Satellite Cell Frequency in Cross-Age Transplanted Rat Extensor Digitorum Longus Muscles

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Abstract

The aim of this study was to find out whether the frequency of satellite cells in cross-age grafts is more influenced by the age of the host or the age of the graft. Extensor digitorum longus muscles (EDL) of young and old animals were excised and cross-age transplanted. M-cadherin-stained (M-cad) satellite cells were counted within stacks of optical images applying the DISECTOR principle. Satellite cell frequency was larger in young than in old control muscles. Data on the frequency of satellite cells in cross-age transplanted muscles indicate that despite a doubling of the satellite cell count and a higher incidence in old-into-young transplants, the effect of the host environment, although seemingly present, is not as strong on this property of the regenerated muscle as it is for certain other characteristics.

Key words: aging, confocal microscopy, optical disector, regeneration, satellite cells, skeletal muscle, stereology.

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Skeletal muscle in old animals is able to regenerate. If the trauma is severe, particularly if it involves motor nerve damage, regeneration is poorer than in younger animals [5, 13, 19], but under certain circumstances, especially if the nerve is not damaged, massively damaged muscles in old rats can regenerate up to control levels [7]. However, control muscles in old individuals are substantially smaller and weaker than those in young animals [3, 16, 17].

Studies on cross-age muscle transplantation have shown that old muscles transplanted into young inbred hosts regenerate *in vivo* as well as autotransplanted young muscles, whereas young muscles transplanted into old hosts regenerate no better than old muscle autografts [4, 5]. Such experiments have led to the conclusion that the environment in which a muscle regenerates is a major determinant of the success of the regenerative process. Subsequent experimentation has shown that the quality of the reinnervation constitutes a major component of the regenerative environment [6, 7]. To date, the only age-related property of muscle that has not been found to be reversed by cross-age transplantation is the percentage and degree of muscle fiber branching in the regenerates [2]. In this case, the high degree of branching that characterizes old regenerating muscle is also seen in old-into-young muscle transplants.

Since satellite cells constitute the major cellular source of regenerating mammalian muscle, we have decided to examine satellite cell populations in regenerating and cross-transplanted young and old muscles in rats. Under normal conditions, the percentage of satellite cells, as compared with the total myonuclear population, steadily declines with age, with the greatest changes seen during the earliest months of life in rats [1, 11, 12]. After regeneration, the satellite cell populations return to the same levels found in control muscles [21]. The principal question underlying this research was whether or not the satellite cell populations of cross-age grafts would conform quantitatively to the pattern seen in autografts in the same hosts or if the numbers would instead conform to autografted muscle of the same age as that of cross-age-grafted muscle.

Material and Methods

Cross-age transplantation

The experiments were performed on WI/HicksCar rats, a strain of 100 generation inbred male Wistar rats, which were 4 and 24 months old at the start of the experiment and 6 and 26 months old at the time of harvesting the tissue [5]. All operations were conducted under ether anesthesia in accordance with the guidelines of the Unit for Laboratory Animal Medicine at the University of Michigan. Two animals per age group were untouched controls. Extensor digitorum longus (EDL) muscles were excised from both legs. In the experiment there were three young and three old surviving rats.

In the young rats, the left EDL muscle was removed and autotransplanted into the site from which it was removed. The EDL muscle from the right leg was excised and placed into the location of the EDL muscle on the right leg from old rats. Conversely, EDL muscles from the right legs of old animals were transplanted into the location of the EDL muscle in young rats. In the old rats, EDL muscle from left leg was removed and autotransplanted to its own position. Two months later, all EDL muscles were excised, frozen in liquid nitrogen and shipped to Slovenia for stereological analysis.

Tissue preparation

Each muscle was cut with Reichert-Jung Frigocut 2800 into five (control muscles) and seven (grafted muscles) 30μm thick transverse sections with an interval of 90 μm for satellite cell counting and three 30μm thick transverse sections with an interval of 80 μm for counting nuclei.

All sections were pretreated in phosphate buffered saline with 0.2% Triton X-100 (PBS-T). They were subsequently fixed in 2% paraformaldehyde in PBS-T for ten minutes and further rinsed in PBS-T. All antibodies were diluted in PBS-T except for M-cadherin (M-cad), which was diluted in PBS/carrageenan solution [15].

(i) Satellite cells were demonstrated by double staining of M-cad and laminin. After rinsing in PBS-T, normal goat serum 1:5 with addition of 3% rat serum was applied for 25 minutes to reduce background staining. Sections were further incubated overnight in primary rabbit polyclonal antibody against M-cad (1:50) (Institute of Physiology, Bonn) and two hours with goat anti rabbit Alexa Fluor 488 (1:500) (Molecular Probes) with 2% rat serum. After rinsing in PBS-T, basal lamina was detected by incubating overnight with monoclonal antibodies against laminin (1:500) (Chemicon) followed by two hours with goat anti mouse Alexa-546 antibody (1:1000) (Molecular Probes) with addition of 2% rat serum. Bisbenzimide was used to stain nuclei.

(ii) On additional sections laminin and myonuclei were demonstrated. After rinsing in PBS-T normal goat serum 1:5 with addition of 3% rat serum was applied for 25 minutes. Sections were further incubated overnight with monoclonal anti laminin antibodies (1:500) (Chemicon) and one hour with goat anti mouse Alexa Fluor 488 antibody (1:1000) with addition of 2% rat serum. Nuclei were stained with propidium iodide for three minutes. Red colored myonuclei beneath the green colored basal lamina were analyzed using a confocal microscope (see below).

Sections were analyzed by the two channel Zeiss LSM 510 confocal microscope using the Zeiss Plan-Neofluar oil immersion 40x objective (NA: 1.3), M-cad in green and laminin in red fluorescence. Green and red fluorescence was excited with an argon (488 nm) and He/Ne (543 nm) laser. The emission signal was filtered using a narrow band (505-530 nm) and an LP 560 nm filter. As it was not possible to use UV excitation in our confocal microscope, the position of nuclei was controlled under a conventional fluorescent Zeiss microscope (in blue color).

Sampling

Stacks of sharp optical sections captured with the confocal microscope were used for counting satellite cells and myonuclei. For satellite cells counting, images were captured systematically from a central square field with x-y dimensions of 230 μm in every second field of view in control muscles, or else in every field of view in grafted muscles.

For counting nuclei, images from every third field of view were captured. Counting of nuclei was reliable only in control muscles. In grafted muscles, due to the uneven staining of basal lamina with laminin, it was not possible to distinguish among myonuclei and nuclei in intercellular spaces.

Stereological analysis

Satellite cells and myonuclei were counted by the optical disector principle [14, 20] under the confocal microscope applying a Disector software [22]. Briefly, in this method the disector probe is represented by a "virtual box" that is placed inside the thick physical section. During focusing up from the bottom we count only those nuclei within the box that disappear during focusing (i.e., they are not intersected by the box upper side) and at the same time are not intersected by the left and front sides of the disector box (i.e., their sections are not intersected by the exclusion lines of the unbiased counting frame). Satellite cells and nuclei were counted within the volume of the disector thus providing the following parameters: number of satellite cells within the disector volume (Nsc/V) and number of nuclei within the volume of the disector (Nnucl/V). Disector x y dimensions were 230 x 230 μm and its height was 15 to 25 μm for satellite cells and 15 μm for nuclei. Within the same volume, number of muscle fiber profiles was counted (Nfib/V). Satellite cell frequency was expressed as number of satellite cells per fiber number, dividing Nsc/V by Nfib/V and recalculated within a 10 μm thick

transverse section. In young and old control muscles the percentage of satellite cell nuclei in all nuclei was calculated by the ratio Nsc/V to Nnucl/V. Additionally, the number of satellite cells in 1cm^3 of muscle tissue was estimated in control and grafted muscles.

Statistics

For each parameter mean, standard deviation and standard error was determined. Differences among groups were tested by the Mann Whitney test.

Results

M-cadherin stained efficiently satellite cells in young and old control EDL muscles and in all four types of grafts: young to young, old to young, young to old and old to old (Fig 1). During the transfer of material from Ann Arbor to Ljubljana several antigens and enzymes deteriorated due to unknown factors, however, morphology remained intact. Consequently, analysis of the existent material was limited to only some immunohistochemical reactions and histological staining. Antibodies to laminin detected basal lamina unevenly (Fig 2), however, background staining enabled us to see clearly outlines of muscle fibers under the confocal microscope, which made the detection of satellite cells according to their position relative to muscle fibers reliable (see Fig 1). On the other hand, in transplanted muscle it was not possible to distinguish myonuclei from nuclei in the interstitial space. Therefore, we counted nuclei only in control muscles; in grafted muscles, however, we were able to relate satellite cells only to the number of muscle fiber profiles within a given section thickness, i.e., 10 µm.

In grafted muscles satellite cells were counted only in larger fibers, i.e., noninnervated fibers that composed 7 to 11 % of the whole muscle cross-sectional area, were excluded from the analysis. Data on satellite cell frequency in young and old control muscles and in transplants are presented in Table 1. Altogether in 10 µm thick sections around 150 satellite cells within 2500 fiber profiles in young control and within 4000 fiber profiles in old control muscles were counted. To reduce interindividual variability in grafted muscles, about 400 to 900 satellite cells within 6000 to 11000 fiber profiles were included in the study.

The frequency of satellite cells differed significantly between young and old control muscles ($p<0.025$). Grafted young muscles into old recipients were significantly different from young control ($p<0.03$) and old control ($p<0.03$) muscles. Grafted old to young muscles were different from grafted young to young muscles ($p<0.03$) and old controls ($p<0.5$). Grafted old to old muscles differed from old control ($p<0.03$) and from young to young grafts ($p<0.05$). Young to old grafts differed from young to young grafts (0.04). Number of satellite cells in 1 cm^3 of muscle tissue was higher in grafted than in control muscles. ($p<0.03$).

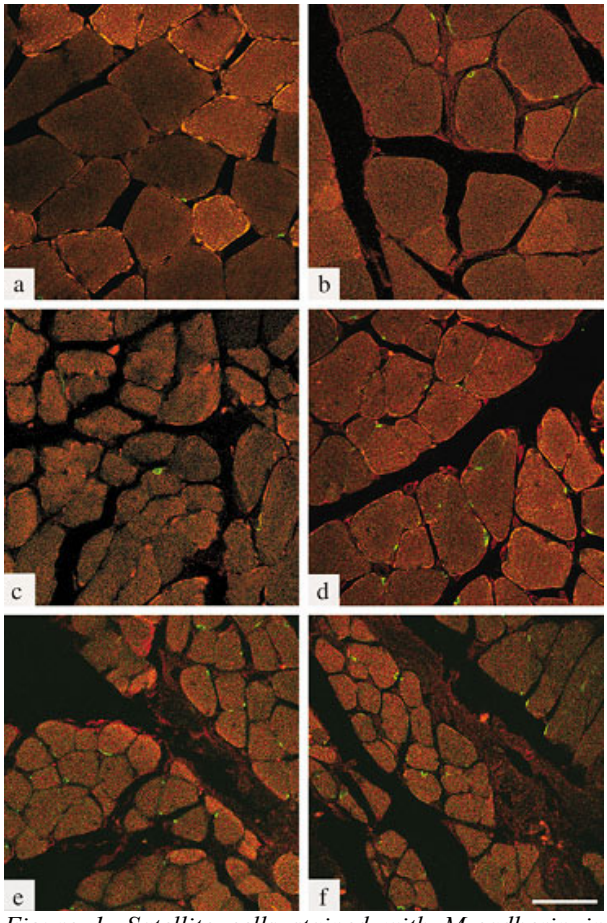

Figure 1. Satellite cells stained with M-cadherin in young (a) and old (b) control muscles and in muscle transplants (c) young to young, (d) old to young, (e) young to old and (f) old to old. Bar is 50µm.

Discussion

The frequency of satellite cells in relation to the number of muscle fibers and the percentage of satellite cells in relation to total number of myonuclei in both young and old control muscles corresponded with those previously noted for this strain of rat [11, 23]. The most striking observation with transplantations was the increase in satellite cell frequency related to number of fiber profiles in the grafted muscle regardless of donor or host age. Obviously, there is an elevation in frequency as a result of or in the process of muscle fiber

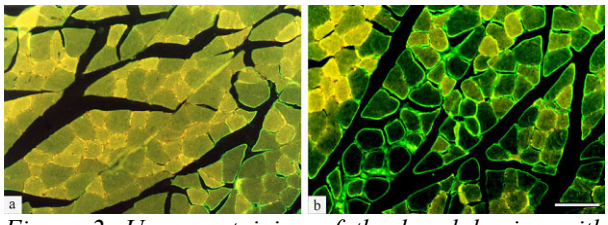

Figure 2. Uneven staining of the basal lamina with laminin seen in different portions (a, b) of the whole muscle cross-section in young controls. Bar is 100µm.

Table 1: Frequency of satellite cells and myonuclei in control and trans-age transplanted muscles of the rat

	Nsc/Nfib profiles*10^{-2}			Nsc/cm^3*10^5			Nsc/Nnucl (%)
specimen	mean	SD	SEM	mean	SD	SEM	
young control	2.55	0.18	0.10	6.03	0.99	0.57	2.96
old control	1.70	0.22	0.11	4.33	0.30	0.15	2.21
young-young	5.06	0.75	0.43	30.39	4.40	2.54	-
old-young	3.88	0.64	0.37	33.93	8.89	5.13	-
old-old	3.45	0.94	0.54	36.16	10.67	6.08	-
young-old	4.08	1.45	0.84	38.30	10.53	6.16	-

Nsc/Nfib profiles – number of satellite cells per fiber within 10 μm thick transverse section
Nsc/cm^3 – number of satellite cells per 1 cm^3 of muscle tissue (connective tissue excluded)
Nsc/Nnucl – percentage of satellite cell nuclei in all myonuclei.

regeneration. Young-into-young and old-into-old grafts underwent a nearly 2 fold increase; with old-into-young the increase was 2.3 fold but was only 1.6 in young-into-old. This can be interpreted as effect of host age, with young hosts providing additional stimuli topping the inherent and donor age characteristic basic increase (This resembles findings in mouse muscles which indicate an increase in satellite cell frequency after severe damage and regeneration in C57bl mice but not in mdx [18].). The frequency of satellite cells in young-into-young and old-into-old grafts was significantly higher than that in control muscles. This contrasts to the report of Schultz [21], who found that the percentage of satellite cells in free grafts of both EDL and soleus muscles of the rat corresponded to that of control muscle. However, differences in experimental design could account for these differences. Schultz performed his grafts on 14-day rats, which were still in a rapid growth and maturation phase, whereas in the present experiments the grafts were performed on sexually mature young adult rats 4 months of age. One possible explanation for the greater frequency of satellite cells in the grafts is that although 60-day grafts are functionally stable, the satellite cell populations have not yet returned to normal levels after an intense regenerative response. Nevertheless, despite the increased frequency of satellite cells in the grafts, that of the old-into-old was still less than that of the young-into-young grafts.

A complicating factor in estimating satellite cell frequency in grafts in mature and especially old animals is the possibility of denervation changes. It is well established that shortly after denervation the population of satellite cells undergoes a sharp rise, which persists for several months after denervation [23]. In muscle grafts in mature rats functional innervation is not established until about three weeks after grafting [8]. Thus the grafts analyzed in these experiments could have still showed the residual influence of both muscle fiber regeneration, and also the period of denervation seen early in the history of a graft. An additional level of complexity occurs because not all muscle fibers in a muscle graft become innervated [10], and in older rats the degree of innervation is less than that in younger adult rats [9]. It is possible that in the grafts the higher frequency of satellite cells could be due to post-denervation responses of regenerated muscle fibers which had not yet begun to show significant signs of atrophy.

In most respects, properties of cross-age muscle grafts correspond more closely to the age of the host rather than to the age of the graft [4, 5]. The main exception to date has been the degree of muscle fiber branching in cross-transplanted muscles. In this case, the increased branching seen in old-into-old muscle grafts carries on into old-into-young grafts, whereas in both young-into-young and young-into-old grafts the degree of branching is much less [2]. The frequency data on satellite cells seen in this study follows a similar pattern, but not to such a strong degree. Since both branching and satellite cell frequency are strongly associated with the muscle fibers themselves, it may be that the effect of the host environment is not as strong on this property of regenerated muscle as it is for certain other characteristics.

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