The genotype of bone marrow-derived inflammatory cells does not account for differences in skeletal muscle regeneration between SJL/J and BALB/c mice

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Abstract. This study determined whether the genotype of bone marrow-derived inflammatory cells contributes to the more pronounced leukocytic exudation and extensive new muscle formation seen in SJL/J compared with BALB/c mice after a crush-injury (Mitchell et al. 1992). Female SJL/J mice were whole-body irradiated and reconstituted with male bone marrow from the BALB/c strain, and irradiated BALB/c females reconstituted with male SJL/J bone marrow. The mice were allowed to recover for 3 weeks and the tibialis anterior muscle (in a leg which had been protected from irradiation) was injured by crushing. At 3 and 10 days after injury the extent of necrotic debris, mononuclear leukocytic infiltration and new muscle formation was assessed in the muscles. The SJL/J mice reconstituted with BALB/c bone marrow showed extensive mononuclear leukocytic infiltration and clearance of necrotic debris when compared with BALB/c mice reconstituted with SJL/J bone marrow, and these strain-specific differences mirrored those seen with control bone marrow reconstituted hosts and non-irradiated hosts. The results show that the genotype of the bone marrow-derived macrophages is not responsible for the superior regeneration of crush-injured skeletal muscle in SJL/J mice, and it appears that factors intrinsic to the muscle tissue may be of central importance.

Key words: Regeneration – Skeletal muscle – Transplantation – Inflammation – Mice (SJL/J, BALB/c)

Introduction

When skeletal muscle is injured it regenerates by proliferation and fusion of muscle precursor cells. This process is closely associated with revascularisation and the infiltration of leucocytes, particularly macrophages. Macrophages are seen in regenerating muscle as early as 3 h after injury (Robertson et al. 1990) and are the cells primarily responsible for the phagocytosis and removal of necrotic muscle (Mastaglia et al. 1975; Papadimitriou and Ashman 1989). The absence of bone marrow-derived leukocytes does not prevent the proliferation and fusion of muscle precursor cells (Robertson et al. 1992) but the formation of new muscle is severely impeded by the persistence of muscle debris. The concentration of mononuclear inflammatory cells within crush lesions of tibialis anterior (TA) muscles at 3 days after injury is twice as high in SJL/J as in BALB/c mice, and at 10 days after injury all necrotic muscle within the lesion has been removed in SJL/J mice, whereas the corresponding region in BALB/c mice has persistent necrotic debris (Mitchell et al. 1992); these differences are associated with more extensive new muscle formation in SJL/J compared with BALB/c mice (McGeachie and Grounds 1987; Grounds and McGeachie 1989, 1990; Mitchell et al. 1992).

This study tested the hypothesis that strain-specific differences in bone marrow-derived leukocytes are responsible for the more successful regeneration of skeletal muscle in SJL/J compared with BALB/c mice. In addition, to examine the possibility that a general systemic difference in vascular function might contribute to a more efficient margination and emigration of leukocytes in SJL/J compared with BALB/c mice, an inflammatory reaction was provoked by two other experimental methods and 3 days later the numbers of inflammatory cells within these sites was determined in both strains.

Materials and methods

Animals

Inbred SJL/J and BALB/c mice (7–10 weeks old) were obtained for the Animal Resource Centre ( Murdoch, Western Australia). All experimental procedures were carried out in strict accordance with the National Health and Medical Research Council of Australia’s guidelines for the care and use of laboratory animals and all experimental protocols were approved by the Animal Welfare Committee of the University of Western Australia.
Irradiation

To eliminate the host bone marrow-derived cells, female SJL/J mice were exposed to an X-irradiation dose of 1000 Rads (10 Gy), whereas female BALB/c mice received 800 Rads (8 Gy). Different (supra-lethal) doses of irradiation were used for the two strains because SJL/J mice are more resistant to X-irradiation than other strains (Roderick 1963; Yuhas and Storer 1969); in addition, BALB/c mice are extremely sensitive to irradiation (Roderick 1963; Yuhas and Storer 1969) and in preliminary trials BALB/c mice did not tolerate the higher dose (10 Gy).

Female (host) mice were anaesthetised with an intraperitoneal injection of pentobarbitone sodium (Nembutal; 30 μg/g body weight), the right hind leg protected with a continuous pliable lead sheet, and the animals irradiated according to the protocol described by Robertson et al. (1992).

Bone marrow reconstitution

Female host mice, either SJL/J or BALB/c, were irradiated to destroy their bone marrow cells, and then reconstituted with donor bone marrow cells (Grounds 1983) from either the unrelated strain (allografting) or from the parental strain (isografting). Male mice of each strain served as bone marrow donors; one donor male mouse was used to reconstitute the bone marrow of 2 female host mice. All procedures were performed aseptically. Bone marrow cells were prepared from cleaned femurs (5x10^6 cells/femur) and injected into the tail vein of pre-irradiated mice as previously described (Grounds 1983).

Assessment of leukocytes in peripheral blood of irradiated/reconstituted mice

Blood was collected from all mice by cardiac puncture and whole blood was transferred to heparinised vials for the determination of total numbers of leukocytes. Peripheral blood smears were made on gelatin-subbed glass slides and stored at -20°C until use. Hybridiisation in situ with the Y chromosome-specific probe 1455C (Nishioka 1987) was carried out as described by Grounds et al. (1992).

Crush-injury and analysis of muscles

At 21 days after irradiation and bone marrow reconstitution, the mid-region of the proxected (right) TA muscle was subjected to a single crush-injury, as described previously (Mitchell et al. 1992). Mice were killed either 3 or 10 days after crush-injury, longitudinal sections prepared and the extent of new muscle and connective tissue assessed. The density of mononuclear leukocytes in a single proximal and distal site (Mitchell et al. 1992) as well as the number of myobute profiles per section was measured in the 3-day post-injured muscle samples.

Cellular response to alternative inflammatory stimuli

Subcutaneous carrageenan injections. Six SJL/J and 6 BALB/c female mice each received a single subcutaneous injection on either side of the back of 0.2 ml of a 1% carrageenan (Seakem Chemical Co.) solution (in PBS; pH 7.2), a known inflammatory agent (Bonney et al. 1978). Three days later, 0.25 ml of a 1% solution (in double distilled water) of trypan blue was injected into the tail vein 1 h before sacrifice in order to visualise the extent of the inflamed site. Tissue surrounding the injection site (approximately 12 mm) was excised to a depth well below the layer of the panniculus carnosus. Samples of tissue were cut through the long axis (containing the site of inflammation), immersed in 10% phosphate-buffered formal saline (pH 7.2) overnight, dehydrated and embedded in paraffin wax. All sections were orientated with the edge containing the carrageenan bolus perpendicular to the face of the block. Sections 5 μm thick were cut, stained with Harris’s haematoxylin and eosin, and the maximum radius of the inflammatory response from the carrageenan bolus measured on a camera lucida projection. In addition, the numbers of mononuclear leukocytes were counted under oil immersion (final magnification, x1200) in 10 random sites within the area of inflammation and external to the carrageenan bolus (Fig. 1).

Intraperitoneal thioglycollate injection. Ten SJL/J and 10 BALB/c female mice were anaesthetised by halothane/oxygen inhalation, their abdomens swabbed with 70% ethanol, and 0.5 ml of a 10% solution of Bacto brewer thioglycollate medium (Difco Laboratories, Detroit, USA) in double distilled water was injected intraperitoneally to elicit a large inflammatory exudate (Withrout et al. 1983), which comprised 85–90% macrophages. Three days later

![Diagram](https://example.com/diagram.png)

Fig. 1. Diagram showing the location of the carrageenan bolus at 3 days after injury and the method used to analyse mononuclear cell density. Maximal radius of inflammation (M) was recorded for each injection site, as the longest distance that an inflammatory response extended from the tip of the carrageenan bolus. All leukocytes (excluding spindle-shaped cells) were counted under oil immersion (x100) in 10 randomly selected sites.
all mice were killed, 3 ml of Modified Eagles Medium containing 100 U/ml heparin was injected intraperitoneally and lavages performed to harvest elicited inflammatory cells (Robertson et al. 1993). Samples were counted on a haemocytometer, averaged over 4 fields, and numbers of exudate cells in peritoneal lavages of SJL/J and BALB/c mice compared using a Student’s t-test, taking P<0.05 as significant.

Results

Survival following irradiation/reconstitution

The proportion of mice surviving for the duration of the experiment (3.5–4.5 weeks) was 81% (13/16) for SJL/J mice allografted with BALB/c bone marrow, and 82% (14/17) for isografted SJL/J mice. In contrast 68% (19/28), of BALB/c mice allografted with SJL/J bone marrow survived compared with 83% (15/18) for isografted BALB/c mice. Using a χ² test none of these values were significantly different.

Leukocytes in the peripheral blood of irradiated/reconstituted mice

The total number of leukocytes in peripheral blood of normal (unirradiated) SJL/L and BALB/c mice was 1.4- to 2.6-fold higher than in irradiated and bone marrow reconstituted mice after 24 days (Fig. 2). Following irradiation/reconstitution there was a decrease in the proportion of lymphocytes and an increase in the proportion of neutrophils, which was not significant in SJL/J mice (P>0.05) but significantly different in all groups of BALB/c mice in comparison to unirradiated controls (P<0.05; Fig. 2). The proportion of Y chromosome-positive leukocytes in peripheral blood smears from transsexually grafted female host mice was greater than 80% in all but one case (which had 54% Y-positive cells), and this mouse was excluded from the study. Female hosts reconstituted with female bone marrow had no Y chromosome-positive cells in the peripheral blood. These results confirmed the success of the irradiation/reconstitut-

![Fig. 2. Absolute numbers and proportions of the major leukocyte cell types in whole body irradiated/bone marrow-reconstituted mice. n Number of mice analysed](image)

Table 1. Numbers of mononuclear leukocytes and myotubes within areas of regenerating tibialis anterior muscles from irradiated and bone marrow-reconstituted SJL/J and BALB/c mice at 3 days after crush-injury. Number of mice sampled in each group is indicated in parentheses. Superscripts to the cell counts refer to the proportion of persistent necrotic muscle debris in the area examined: ^ none; b 0–10%; c 10–30%; d 30–60%; e >60%. The average number of myotubes (per longitudinal section) in each group as seen at 3 days after injury is denoted as: 0=none, ±=1 or less, ++=1–5, +++=5–10, ++++=>11.  * This group contained only one sample.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mononuclear leukocytes cell/field (mean±SEM)</th>
<th>Myotubes</th>
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<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Distal</td>
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<tr>
<td></td>
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<tr>
<td>SJL/J (6)</td>
<td>BALB/c male</td>
<td>310±41</td>
</tr>
<tr>
<td>SJL/J (5)</td>
<td>SJL/J male</td>
<td>152±22</td>
</tr>
<tr>
<td>SJL/J (1)</td>
<td>SJL/J female*</td>
<td>224^</td>
</tr>
<tr>
<td>BALB/c (6)</td>
<td>SJL/J male</td>
<td>106±20</td>
</tr>
<tr>
<td>BALB/c (6)</td>
<td>BALB/c male</td>
<td>146±10</td>
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<tr>
<td>BALB/c (5)</td>
<td>BALB/c female</td>
<td>154±30</td>
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Quantitative histology of tibialis anterior muscles 3 days after injury

At 3 days after injury the concentration of mononuclear leukocytes in muscle lesions of bone marrow-allografted SJL/J mice was significantly higher ($P<0.05$) both proximal and distal to the centre of the crush lesion, than in BALB/c mice allografted with SJL/J bone marrow. In addition, there was much less persistent necrotic debris in muscles of all SJL/J mice than in BALB/c mice (Table 1). The numerical density of mononuclear leukocytes was greatest 250–500 µm distant from surviving myofibres in SJL/J muscle, in contrast to BALB/c muscle where mononuclear leukocyte density was highest adjacent to surviving muscle fibres (Table 1; Fig. 3). There were no significant differences between the numbers of mononuclear cells in muscles of any of the BALB/c hosts ($P>0.05$). Although mononuclear leukocyte counts in some of the SJL/J muscles were as low as those of BALB/c mice, the areas devoid of necrotic muscle debris (principally between surviving myofibres) were much larger than those in the corresponding BALB/c hosts (Fig. 3). In all groups examined, the SJL/J muscles had many more myotubes than the BALB/c hosts (Table 1; Fig. 3).

At 10 days after injury, there were many myotubes and minimal connective tissue in lesions from all reconstituted SJL/J mice (Fig. 4). In allografted BALB/c mice there was a marked variation in the histology of regenerated muscles at 10 days; this ranged from a histological...
appearance similar to that seen in SJL/J muscles to lesions characterised by large patches of necrotic muscle, extensive fibrosis and minimal myotube formation (Fig. 4). The two BALB/c mice isografted with BALB/c bone marrow and sampled at 10 days after injury both had very poor regeneration.

Cellular response to alternative inflammatory stimuli

The average number of leukocytes per field (final magnification, ×1200) in mice injected subcutaneously with carrageenan (Fig. 5) was 302±64 (mean±1 SD) in SJL/J mice (n=11), compared with 264±59 in BALB/c mice (n=12). The maximal radius of inflammation was 672±199 μm in SJL/J mice and 596±191 μm in BALB/c mice. The total number of leukocytes elicited by intra-peritoneal injection of thioglycollate was 8.8±1.4×10⁶ in SJL/J mice and 11.1±4.0×10⁵ in BALB/c mice. None of these values were significantly different.

Discussion

Irradiation and subsequent reconstitution (I/R) with either allogeneic or isogeneic bone marrow resulted in a reduction by approximately half in the total number of leukocytes of normal control mice of both strains. There was also an increase in the numbers of neutrophils (neutrocytosis) in both strains, which was mild in SJL/J but marked in BALB/c mice. This neutrocytosis seems to be a consequence of the greater susceptibility of BALB/c mice to irradiation (Roderick 1963; Yuhas and Storer 1969), as either allogeneic or isogeneic reconstitution of
bone marrow in BALB/c mice resulted in similar numbers and proportions of the major leukocytic cell types. With one exception, all of the female host mice irradiated and reconstituted with male bone marrow, had greater than 80% Y chromosome-positive (i.e. male) bone marrow-derived cells in their peripheral blood at the conclusion of the experiment, confirming the success of reconstitution with male donor bone marrow.

The success of bone marrow reconstitution between different strains of mice is largely dependent upon the major histocompatibility complex (MHC) antigens, specifically the H2 class in mice. Mice with similar H2 haplotypes are generally susceptible to I/R, while those with differing H2 haplotypes demonstrate varying degrees of resistance to reconstitution with bone marrow (Lotzova et al. 1977). The 30 day survival rate after I/R is determined by both the H2 compatibility and the number of injected bone marrow cells, with H2 incompatible donors requiring a higher number of cells (Vriensendorp et al. 1976). The numbers of cells used to reconstitute irradiated host mice in this study (5x10^6) is sufficient to ensure approximately 71% survival for 30 days (Vriensendorp et al. 1976), and this corresponds closely to the observed survival rate (68%) for the allografted BALB/c hosts. In contrast, SJL/J mice allografted with bone marrow from non-H2 compatible strains have a longer survival time, as previously demonstrated (Cläesson et al. 1978). Although various combinations of I/R between mice which differ in their H2 haplotype have been reported, allodemeic bone marrow transfer between mice with H2a (SJL/J) (Bubbers 1983) and H2b (BALB/c) ( Staats 1976) haplotypes has not (to the authors' knowledge) been documented. Thus the effect of H2 haplotype or possible effects of graft-versus-host reaction on the decreased survival seen in allografted SJL/J or BALB/c mice is unknown. In this study, no evidence of graft-versus-host reaction was found in samples of gastrocnemius muscles taken from the protected legs of I/R mice of either strain (data not shown).

In the regenerating muscles, the results clearly show that the increased infiltration of mononuclear inflammatory cells (Mitchell et al. 1992) and removal of necrotic tissue (Grounds et al. 1992; Mitchell et al. 1992) seen in the SJL/J strain is not dependent upon any genotypic property of SJL/J leukocytes. Both allografted and isografted SJL/J mice had very little persistent necrotic muscle debris, although the allografted SJL/J mice had significantly more mononuclear inflammatory cells within the sampling area than the isografted SJL/J mice. While the number of inflammatory mononuclear cells in the areas studied were similar in muscles of isografted SJL/J and BALB/c hosts (Table 1), the SJL/J mice had more extensive removal of necrotic debris and a much larger area of the lesion occupied by infiltrating inflammatory cells than BALB/c mice. These data are consistent with the response seen in unirradiated muscles of SJL/J and BALB/c hosts at 3 days after crush-injury (Mitchell et al. 1992).

Similarly there was no difference in the number of myotubes at 3 days after injury between allografted and isografted SJL/J hosts. Myotube formation occurs earlier, at 2.5 days after injury (Mitchell et al. 1992), and is more extensive (Grounds and McGeachie 1989) in SJL/J compared with BALB/c mice, and tissue culture studies indicate that there are intrinsic differences in the myogenicity of muscle precursor cells from the two strains which is independent of the host environment (Maley et al. 1994). Myotube numbers were much higher in the proximal end of the SJL/J muscles, than in the same regions of all I/R BALB/c mice. The blood supply to the distal (tendinous) portion of the TA is frequently interrupted by the crush, which most likely accounts for the variation in mononuclear cells numbers and extent of phagocytosis, as well as lower myotube numbers in comparison with the proximal region in both strains.
The more prominent accumulation of emigrated leukocytes in SJL/J mice in response to inflammatory stimuli such as crush-injury might be due, at least in part, to a more generalised blood-organ “leakiness” (Teuscher 1985) such as the “leaky” blood thymus barrier of SJL/J mice (Clässon et al. 1978) compared with other strains, enhancing the number of emigrated leukocytes. Furthermore, strain-specific differences in monocyte migration in response to inflammatory stimuli have been described for SJL/J mice (Crowle and May 1978) and other strains (Goichot and Joyeux 1977). However, the results from this study where the numbers of leukocytes elicited in response to intraepidermal thiglycollate injection or subcutaneous injections of carragenan were similar for both strains, do not support the hypothesis of a more generalised difference in the response to inflammatory stimuli between SJL/J and BALB/c mice. It therefore seems likely that the strain-specific differences in the inflammatory response of injured muscle is due to factors associated with the muscle itself: for example, chemotactic factors produced by the injured muscles (Robertson et al. 1993) or local differences in leukocyte margination due to the generation of inflammatory mediators at the injury site [e.g. increased histamine levels (Yong et al. 1993)] associated with the greater numbers of mast cells reported in SJL/J muscle (Rosenberg 1993)] or the nature of the microcirculation in striated musculature in the two strains.

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