Intrinsic Differences in MyoD and Myogenin Expression between Primary Cultures of SJL/J and BALB/C Skeletal Muscle

MOIRA A. L. MALEY,1 YING FAN,2 MANFRED W. BEILHARZ,2 AND MIRANDA D. GROUNDS

1Departments of Pathology and Microbiology, University of Western Australia, and the 2Australian Neuromuscular Research Institute, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia

The time course of expression of the skeletal muscle-specific regulatory genes MyoD and myogenin was studied in primary cultures of skeletal muscle from adult SJL/J and BALB/c mice. In situ detection of expression with MyoD and myogenin riboprobes and myogenin antibody showed that the onset of expression of these genes occurred earlier in cells from SJL/J mice. Progenitor cells and myotubes were also more frequent in cultures from SJL/J mice than in BALB/c. The onset of expression of MyoD and myogenin was delayed in cultured cells relative to the time course seen following injury in vivo. Myogenin protein was demonstrated in replicating cells and all myogenin-positive cells expressed desmin. The observed strain-specific differences infer a greater intrinsic myogenicity of cells in SJL/J muscle in vitro and reflects the superior capacity for new muscle formation previously reported in SJL/J mice in vivo. © 1994 Academic Press, Inc.

INTRODUCTION

Formation of new skeletal muscle in regenerating tibialis anterior muscles of adult animals is much more extensive in SJL/J mice than in BALB/c mice in crush-injured muscles [1] and isografts of minced muscle fragments [2]. The superior regeneration in SJL/J mice is associated with a more rapid onset of muscle precursor cell (mpc) replication, which occurs 6 h earlier (at 24 h) in crush-injured muscles [1] and 18 h earlier (at 30 h) in minced isografts [3] of SJL/J compared with BALB/c muscle. In addition, about twice as many inflammatory cells are seen at 3 days in SJL/J crush lesions and myotube formation is earlier and much more extensive in this strain [1, 4]. The marked differences in the pattern of muscle regeneration are independent of the genotype of the bone marrow-derived inflammatory cells [5] and, therefore, must be either a function of the muscle itself or a function of other factors in the host environment. SJL/J mice appear to be markedly different immunologically than BALB/c mice and other strains as shown by their susceptibility to the induction of autoimmune diseases like myositis and encephalomyelitis [6–9]. This has been linked at the local tissue level to large numbers of mast cells and a histamine sensitivity of the vasculature in SJL/J but not BALB/c mice [10]. Furthermore, a spontaneous inflammatory myositis has also been described in SJL/J muscles [8, 11, 12]: this is marked in older (40 week) mice [12] where it is most pronounced in the quadriceps muscles [Mitchell et al., unpublished data]. The extent to which these host factors might be responsible for the large number of inflammatory cells and superior muscle formation seen in regenerating SJL/J muscles has yet to be determined.

In this paper we test the hypothesis that there are intrinsic differences between mpc of SJL/J and BALB/c mice and conclude that there is a differential timing in the induction of transcription of the skeletal muscle specific regulatory genes MyoD [13] and myogenin [14] between the strains. In normal uninjured adult skeletal muscle, there is generally very low level expression of these genes. The transcription of MyoD and myogenin is induced rapidly in vivo in response to denervation [15] and injury, where in situ hybridization studies on regenerating skeletal muscles of adult SJL/J mice show that mRNAs for MyoD and myogenin are present in mononuclear cells by 6 h after crush injury and are pronounced by 24 h [16]. A vast amount of work on myogenesis and associated gene control has been carried out using established skeletal muscle cell lines [for review see 17]; however, the time course of induction of MyoD and myogenin genes in primary cultures of mpc extracted from mature muscle is unknown.

The present study examines the time course of expression of these genes in primary cultures of SJL/J and BALB/c muscles. Parallel primary cultures were established both on gelatin and on the reconstituted basement membrane Matrigel, using the time of death for muscle harvest as equivalent to the time of crush injury (Time 0) in vivo [16]. Myogenin and MyoD tran-
scription was detected with digoxigenin-labeled riboprobes. Double immunofluorescent labeling for the incorporation of thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) and for myogenin was used to determine whether replicating mpc can express myogenin protein. The expression of the cytoskeletal protein desmin was examined, as this is used widely as a marker for skeletal mpc and is present in replicating rodent [18, 19] and avian [20] mpc.

**MATERIALS AND METHODS**

**Animals**

Four- to 6-week-old male and female inbred SJL/J and BALB/c mice were obtained from specific pathogen-free colonies of the Animal Resource Centre (Murdoch, Western Australia).

**Preparation of Primary mpc Cultures**

As a comparison of cells from two strains of mice was required, muscle was removed from SJL/J and BALB/c mice sequentially and processed in parallel throughout all experiments. Mice were anaesthetized with halothane and killed by cervical dislocation, and muscles were carefully removed from the hind limbs and lower back. The time from death to removal of all muscles was less than 10 min, and the time of death was taken as Time 0. Muscle tissue was placed immediately in Dulbecco’s modified minimal essential medium (DMEM, ICN-Flow Cat. No. 10-331-22) supplemented with 10% (v/v) horse serum (HS; donor horse serum, CytoSystems, Australia, Cat. No. 15-040) and 30 μg/ml gentamicin sulfate (gent; Delta West, Australia). Further processing was commenced within 45 min of Time 0 for the first animal, and all subsequent manipulations were performed in a laminar flow cabinet using aseptic techniques. The muscle was debrided and then minced continually for 5 min in a minimal volume (approx 0.5 ml) of collagenase solution (300 units/ml; Type 1A, Sigma C9891). More collagenase solution (10 ml/g muscle) was added and the slurry was stirred moderately rapidly in a 75-ml tryspinizing flask at 35°C for 35 min and centrifuged (500g 10 min). The pellet was washed in phosphate-buffered saline, pH 7.2 (PBS), and resuspended in a trypsin/EDTA solution (0.1%/0.001% in PBS, 10 ml/g of muscle) by trituration. This suspension was digested for 20 min and stopped with addition of an equal volume of 20% HS/DMEM. The pellet was collected by centrifugation (as above), washed twice in 10% HS/DMEM, resuspended in 10% HS/DMEM/gent, and filtered through 100-μm nylon gauze. Four hundred microliters of cell suspension was plated into each well of an 8-well tissue culture slide (Nunc Cat. No. 172402) at an initial apparent concentration of 1 × 10⁶/ml. Slides were either precoated with 0.1% gelatin by air drying or thinly coated with a 1/10 dilution of Matrigel (Collaborative Research) according to manufacturer’s instructions. The final culture medium was DMEM supplemented with 10% HS/gent, 1 μg/ml linoleic acid (Sigma L8884), and 40 μg/ml dexamethasone (David Bull Labs). Cultures were incubated in a humidified 5% CO₂/air incubator at 37°C.

**Preinjured Muscles**

In one experiment the TA and quadriceps muscles of SJL/J mice were crush-injured with grooved artery forceps [1, 4] to activate the mpc, 3 days prior to harvesting the muscles for culture. This was designed to examine the pattern of myogenin detection in cultures derived from muscles where many mpc were replicating [2] and expressing myogenin [16, 21] at Time 0. These cultures were grown on gelatin-coated slides as described above.

**Detection of mRNA for MyoD and Myogenin**

*In situ* hybridization studies were carried out on cultured mpc using riboprobes for the detection of MyoD and myogenin mRNAs [16]. The culture plates were processed at 12, 24, 48, and 72 h. Culture medium was decanted, and slides were washed with PBS to remove debris, left to air dry for 30 min, and stored at −20°C in aluminum foil. Cells were fixed in 4% paraformaldehyde for 30 min, treated with proteinase K (2 μg/ml) at 37°C for 20 min, and postfixed in 4% paraformaldehyde. Hybridization was performed at 42°C overnight in hybridization mixture which contained 40% formamide, 5× saline sodium citrate (SSC), 10% dextran phosphate, and 0.25 ng/ml digoxigenin-labeled (Boehringer Mannheim) MyoD or myogenin antisense probes. Control hybridizations were carried out with sense riboprobes and RNase control sections were also used. After hybridization, slides were washed with 2× SSC, 1× SSC, and 0.1× SSC at 37°C and incubated with antibody conjugate, and color development was performed in the dark according to manufacturer’s specifications. Slides were counterstained with methyl green and a set area was viewed using a ×16 NP1 objective on a Leitz Ortholux II microscope. The numbers of labeled and unlabeled cells in 12 to 15 fields were recorded, counting a total of approximately 5.5–8.0 × 10⁶ cells/well. Cells were photographed using Kodak Ektachrome 64 tungsten film. Statistical comparisons between groups were made using the General Linear Model procedure of the SAS statistical package (SAS Institute, NC).

**Immunofluorescence**

**Antibodies.** The monoclonal mouse antibody to myogenin, 5PD, was generously provided by Professor Wooding E. Wright (Department of Cell Biology and Neuroscience, University of Texas, Dallas, Texas). It was prepared against rat myogenin recognizing the 144– to 170-amino acid epitope [22]. A 1:150 dilution of Texas red-conjugated sheep anti-mouse immunoglobulin (lg) (Amersham N2031) was the secondary antibody used with 5PD. Desmin was detected by a rabbit primary antibody prepared against chicken gizzard smooth muscle (Immunon Cat. No. 490410) and an FITC-conjugated donkey anti-rabbit lg (Amersham N1054). Cells synthesizing DNA were detected using a modification of a kit (Amersham RPN20) in which BrdU (3 ng/ml) was included in cultures for 1 h prior to fixation. The primary monoclonal mouse anti-BrdU antibody was from the kit but a Texas red-conjugated sheep anti-mouse Ig (Amersham N2031) was used as the secondary antibody.

**Immunofluorescent detection of myogenin, desmin, and BrdU.** The eight-well slide cultures were fixed in 2% paraformaldehyde/PBS for 20 min, washed with PBS, and permeabilized for 10 min with 0.15% Triton X-100/PBS, washed with PBS (0.05% NaN₃), and stored briefly at 4°C. Before every immunofluorescent run the cultures were preincubated with 10% HS/1% bovine serum albumin/PBS for at least 2 h. The staining protocol comprised overnight incubation with 100 μl of primary antibody/well at 4°C, 5× PBS washes, and a 1-h incubation with the appropriate secondary conjugate at room temperature in the dark, followed by five washes in PBS. Where dual labeling (myogenin/desmin, myogenin/BrdU, or desmin/BrdU) was performed, the second labeling sequence (in the dark) was commenced immediately and incubations with the primary antibody were reduced to at least 4 h at 4°C and with the secondary conjugate were extended to 2 h at 4°C with all the appropriate washes. Finally, the wells and gaskets were removed, and the slides were mounted in a nonfade aqueous mountant (polyvinyl alcohol). Control wells which had no primary or nonimmune serum or, in dual labeling runs, omission of the first secondary conjugate were performed and found to be negative. The specimens were viewed on a Leitz Ortholux microscope under incident ultraviolet illumination using N2 (Texas red) and K2 (FITC) filter blocks. At least 1500 cells were counted in 12–15 fields in a set area of each well under phase contrast using a ×40 Fl oil objec-
tive and a stage micrometer. Cells were photographed using Kodak Ektachrome 400 film. Statistical comparisons were made as above.

**Electron Microscopy**

The primary cultures were trypsinized and the cell pellets were processed and viewed in a Philips 410LS transmission electron microscope as described by Robertson et al. [23].

**RESULTS**

**General Observations on Primary mpc Cultures**

It was noted that more "apparently viable" cells (tested by trypan blue exclusion) were extracted per gram of muscle from SJL/J compared with BALB/c mice: approximately 3.5 and 1.8 × 10^6 cells/g, respectively. Furthermore, when cells from both strains were plated at the same initial density (1 × 10^6 cells/ml) this resulted in about 50% more adherent cells in SJL/J compared with BALB/c cultures at 24 h. To compensate for this difference, 50% more apparently viable BALB/c cells were plated to attain the same number of attached cells.

In 12-h cultures, most cells had a rounded appearance. By 24 h, some bipolar cells were evident and at 48 h most cells had a flattened or bipolar morphology. A few multinucleated cells (myotubes) were first seen at 48 h and were frequent by 72 h in SJL/J cultures on Matrigel, but were not seen until 72 h in cultures on gelatin. In contrast, myotubes were not seen in any BALB/c cultures at 48 h and were rare at 72 h in BALB/c cells grown on gelatin, although they were frequent at this time on Matrigel.

Electron microscopic examination of cell pellets of cultures from both strains trypsinized at 5 days showed that only about 30% of the cells had an appearance similar to that of mononuclear cells of the murine myogenic cell line C2C12 [23] and were therefore considered to be mpc (Fig. 1A). Thick (myosin) and thin (actin) filaments were not seen in the mononuclear cells but were present in myotubes (Fig. 1B). About half of the remaining cells were identified as fibroblasts on the basis of abundant endoplasmic reticulum and morphological shape. The remaining 35% of cells were essentially undistinguishable (and some could have been mpc), although clumps of endothelial cells and an occasional macrophage or lipocyte were noted. Examination of cultures prior to this time was inconclusive as most cell types did not manifest distinctive morphological features.

**Detection of MyoD and Myogenin**

Within each mouse strain, in situ hybridization studies showed a very similar pattern for both MyoD and myogenin riboprobes. Probe-positive cells were not seen in any 12-h cultures established from uninjured muscles. On gelatin-coated slides at 24 h there was definite hybridization signal with both probes in the cytoplasm of a few cells (only 2 cells in the same field on the entire slide) in SJL/J, but not in BALB/c cultures. However, in mpc grown on Matrigel, expression of MyoD and myogenin was more frequent at 24 h in SJL/J cultures (7 myogenin-positive cells of 8601 cells counted) than in BALB/c cultures (a single positive cell on the entire slide) (Table 1). Immunofluorescent studies with myogenin antibody generally correlated with the mRNA data, and myogenin antibody-positive cells were detectable at higher frequencies in SJL/J than in BALB/c cultures (P < 0.05) (Table 2).

By 48 h, transcripts (Fig. 2 and Figs. 3A, 3C, and 3D) and protein (Figs. 3B and 3F) were readily detected in all SJL/J cultures (Tables 1 and 2). The proportions of riboprobe and myogenin protein-positive cells were far higher in SJL/J than BALB/c cultures at 48 h (P < 0.05). The proportion of probe-positive mononuclear cells at all times never exceeded about 30% of the cell population. Myotubes also showed positive hybridization for both probes (Figs. 3C, 3D, and 3J) and stained positively with myogenin antibody (Fig. 3I). Control hybridizations with sense riboprobes for MyoD or myogenin produced no signal (data not shown).

It was noted that closely paired cells were often myogenin positive, and this is clearly shown in Figs. 3B and 3I. Approximately 1 in 20 myogenin-positive cells in 48-h SJL/J cultures were also positive for BrdU; i.e., these myogenin-positive mpc were synthesizing DNA. However, at 72 h, none of the myogenin-positive cells were positive for BrdU. Control cultures indicated that there was no interference of the primary antibodies with one another in the double labeling experiments even though both had nuclear localization. BrdU stained brightly in a discrete speckled pattern, whereas myogenin labeled evenly and diffusely over the entire nucleus.

**Detection of Desmin**

The proportion of desmin-positive cells (30–40% of the cell population) did not change significantly with time during the 72-h culture period in either strain (Table 2). However at 24 h SJL/J muscle cultures had a greater proportion of desmin-positive cells than BALB/c cultures (P < 0.05), although at 48 and 72 h there was no such difference. Many of the desmin-positive cells were synthesizing DNA, as shown in double-labeled cultures where cells positive for BrdU and desmin were present (data not shown). Desmin was present in all myogenin-positive cells (Figs. 3F and 3G), but myogenin was not present in all desmin-positive cells.

**Cultures of Preinjured SJL/J Muscles**

Cells strongly positive for myogenin protein were detected at all times examined. The mean proportion of myogenin-positive cells per 1000 total cells (± SEM) in the cultures (grown on gelatin) at 12 h was 10 ± 1. This frequency represented some positive cells in about every second field examined, contrasting with that in cultures of uninjured muscle where no positive cells
were seen. This proportion increased to 399 ± 27 and 678 ± 33 at 24 and 48 h, respectively. All myogenin-positive cells also stained strongly for desmin, although all desmin-positive cells were not necessarily myogenin positive.

**DISCUSSION**

**In Vitro versus in Vivo—Onset of MyoD/Myogenin Expression**

Primary cultures of mpc extracted from uninjured muscles of 4- to 6-week-old mice are derived from a population of essentially quiescent synchronized mpc [1, 16, 21] which makes it possible to determine the time course and sequence in which genes are switched on during mpc activation. In primary cultures of mpc obtained from either SJL/J or BALB/c adult mice, the onset of transcription of MyoD and myogenin genes was not seen before 24 h. This contrasts with the *in vivo* situation, where transcripts of these genes could be detected at 6 and 12 h in regenerating muscle of SJL/J mice aged 6 to 8 weeks and were conspicuous by 24 h [16].

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Myogenin</th>
<th>MyoD</th>
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<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
</tr>
<tr>
<td>SJL/J</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrigel</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Gelatin</td>
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<td>0.1</td>
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<tr>
<td>BALB/c</td>
<td></td>
<td></td>
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<tr>
<td>Matrigel</td>
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<td>0.1</td>
</tr>
<tr>
<td>Gelatin</td>
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</tbody>
</table>

*Note. The mean ± SEM proportion of labeled/total cells per field (×10³) is shown.

* Myotubes were present.
TABLE 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Myogenin*</th>
<th>BrdU*</th>
<th>Desmin*</th>
<th>Myogenin/desmin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SJL/J</td>
<td>BALB/c</td>
<td>SJL/J</td>
<td>BALB/c</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>79 ± 28</td>
<td>8 ± 2</td>
<td>147 ± 16</td>
<td>199 ± 12</td>
</tr>
<tr>
<td>72</td>
<td>250 ± 25</td>
<td>148 ± 19</td>
<td>150 ± 20</td>
<td>111 ± 18</td>
</tr>
<tr>
<td></td>
<td>450 ± 26</td>
<td>300 ± 17</td>
<td>0.18 ± 0.04</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>380 ± 45</td>
<td>300 ± 16</td>
<td>380 ± 28</td>
<td>320 ± 23</td>
</tr>
<tr>
<td></td>
<td>0.67 ± 0.05</td>
<td>0.44 ± 0.05</td>
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</tr>
</tbody>
</table>

* Values represent the mean proportion of positive/total cells ± SEM × 10^3 per 15 fields counted in each well.

Index relating expression of muscle-specific markers, i.e., proportion of myogenin-positive cells/proportion of desmin-positive cells.

Factors which may contribute to the observed delayed onset in vitro include the absence of exogenous tissue factors that are normally present following injury in vivo and the need to repair cell damage as a result of the enzymatic tissue disaggregation procedures. That the extraction procedure itself did not affect detection of myogenin that was already expressed is shown by the proportion of myogenin-positive cells in 12-h cultures of preinjured muscles. The age difference between the mice used in the in vitro and in vivo experiments (i.e., 5 and 8 weeks) is not relevant as mpc from old animals (rats) have been shown to enter the cell cycle more slowly than those from younger animals [24].

SJL/J versus BALB/c—Onset of MyoD/Myogenin Expression

The earlier onset of MyoD and myogenin transcription in mpc from SJL/J compared with BALB/c mice in vitro shows that SJL/J mpc are activated more rapidly from quiescence. This parallels differences in the onset of DNA synthesis in vivo where the onset of DNA synthesis could always be detected at 24 h in some mpc of SJL/J mice regenerating after crush injury [1; plus unpublished data], whereas in BALB/c mice it was never seen before 30 h in crush-injured muscles [25] and only rarely before this time in BALB/c muscles regenerating in response to metal implants [26].

FIG. 2. Low-power views of BALB/c cultures hybridized with riboprobes. (A) Forty-eight-hour culture grown on Matrigel showing an area with a high proportion of MyoD-positive cells. (B) Seventy-two-hour culture grown on gelatin hybridized with myogenin riboprobe (a higher power view is shown in Fig. 3H). The scale bar is 5 μm.
FIG. 3. Expression of MyoD, myogenin, and desmin in primary cultures of SJL/J and BALB/c muscle. The scale bar is 10 μm. (A and B) Forty-eight-hour SJL/J cultures grown on gelatin. (A) Myogenin mRNA is shown in the cytoplasm of mpc by in situ hybridization and resultant brown alkaline phosphatase reaction product. (B) Myogenin protein is shown by Texas red immunofluorescence localized to the nucleus of mpc. (C and D) Forty-eight-hour SJL/J cultures grown on Matrigel. Both myogenin (C) and MyoD (D) transcripts are present in the cytoplasm of mpc and myotubes. (E) Seventy-two-hour SJL/J culture grown on gelatin and hybridized with myogenin riboprobe. Note apparently fused cells. (F and G) Forty-eight-hour (F) and seventy-two-hour (G) SJL/J cultures grown on Matrigel. Desmin (shown by green FITC in the cytoplasm) is present in all myogenin-positive (red nucleus) cells. (H, I, and J). Seventy-two-hour BALB/c (H, I) and SJL/J (J)
The presence of a slight myositis in some of the SJL/J muscles used for culture, particularly the quadriceps (see Introduction), could contribute to the differential onset of MyoD/myogenin expression if some activated/replicating myogenin-positive cells had been present in the SJL/J but not BALB/c muscles at the time of extraction. Had this played a role in the earlier onset observed, at least some myogenin-positive cells would have been present at 12 h but there were none. The experimental system allowed for the detection of such preactivated mpc as the preactivated mpc (deliberately generated by preinjury of muscle prior to culture) were shown to be strongly positive for myogenin protein (1% of total attached cells) at 12 h, increasing to 40% by 24 h.

Coexpression of MyoD and Myogenin and Replication of mpc

The coexpression of MyoD and myogenin in vitro in the present study is in accord with the reported expression of these proteins after 24 h in cultured mpc derived from adult or embryonic mouse muscle [22]. The onset of MyoD expression at 24 h for SJL/J mpc in vitro also corresponds with data for MyoD expression in cultured mpc from adult (9 month old) rats, where polymerase chain reaction amplification showed that transcripts of MyoD were present at 24 h, although Myf5 and MRF4 mRNAs were not detected until 48 h and myogenin mRNA was delayed until 72 h [R. Allen, Arizona, personal communication]. This delay in myogenin expression in rats clearly contrasts with the situation for adult murine mpc where transcripts and protein could be detected as early as 24 h, at the same time as MyoD in our study. However, it is of interest that Cusella-de-Angelis et al. [22] noted many more mouse cells positive for MyoD than cells positive for myogenin at this time. The apparent coexpression of MyoD and myogenin in populations of mpc in vitro parallels the situation in vivo in mature muscle regenerating after injury [16, 27] and during limb development in mouse embryogenesis [28].

In vivo observations suggest that MyoD and myogenin transcription can occur in populations of replicating mpc [16] and the present study showed that, in 48-h cultures, myogenin protein was present in some cells also labeled with BrdU after exposure for 1 h. Since BrdU is incorporated into nuclei of cells which are synthesizing DNA, this confirms that myogenin is present in some replicating myogenic cells. Only a small proportion of the total replicating mpc population would have been detected in the 1-h BrdU labeling period as S-phase for mpc is approximately 6–8 h [29].

Transcripts of MyoD and myogenin have been observed with digoxigenin-labeled riboprobes in early myotubes in vivo [30], although the transcription of these genes appears to be rapidly down-regulated after myotube formation, as Northern analysis of regenerating mouse muscle showed that mRNA levels for both genes have essentially returned to those of uninjured mature muscle by 2 weeks after crush injury [21].

Proportion of Myogenic Cells in Primary Cultures

It was noted that more adherent cells were extracted per gram of muscle from SJL/J mice, although the extent to which these represent mpc is not known. Although starting cultures were equivalently dense it cannot be excluded that there was a greater absolute number of mpc present in SJL/J cultures. Identification of mpc in primary cultures by staining with antibody to desmin, a cytoskeletal protein, showed that desmin-positive cells represented less than 40% of the total cell population in accord with the results from desmin staining of cultured rat mpc [31]. The lower proportion of desmin-positive cells in BALB/c cultures at 24 h probably represents a relatively delayed desmin expression in these mpc, as at 12 h desmin staining is very weak and increases in intensity up to 48 h [Maley, unpublished]. This indicates that desmin is not a definitive marker for early mpc as the level of expression increases with time after activation. Since only about 30% of the cultured mononuclear cells showed expression of MyoD or myogenin this also suggests that only about a third of the cultured cells were skeletal mpc if, indeed, these genes were expressed in all of the extracted mpc. Similar results were seen in muscle cultures derived from fetal, embryonic, or newborn mice, where the proportion of cells immunopositive for myogenin (or other helix-loop–helix muscle proteins) was always less than 30% [32]. It seems likely that only a subset of mpc extracted from mature mouse muscle might be expressing these genes under the culture conditions as has been demonstrated in developing mouse muscles, where clonal cells isolated from 9-day somites showed after 7 days that only 60% of clones were positive for myogenin [22]. Immunocytochemical studies in our laboratory on the murine skeletal muscle cell lines C2C12 [33] and H-2K, derived from limb muscles of the H-2K<sup>cu</sup>-tsA58 transgenic mouse [34, 35]; a kind gift from Dr T. Partridge, London, UK) confirm that desmin- or myogenin-positive cells also represent less than 40% of these mononuclear mpc populations. As seen in the primary cultures, while all myogenin-positive cells were desmin positive, the reverse was not true [M. Maley, unpublished observations].

The problem of determining the relative proportions of mpc and fibroblasts in primary cultures of skeletal muscle is well reviewed by Yabloinka-Reuveni et al., 1988 [36]. Even on the basis of ultrastructure these cell types could not be quantitated at the early times in either strain. In 5-day cultures, only about 30% of the cells grown on gelatin. Myogenin protein is present in mpc and myotubes (I) and myogenin mRNA is distributed unevenly throughout a large myotube (J).
could be considered to be mpc on the basis of ultrastructural features. While cells containing abundant endoplasmic reticulum and with the appearance of fibroblasts were seen in 5-day primary cultures, cells with the same "fibroblastic" features were also observed [Robertson, unpublished observations] in cultures of the clonal myogenic cell line H-2K [34, 35] (where fibroblasts should not be present). While fibroblasts which may be present in the primary cultures might influence the proliferation of the mpc [37] there is no evidence that they play a role in the onset of MyoD/myogenin transcription.

Effect of Substrate

A comparison was made between cells grown on gelatin (which consists of type I collagen) and Matrigel (which consists mainly of laminin, type IV collagen, heparan sulfate proteoglycan, and entactin [38]) since tissue culture conditions can influence the behaviour of cultured cells [discussed in 39]; it has been shown that mpc adhere faster to matrices of laminin/type IV collagen [40] or Matrigel [41], that laminin stimulates mpc proliferation [42, 43] and differentiation [44], and that Matrigel [41] and entactin [45] promote the maturation and long term maintenance of cultured myotubes. In the present study, the earliest myotubes were seen at 2 days in SJL/J cultures on Matrigel and at 3 days on gelatin, whereas myotubes were less frequent (data not shown) and later in cultures of BALB/c mpc. These observations were confirmed in another study where quantitation of myotube formation at 4 days in equivalent SJL/J and BALB/c cultures grown on gelatin showed that 50% more myotubes were found in SJL/J compared with BALB/c cultures, and myotubes with more than four nuclei were also three times more frequent in SJL/J cultures [Maley et al., in preparation]. The earlier gene expression and earlier myotube formation seen on the Matrigel versus gelatin substrates in both strains was anticipated as Matrigel substrate more closely resembles the composition of the extracellular matrix surrounding the muscle cell in vivo.

Apart from the delay in onset of gene expression, the tissue culture observations appear to reflect the in vivo differences between the strains. The earlier expression of MyoD and myogenin in vitro in SJL/J mice correlates with the earlier onset of mpc replication in SJL/J mice after crush injury in vivo and the earlier myotube formation seen in SJL/J compared with BALB/c muscles both in vitro and in vivo. The results suggest that intrinsic genetic differences in cells of the muscle cultures contribute to the superior myogenesis seen in SJL/J mice.

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