Cellular differences in the regeneration of murine skeletal muscle: a quantitative histological study in SJL/J and BALB/c mice

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Summary. Skeletal muscle regeneration in SJL/J and BALB/c mice subjected to identical crush injuries is markedly different: in SJL/J mice myotubes almost completely replace damaged myofibres, whereas BALB/c mice develop fibrotic scar tissue and few myotubes. To determine the cellular changes which contribute to these differential responses to injury, samples of crushed tibialis anterior muscles taken from SJL/J and BALB/c mice between 1 and 10 days after injury were analysed by light and electron microscopy, and by autoradiography. Longitudinal muscle sections revealed about a 2-fold greater total mononuclear cell density in SJL/J than BALB/c mice at 2 to 3 days after injury. Electron micrographs identified a similar proportion of cell types at 3 days after injury. Autoradiographic studies showed that the proportions of replicating mononuclear cells in both strains were similar: therefore greater absolute numbers of cells (including muscle precursors and macrophages) were proliferating in SJL/J muscle. Removal of necrotic muscle debris in SJL/J mice was rapid and extensive, and by 6 to 8 days multinucleated myotubes occupied a large part of the lesion. By contrast, phagocytosis was less effective in BALB/c mice, myotube formation was minimal, and fibrotic tissue conspicuous. These data indicate that the increased mononuclear cell density, more efficient removal of necrotic muscle, together with a greater capacity for myotube formation in SJL/J mice, contribute to the more successful muscle regeneration seen after injury.

Key words: Regeneration – Skeletal muscle – Injury – Autoradiography – Morphometry – Electron microscopy – Mouse (SJL/J; BALB/c)

The process of skeletal muscle regeneration is similar regardless of the method of injury. It is characterized by necrosis of muscle tissue, revascularization, the infiltration of inflammatory cells, phagocytosis of necrotic muscle tissue, proliferation of mononuclear muscle precursors, and their fusion to form multinucleated young muscle cells called myotubes (Carlson 1973). Grounds and McGeeachie (1989) reported major differences in the effectiveness of muscle regeneration between SJL/J (Swiss) and BALB/c mice, 10 days after injury. Their autoradiographic studies showed that replication of muscle precursor cells begins at 1 day after muscle crush injury in SJL/J mice, and by 1.25 days in BALB/c mice, peaks from 2.5–3.5 days and is essentially complete at 5 days. By 10 days the injured muscle in SJL/J mice is replaced by newly-formed myotubes with minimal fibrous scar tissue. By contrast, in BALB/c mice the injured area of muscle is replaced by large areas of fibrous scar tissue with few myotubes. A similar pattern of regeneration was reported in minced muscle isografts of these two strains of mice (Grounds 1987). The crush-injury studies show differences at the "beginning" and "end" of myogenesis in these two strains, however there are no data to account for this differential response to injury, or what happens in these lesions between the initiation of muscle precursor cell replication (at 1 day after injury) and the consolidation of myotubes (at 10 days after injury).

Could these major differences in the muscle regenerative response in these two strains of mice be attributable to differences in: the efficiency and removal of necrotic muscle, the numbers and proliferative activity of inflammatory and myogenic cells, and/or differences in the formation of myotubes within lesions? The present investigation was designed to quantitate these events in skeletal muscle of SJL/J and BALB/c mice regenerating after crush injury.

Materials and methods

Animals and muscle injury

Forty three male (22–30 g body weight) inbred SJL/J and 41 male inbred BALB/c mice (20–26 g body weight) mice aged 6–10 weeks
were used in this study. Of these, 16 (8 BALB/c and 8 SJL/J mice) were used in the autoradiographic experiments. All mice received a single severe crush injury to the TA muscle of both legs, as described by McGeachie and Grounds (1987). Briefly, mice were anaesthetized with ether, both legs shaved and a 4 mm longitudinal skin incision made over the TA. The middle 2 mm of the TA was dissected free of the tibia, without disturbing the nutrient vessel or nerve supply, allowing one arm of a pair of artery forceps to be inserted between the TA and the tibia in the mid-belly of the muscle. A single severe crush applied for 6 seconds across the muscle, including the epimysium, resulted in an injury 4 mm wide and 2 mm long which did not disturb overall muscle continuity. No antibiotics were administered.

**Muscle sampling and processing**

Animals were anaesthetized with ether and sacrificed by cervical dislocation. The intact legs with the muscles undisturbed were stripped of skin, removed and immersed in 0.1 M sodium phosphate buffered 10% formal saline (pH 7.2) for 3 days. Thus the TA muscles were fixed at their in vivo length with attachments intact. The TA of the right legs were cut in longitudinal section (LS) vertically through the centres of the lesions. For histological analysis, muscle samples were embedded in Araldite, 2-μm-thick sections cut, deplasticized for 2 min in sodium ethoxide (2% NaOH in absolute ethanol; Imai et al. 1968) stained with haematoxylin and eosin and examined with a light microscope.

**Autoradiography and analysis**

 Autoradiographic analyses to quantitate the proportions of replicating cells in regenerating lesions were carried out on TA muscles of 8 SJL/J and 8 BALB/c mice. At 2 or 3 days after crush injury (4 mice of each strain per time), tritiated thymidine (3H-TdR; specific activity 5 Ci/mmol, Amersham International) was injected intraperitoneally at a dose of 1 μCi/g body weight, 1 h prior to sampling. Thus, all labelled cells were premitotic when sampled. Each mouse received a single injection only of 3H-TdR. Muscle samples for autoradiography were fixed as above, postfixed in 1% OsO4 and block-stained with 1% paraformaldehyde in 70% ethanol (Dilley and McGeachie 1983). Araldite sections, 2 μm thick, were placed on glass slides and coated with AR10 stripping film (Kodak). Slides were exposed in light-tight boxes at −20°C for 8 weeks, developed in D19 (Kodak), fixed in acid hardener fixer, washed, and dried. Sections were viewed and analysed by use of a light microscope with a 100× oil immersion lens. Nuclei were considered to be labelled if 3 or more silver grains were seen in the emulsion above (McGeachie and Grounds 1987).

**Cell density analysis**

Cell densities were measured morphometrically in LS from crush-injured muscles of 4 SJL/J and 4 BALB/c mice sampled at 2 and 3 days after injury. Morphometric measurements were made by projecting a camera lucida image of the section (final magnification 85x) onto a square representing 200 μm sides (total area 40,000 μm²). Twelve test areas within the LS were chosen to assess regional variations in mononuclear cell densities of regenerating lesions (Fig. 1). The total numbers of mononuclear cells, including presumptive myoblasts, connective tissue and inflammatory cells, within the confines of the test area were counted. In many samples, a proportion of the test area was occupied by persisting necrotic muscle tissue (Table 1). In all cell density measurements the proportion of the test area occupied by myofibres was less than 10%.

Fig. 1. Locations of test areas for measurements of cell densities in the TA. Areas 1 to 4 are located in the proximal adjacent zone, start 100 μm from the anterior surface, and are successive measurements 100 μm apart. Areas 5 to 7 are separated from areas 1 to 4 by 200 μm, and begin 200 μm from the anterior surface. The cell density in area 8 was obtained by placing the test square 200 μm from the anterior border at the midpoint of the crush zone (CNZ). Areas 9 and 10 are located in the distal adjacent zone of the lesion, and areas 11 and 12 are midway between the CNZ and 9 and 10. Results of cell density measurements are summarized in Table 1.

**3H-TdR labelling of mononuclear cells**

Three areas of the lesions (proximal, distal and central) were examined in LS for 3H-TdR labelling of premitotic mononuclear cells at 2 and 3 days. In the proximal and distal areas at least 500 mononuclear cells were counted, the numbers of labelled and unlabelled cells recorded, and the percentages of labelled cells calculated (Table 2). In the central area (where there was more necrotic tissue) at least 200 mononuclear cells were analysed. All samples used for mononuclear cell labelling were assessed by blind analysis.

**Electron-microscopic quantitation**

Six mice (3 SJL/J and 3 BALB/c) were crush-injured and allowed to recover for 3 days, when they were deeply anaesthetized with halothane and perfused via the heart with 20 ml of heparinized normal saline followed by 20 ml of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.4). After perfusion the lower leg was stripped of skin and the TA muscle was further fixed at room temperature overnight.

The TA was divided longitudinally through the centre of the lesion and transverse cuts made between the central crush site and adjacent tissue (clearly visible upon gross examination), resulting in 6 blocks for each animal. These samples were postfixed in 1% OsO4, dehydrated in graded concentrations of ethanol, infiltrated with Araldite and embedded in flat rubber moulds. Critical orientation of each specimen was carried out under a dissecting microscope. Longitudinal semi-thin sections were prepared from each
Table 1. Cell density (cells per 0.04 mm²) measurements from longitudinal sections of crush injured tibialis anterior muscle. Values represent mean ± standard error for samples from 4 mice from each test point. Subscripts refer to the mean proportion (% of the remaining necrotic muscle tissue within the test area; 0: No remaining necrotic tissue, 1: 10%, 2: 30%, 3: 50%, 4: >60%.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Sampling time after injury (days)</th>
<th>Area</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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</tr>
</thead>
<tbody>
<tr>
<td>SJL/J</td>
<td>1</td>
<td></td>
<td>184±18</td>
<td>213±18</td>
<td>160±18</td>
<td>184±24</td>
<td>169±56</td>
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<td>171±48</td>
<td>169±36</td>
<td>149±49</td>
<td>154±28</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2</td>
<td></td>
<td>106±38</td>
<td>125±37</td>
<td>109±31</td>
<td>139±39</td>
<td>124±49</td>
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<td>146±53</td>
<td>154±53</td>
<td>146±53</td>
<td>146±53</td>
</tr>
<tr>
<td>SJL/J</td>
<td>3</td>
<td></td>
<td>224±38</td>
<td>206±36</td>
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<td>236±39</td>
<td>266±64</td>
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<td>226±39</td>
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<tr>
<td>BALB/c</td>
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<td>146±53</td>
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Table 2. Autoradiographic data showing the proportion (%) of labelled mononuclear premitotic cells within longitudinal sections of crush-injured muscle from SJL/J and BALB/c mice sampled at 2 and 3 days after injury. Percentage values are shown as means ± SEM, n = 7 or 8 samples per group. Statistical analyses were performed on arcsine transformed data by use of Student’s t-test. There was a difference between central and proximal or distal mononuclear cell labelling in both strains at 2 and 3 days. In addition statistical analysis revealed a significant reduction in the proportion of labelled cells between proximal and distal regions in SJL/J mice at 2 days after injury (P<0.005) and in the central region of BALB/c mice from 2 to 3 days. Statistical comparisons between other groups were not significant (P>0.001).

<table>
<thead>
<tr>
<th>Region</th>
<th>SJL/J</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Distal</td>
<td>7.5±0.6</td>
<td>7.1±1.1</td>
</tr>
<tr>
<td>Proximal</td>
<td>10.1±0.5</td>
<td>9.6±1.3</td>
</tr>
<tr>
<td>Central</td>
<td>4.3±1.3</td>
<td>5.2±1.5</td>
</tr>
</tbody>
</table>

block, stained in 0.1% toluidine blue in 5% borax and examined by use of a light microscope. Ultrathin sections (50 nm) were mounted on 200 mesh thin-bar copper grids, stained with lead citrate, coated with carbon, and examined in a Philips 301 transmission electron microscope at an accelerating voltage of 60 kV. At least 100 cells were identified ultrastructurally in the sections of muscle tissue adjacent to the central crush site from randomly-chosen grids within the section. However, in the central sections fewer cells were observed and between 50 and 100 cells were identified.

Results

A diagrammatic representation of the sequence of cellular changes occurring in the regenerating muscle lesions at 2, 3, 5 and 10 days is shown in Fig. 2. By 1 day after injury the centre of the crush lesion in both strains of mice consisted of a large zone of necrotic myofibres and this is referred to as the central necrotic zone (CNZ). The CNZ contained a few polymorphonuclear leucocytes, monocytes and scattered erythrocytes but was otherwise devoid of mononuclear cells until the third day. In muscle adjacent to the direct crush injury site (termed the adjacent zone; AZ), mononuclear cells were conspicuous in SJL/J mice, but scarce in BALB/c mice. The most common inflammatory cell at 1 day was the polymorphonuclear leucocyte.

After 2 days there were twice as many mononuclear cells in the AZ of SJL/J mice compared with the same area in BALB/c mice (Table 1). In addition, SJL/J mice had much less necrotic muscle in the lesions than their BALB/c counterparts at this time (Table 1). Autoradiographic analyses of the proportions of replicating mononuclear cells within the 3 areas examined (proximal, central and distal) revealed a consistent 6-10% nuclear labelling index in both strains at 2 and 3 days (Table 2). A distinct alignment of fusiform mononuclear cells (identified ultrastructurally as myogenic precursors) along surviving basal laminae was evident in SJL/J mice at 2 days but was rarely seen in BALB/c mice at this time (Fig. 2).
By 3 days all necrotic material within the AZ of SJL/J mice had been removed and numerous macrophages were found at the borders (proximal, distal and medial) of the CNZ (Fig. 3A). By contrast necrotic muscle persisted in BALB/c mice, and there were few mononuclear cells within the CNZ (Fig. 3B). A conspicuous feature of 3 day lesions in SJL/J mice was the presence of long rows of fusiform myogenic cells, some of which had fused into immature myotubes (Fig. 3A) containing myofilaments and 2–11 nuclei. Myotube formation in SJL/J mice was seen 2 days after injury, whereas in BALB/c it was evident at 2.75 days.

Quantitative electron microscopy of 3-day lesions showed that, in the AZ of SJL/J mice, 55–71% of all mononuclear cells were macrophages (Fig. 4), and between 7–26% were myogenic, as identified by the presence of thick and thin filaments within the cytoplasm (Fig. 4A) or by exclusion of other cell types (Fig. 4B). Similarly in the AZ of BALB/c mice, 47–66% of the mononuclear cells were macrophages and 6–33% myogenic cells. In both strains the proportion of myogenic cells in the proximal AZ of the lesion was higher than in the distal. Fibroblasts (Fig. 4A) accounted for between 6 and 22% of mononuclear cells in SJL/J mice and 0–17% in BALB/c mice. Endothelial cells (identified by tight junctions and the presence of a capillary lumen) accounted for 4–9% of cells, and polymorphonuclear leucocytes (which have multilobular, mostly heterocho-
mastic nuclei and scant cytoplasm) constituted 0–5% of the remaining cells.

At 4–5 days the AZ of SJL/J mice were completely filled with multinucleated myotubes, in comparison with AZ of BALB/c lesions which had fewer myotubes and conspicuous connective tissue deposition (Fig. 2). Numerous mononuclear cells were present within the CNZ of SJL/J mice and the areas containing these cells were devoid of necrotic muscle tissue. In contrast, BALB/c mice had fewer mononuclear cells at the border of the CNZ, and persistent necrotic muscle and connective tissue (Fig. 2).

From 6–10 days the central necrotic tissue in SJL/J mice had been completely removed and replaced by regularly-aligned myotubes and a small amount of connective tissue (Fig. 2). In BALB/c mice, as in the 5 day sample, necrotic muscle tissue persisted, myotubes were restricted to the periphery of the CNZ and connective tissue was conspicuous throughout the CNZ (Fig. 2).

Discussion

This sequential histological study revealed several factors which contribute to differences in the regenerative responses seen in crush-injured muscles of SJL/J and BALB/c mice (Grounds and McGeachie 1989). Briefly, in SJL/J mice the cellular infiltration into the central narrow AZ seen in many BALB/c mice. In the AZ of SJL/J mice there is little necrotic muscle and many mononuclear cells are often aligned (open arrows) with healthy muscle fibres. In BALB/c muscle, the myofibre damage does not extend far beyond the CNZ. Necrotic muscle still persists within the relatively small AZ (arrows).
Fig. 4A, B. Transmission electron micrographs of an area within the central portion of an SJL/J lesion at 3 days after injury. Bar: 5 μm. A A myoblast (Mb) containing filaments and early z-band formation (arrows) at the periphery of the cell. In addition a macrophage (M) and fibroblasts (F) are seen in this field. B By far the most common cell within lesions in both strains of mice was the macrophage (M) seen clearly in this micrograph. A presumptive myoblast (PMB), closely apposed to the cytoplasm of another (arrows), has a large euchromatic nucleus, mitochondria and some endoplasmic reticulum, but is identified principally by exclusion of other cell types.
portion of the lesion was faster and more extensive, removal of necrotic muscle tissue more efficient, and myotube formation better than in BALB/c mice.

Cellular infiltration was much faster in SJL/J than in BALB/c mice. This difference was evident at 1 day and pronounced by 2 days after injury. The analysis of cellular density confirmed the histological findings and revealed a general doubling of total mononuclear cell numbers in SJL/J compared with BALB/c mice at 2 days after injury. The higher cell densities in SJL/J mice did not appear to be accounted for by an increased rate of cell division, as autoradiographic studies showed little difference in levels of cell replication in the two strains at 2 and 3 days. This suggests that the higher cell densities were primarily due to a greater migration of inflammatory cells into lesions in SJL/J mice. This may reflect a difference between these strains in the amount or types of chemotactic agents (such as complement components or growth factors) produced after injury.

The greater cellularity in SJL/J muscle lesions was clearly associated with earlier and more effective phagocytosis. Removal of necrotic tissue from the CNZ began at 3 days and was essentially completed in SJL/J mice by 6 to 8 days, whereas in BALB/c mice phagocytosis was poor and large tracts of necrotic debris persisted at 10 days. Removal of necrotic tissue is a prerequisite for effective new muscle formation (Grounds 1991). Where phagocytosis is incomplete (Markley et al. 1978; Grounds 1987), muscle regeneration is unsuccessful.

The ischaemic CNZ was virtually devoid of mononuclear cells from 1 to 3 days after injury in both strains. This concurs with results of Phillips et al. (1987) and Schultz et al. (1988) who showed that the ischaemic central portion (equivalent to the CNZ in this study) of autotransplanted muscles does not give rise to viable myogenic cells. The cells originally in this muscle tissue, which include muscle precursor "satellite" cells (Mauro 1961; Campion 1984) have either died or migrated away from the ischaemic area (Schultz et al. 1988; Phillips et al. 1990). This means that successful regeneration of muscle within the CNZ requires repopulation with muscle precursors from the extremities of the lesion.

Myotubes appeared earlier and were more widespread in muscle lesions of SJL/J mice in comparison with BALB/c. The striking myotube formation in SJL/J mice (Robertson et al. 1990) may be the result of more effective removal of necrotic muscle and higher absolute numbers of mononuclear cells (including macrophages and muscle precursors) within the regenerating lesion. The intimate association of macrophages and muscle precursors during regeneration may be critical to successful myogenesis, as macrophages secrete a wide variety of bioactive factors and enzymes (Nathan 1987). Factors such as basic fibroblast growth factor (bFGF) are implicated in processes affecting muscle regeneration, including angiogenesis (Knighton et al. 1983; Folkman and Klagsbrun 1987) and muscle precursor proliferation (Bischoff 1986; Allen and Rankin 1990; Grounds 1991). Some cytokines appear to be specific mitogens for muscle precursors (Austin and Burgess 1991), and enzymes such as heparinase can liberate endogenous growth factors complexed to the extracellular matrix (Baird and Ling 1987).

In addition, muscle tissue of SJL/J mice may produce larger quantities of, or more effective, skeletal muscle mitogens than that of BALB/c mice: these may include muscle specific growth factors which increase in response to injury (Bischoff 1986, 1990), bFGF (Anderson et al. 1991) and insulin-like growth factors (Jennische et al. 1987) which are present within myofibres and muscle precursors. In addition to these growth factors, the adrenocorticotropic-like neuropeptides have been shown to be specific muscle mitogens for embryonic muscle (Cossu et al. 1989) although their role in regenerating mature muscle is not known.

The overall process of regeneration in crushed muscles of SJL/J and BALB/c mice is similar, although major quantitative differences are apparent with respect to cell numbers, removal of necrotic tissue and myotube formation. The factors responsible for the different cellular responses in these two strains are presently unknown, and are the subject of further investigation.

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