days 5-7 after implantation. Student's t-test was used to compare the average distance covered by new blood vessels at each time point (*$p<0.05$).

Figure 4 H&E stained sections of rat corneas implanted with young (A, C, E) and old (B, D, F) BALB/c muscle and sampled at 3 (A, B), 5 (C, D) and 9 (E, F) days. At day 3 (A, B) there were no differences in the appearance of the corneas implanted with young (A) or old (B) muscle, and in both cases many inflammatory cells were present (white arrows). In contrast at day 5 (C, D), corneas implanted with young muscle (C) contained many newly formed blood vessels (black arrows), while corneas implanted with old muscle were still devoid of blood vessels (D). By day 9 (E, F), all corneas

were characterised by many capillaries growing towards the muscle implant (black arrows). Bar = 50 μm.