Extracellular Matrix, Growth Factors, Genetics: Their Influence on Cell Proliferation and Myotube Formation in Primary Cultures of Adult Mouse Skeletal Muscle

MOIRA A. L. MALEY, Marilyn J. DAVIES, AND MIRANDA D. GROUNDS

*Research Officer (NH&MRC), †Research Officer (Raine Foundation), ‡Senior Research Fellow (NH&MRC),
Department of Pathology, the University of Western Australia, Nedlands, Western Australia

INTRODUCTION

Following an injury to skeletal muscle, quiescent muscle precursor cells (mpc) become activated and migrate toward the injured area (1) eventually fusing with other mpc to form new multinucleated cells (myotubes) and replace the damaged tissue (2). This process is achieved by a sequence of chemotactic, proliferative, and differentiating signals. While many growth factors, hormones, cytokines, and extracellular matrix (ECM) components have been shown in vitro to regulate the proliferation and differentiation of mpc (reviewed in [3]), the signals regulating myogenesis in vivo both during development and in the adult regenerative situation remain ill-defined.

Inbred SJL/J mice show more vigorous muscle regeneration in vivo compared with BALB/c mice following crush injury [4]. That there are intrinsic differences between mpc from these two strains has already been demonstrated in tissue culture in the form of earlier expression of the skeletal muscle-specific genes MyoD and myogenin in the SJL/J mice [5]. It is now proposed that a difference in responsiveness to proliferative or differentiating stimuli may underlie the superior regeneration seen in vivo in SJL/J mice. In this paper, we have examined the in vitro characteristics of primary cultures from SJL/J and BALB/c skeletal muscle with respect to their proliferation in response to growth factors and various extracellular components (ECM) and their relative capacity to form myotubes in a controlled environment.

As basic fibroblast growth factor (bFGF) [6], the platelet-derived growth factors (PDGF-AB, PDGF-BB) [7], transforming growth factor β (TGF/β) [8], and leukaemia inhibitory factor (LIF) [9] have been previously attributed a role in myogenesis in vitro, it seems likely that they might be involved in muscle regeneration in vivo (reviewed in [3, 10]). Similarly, various ECM components (i.e., collagens I and IV, laminin, fibronectin, and the synthetic basement membrane Matrigel) are also known to selectively influence myogenesis (re-
viewed in [10], [11, 12]). Laminin and fibronectin are reported as mitogenic in various cell types including fibroblasts, epithelial cells, and myoblasts [13–17]. Since an external lamina, composed of these substances is in intimate contact with mature myofibers in vivo [18] it seems likely that the interaction of such ECM components and growth factors may be of central importance in vivo [10]. Thus, primary muscle cultures from SJL/J and BALB/c mice were used (i) to determine whether the proliferative response of mpc to bFGF, PDGF, and TGFβ, was comparable and whether it was affected by ECM components, and (ii) to examine the extent of myotube formation on various ECM components and the influence of LIF on myotube formation.

MATERIALS AND METHODS

Preparation of Primary Cell Cultures

Four- to six-week-old inbred SJL/J and BALB/c specific pathogen-free male mice were purchased from the Animal Resources Centre, Murdoch, Western Australia. Mice were anesthetized with Fluothane (ICI Pharmaceuticals, England), killed by cervical dislocation, and muscles dissected from the lower back and hind limbs. The external fascia was carefully removed from the muscle tissue before immersion in DMEM supplemented with 10% donor horse serum (CytoSystems, Australian Cat. 15-040-100V) (HS) and 60 μg/ml gentamicin sulphate (gent.) (Delta West) until further processing which was commenced within 45 min of the death of the first animal (time 0). All subsequent manipulations were performed in a laminar flow cabinet using aseptic technique and cultures were incubated in a 37°C humidified incubator in a 5% CO₂/air atmosphere.

After further debridement in DMEM the muscle was minced continuously for 5 min in a minimal volume (approximately 1 ml) of collagenase solution (300 units/ml Type 1A Sigma C9891). This slurry was made up to 7 ml/kg of muscle with collagenase solution and stirred moderately fast in a 75-ml trypsinizing flask at 36°C for 35 min and then centrifuged (500g for 10 min). The pellet was washed in phosphate-buffered saline (pH 7.4) (PBS) and resuspended in a trypsin/EDTA solution (0.1%/0.001% in PBS, 7 ml/kg muscle) by trituration. This suspension was stirred at 36°C for 20 min and digestion stopped by the addition of an equal volume of 20% HS/DMEM. The pellet was harvested by centrifugation (as above), washed twice in 10% HS/DMEM, resuspended in 10 ml 10% HS/DMEM/gent., filtered through 45-μm nylon gauge, centrifuged, and the pellet resuspended at an apparent initial concentration of 1.5 × 10⁶/ml for BALB/c muscle and 1 × 10⁷/ml for SJL/J muscle in 10% HS/DMEM/gent., 1 μg/ml linoleic acid (Sigma L8384), and 40 μg/ml dexamethasone (David Bull Labs) (“complete medium”).

As cultures from two strains of mice were to be compared, muscle was harvested from SJL/J and BALB/c mice concurrently and processed in parallel throughout all experiments. The only difference in handling was that 1.5 times as much weight of muscle was required from BALB/c mice as from SJL/J to achieve the same attached cell density. Cultures were incubated at 37°C in a humidified, 5% CO₂ atmosphere. Where a time course of expression of an antigen was to be studied, cultures were removed from incubation and fixed at the appropriate time intervals and stored at 4°C as indicated.

For [³H]thymidine incorporation studies, 100 μl of cell suspension was plated into each well of a 96-well tissue culture plate (Corning 25860) and for immunostaining studies, 400 μl of cell suspension was plated into each well of an 8-well tissue culture slide (Nunc, Cat. 177402).

ECM Components

The surface of culture wells were precoated with gelatin (Sigma G1890) (Collagen I), collagen IV (Sigma C0543), fibronectin (Sigma F4759), or laminin (entactin-free) (Collaborative Biomedical 40230) by incubation at room temperature overnight, air-dried, and sterilized by ultraviolet irradiation. A 1/10 dilution of Matrigel (Collaborative Biomedical 40234) in DMEM was also used to “thin-coat” culture wells.

Cell Lines

The C57Bl/6J mouse C2C12 myogenic cell line (CRL1772) was obtained from the American Type Culture Collection (ATCC) and cells were grown in DMEM (Trace 50-115-PB) supplemented with 10% fetal calf serum (FCS) (CytoSystems 15-010-0100V, Sydney, Australia), 1% penicillin/streptomycin (p/s) (Flow-ICN 16-700-49). Another murine myogenic cell line has been derived from the H-2K<sup>b</sup><br>tsA58 mouse [19], and its clone 27, referred to hereafter as H-2K<sup>b</sup>(27) cells [20], was a kind gift from Professor T. A. Partridge (London, UK). These were grown at 37°C under nonpermissive culture conditions (as described below) to allow differentiation in response to experimental conditions. The mouse 3T3 fibroblasts used were originally from ATCC (CCL163) and were grown in RPMI 1640 (Trace 50-022-PB) supplemented with 5% FCS, 1% p/s.

Growth Factors

The recombinant peptide growth factors used were human bFGF (a generous gift from Synergy, Co., U.S.A.), AA-PDGF, AB-PDGF, and BB-PDGF (Upstate Biotech, NY), TGFβ, (Genzyme), and mouse LIF (a generous gift from Amrad, Australia). Each factor was thawed and diluted in serum-free tissue medium immediately before addition to cultures.

Quantitation of Cell Replication by [³H]Thymidine Incorporation

When cells synthesizing DNA are exposed to [³H]thymidine it is incorporated into DNA and the amount of incorporated isotope (detected by β-scintillation counting) can be used as a measure of the amount of replication.

Assay protocol. Cells were allowed to attach for the first 24 h of culture in complete medium after which each well was washed with PBS and then refed with 1 part complete medium to 9 parts DMEM supplemented with gent. At this time, growth factors (in 5 μl), or 10% horse serum (positive control), or DMEM only (negative control) were added to each well in quadruplicate. Twenty hours later, each well was pulse-labeled with 0.1 μCi of [³H]thymidine (Amersham TRK926) and incubated for a further 4 h when the assay was stopped (at 48 h after time 0) by the addition of 60 μl of 1 M NaOH to every well and stored at 4°C until harvest. The contents of each well were harvested onto glass fiber filters (Titertek Cat 78-115-05) using a Titertek Cell Harvester and counted by β scintillation. The data downloaded from an LKB-Wallace Rackbeta Counter was imported into the SAS statistical package (SAS Institute, NC) and experimental groups were compared by analysis of variance using the general linear models and ANOVA procedures of SAS.

Immunostaining of Primary Cultures

Expression of the intermediate filament desmin (a cytoplasmic marker for myogenic cells) [21–23] was used to distinguish mpc from fibroblasts in the primary muscle cultures. Desmin was used instead of myogenin (a highly specific marker for skeletal mpc) [5] since double staining for myogenin and 5-bromo-2′-deoxyuridine (BrdU) is unsatisfactory as both are nuclear markers. Culture slides were stained sequentially with a rabbit anti-desmin antibody (Immunon 490410), a biotinylated anti-rabbit IgG (Boehringer-Mannheim
Assessment of Myotube Formation in Vitro

Myotube formation was compared by plating cells at moderate (10⁶ cells/ml) and high (3 × 10⁶ cells/ml) density in 8-well culture slides. Cells were grown in complete medium for 96 h before fixing and staining with a light hematoxylin and eosin. Image analysis (see below) was used to quantitate the number of myotubes with 2–3 nuclei, or 4 or more nuclei, and statistical comparisons were made. These preparations were later restained for desmin using a peroxidase substrate (as above).

Quantitation by Image Analysis

The data collection sampling parameters, object classes, and color thresholding categorization of the Optimas image analysis system (Bioscan, Inc., WA) were used to quantitate the following: the number of blue nuclei (i.e., hematoxylin stained); the number of brown nuclei (i.e., peroxidase-labeled, BrdU-positive); the number of myotubes (i.e., eosin stained where the density of the preparation allowed discrimination); and the total area of brown cytoplasm (i.e., peroxidase-labeled, desmin-positive) per field, in 50 fields of each well of the appropriate 8-well slide culture. Slides were viewed on a Zeiss microscope using a 25× objective and images captured with a Pulnix TMC-76 camera. The tallies were output as an Ascii file and experimental groups compared by analysis of variance using SAS. This semi-automation of cell counting allowed larger data samples to be collected while still having the reliability of the experimenter viewing every field and validating the categorization made by the image analysis software.

RESULTS

Response to Growth Factors

Primary cultures of BALB/c and SJL/J muscle were prepared in parallel and their proliferation on gelatin was compared over dose–response curves for bFGF, PDGF-AA, PDGF-AB, PDGF-BB, at 10, 4, 0.8, and 0.016 ng/ml, and for TGFβ₁ at 5, 1, 0.2, and 0.004 ng/ml. bFGF and each of the PDGF isoforms were mitogenic for primary cultures of both SJL/J and BALB/c muscle; however, TGFβ₁ was not mitogenic (Fig. 1). Comparison of SJL/J and BALB/c dose–response curves by two-way analysis of variance (using strain and growth factor concentration as effects) confirmed that the SJL/J cultures were more responsive to bFGF than BALB/c cultures (P < 0.05) but there was no such difference for any of the other growth factors tested. These primary cultures comprise both mpc and fibroblasts although the actual proportion is difficult to determine [5]. The potency of the growth factors used was examined using the established myogenic cell line C2C12 and mouse 3T3 fibroblasts in a similar experimental protocol (data not shown). Each of bFGF, PDGF-AB, and PDGF-BB were mitogenic for primary muscle, 3T3 fibroblasts, and C2C12 cells; PDGF-AA was not mitogenic in 3T3 fibroblasts or C2C12 cells, whereas TGFβ₁ was mitogenic in these cells but not in the primary cultures; LIF was not mitogenic for any cell type (data not shown). Similar experiments carried out with the myogenic H-2Kb(27) cells under both permissive and nonpermissive culture conditions [20] showed minimal response to any of the growth factors in three separate experiments (data not shown). The proliferative response of SJL/J primary cultures to a range of concentrations of bFGF and TGFβ₁, was compared in parallel for cultures grown on gelatin versus Matrigel. The only statistically significant difference seen in these parallel cultures was at the lower end of dose–response curves, where there was more proliferation in response to 0.2 ng/ml bFGF on gelatin than on Matrigel (P < 0.05) (Fig. 2).

The effect of a range of ECM substrates was tested in BALB/c cultures. The proliferation by BALB/c primary
cultures to low dose (0.1 ng/ml, i.e., submitogenic) and high dose (50 ng/ml, i.e., supramitogenic) bFGF was compared in parallel between cells grown on collagen I (gelatin), Matrigel, laminin, fibronectin, and collagen IV. These cultures were seeded at a lower cell density (2 x 10^4/ml) with the aim of reducing the contribution of endogenously secreted extracellular matrix. The proliferative response was not affected when the cells were plated on a selection of ECM components, i.e., collagen I, Matrigel, laminin, fibronectin, and collagen IV (Fig. 3).

Visual identification of proliferating cells in the cultures was also carried out. Primary cultures of SJL/J muscle were plated on Matrigel and assayed in the presence of 1 ng/ml bFGF or PDGF-BB and the slides double-stained for BrdU (identifying cells synthesizing DNA) and desmin (identifying myoblasts). Positive and negative controls were also run according to the assay protocol (above) for measurement of cell replication. The frequency of BrdU-positive cells was significantly increased (P < 0.05) in the presence of bFGF or PDGF (negative control 7.8 ± 0.6%, 1 ng/ml bFGF 17.8 ± 1.1%, 1 ng/ml PDGF-BB 16.2 ± 0.9%, positive control (10% HS) 20.3 ± 1.2%). Although cells with a myoblast-like phenotype, i.e., bipolar and lined up in parallel with each other, were frequently BrdU positive, very few of these stained appreciably positive for desmin as detected by peroxidase labeling. These are not illustrated because of the difficulty in distinguishing between BrdU-positive nuclei (peroxidase-labeled) and BrdU-negative nuclei (only hematoxylin stained) in black and white photography. Myotubes were present in the positive and negative control wells but were rarely seen in the wells with either bFGF or PDGF in these 48-h cultures. Similarly, fewer desmin-positive mononuclear cells were seen with either bFGF or PDGF in contrast with either of the controls (data not shown).

Comparison of Myotube Formation in SJL/J versus BALB/c Mice

Parallel cultures of SJL/J and BALB/c mouse muscle were plated on gelatin and Matrigel at moderate and high densities. These culture slides were fixed and stained for desmin after 4 days. The number and size of myotubes in each of 50 fields examined was compared between strains in cultures grown for 4 days on gelatin (collagen I) or Matrigel (Table 1). In the moderately dense cultures (initial plating density 1 x 10^4/ml) it was possible to discriminate between myotubes with 2-3 nuclei and those with 4 or more nuclei; however, in the high-density cultures (initial plating density 3 x 10^4/ml) it was difficult to make this discrimination with confidence because of the very dense appearance of the preparation. Therefore, moderately dense cultures were used for these studies.

There was a statistically significant difference between the strains in both the number and size of myotubes grown on gelatin. In the moderately dense cultures, more than twice as many myotubes were formed in SJL/J than BALB/c cultures on both gelatin and Matrigel (P < 0.05). Similarly, on gelatin there was a greater than 3-fold higher frequency of myotubes with 4 or more nuclei in SJL/J compared with BALB/c cultures (P < 0.05) (compare Figs. 4C with 4A and 4G with 4E). On Matrigel, the proportion of myotubes having greater than 3 nuclei was similar between the strains.


<table>
<thead>
<tr>
<th></th>
<th>Moderately dense cultures</th>
<th>Very dense cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SJL/J</td>
<td>BALB/c</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.035 ± 0.002</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>Matrigel</td>
<td>0.053 ± 0.003</td>
<td>0.043 ± 0.003</td>
</tr>
<tr>
<td>Myotubes with 4 or more nuclei per total nuclei/field</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.031 ± 0.002</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>Matrigel</td>
<td>0.047 ± 0.003</td>
<td>0.042 ± 0.002</td>
</tr>
</tbody>
</table>

(compare Figs. 4D with 4B and 4H with 4F). The largest myotubes in BALB/c cultures were seen on Matrigel (Figs. 4B and 4F) but these were not as frequent as in SJL/J cultures where many myotubes, in cultures grown on Matrigel (Figs. 4D and 4H) and to a lesser extent on gelatin (Figs. 4C and 4G), were far larger sometimes having more than 20 nuclei per myotube. In very dense cultures, the numbers of myotubes formed by SJL/J versus BALB/c cultures was similar on gelatin, but on Matrigel the SJL/J cultures formed more myotubes than did BALB/c (P < 0.05) (Table 1).

**Effect of LIF on Myotube Formation**

A similar experiment was run using moderately dense cultures with and without LIF (3 units/ml). In SJL/J muscle cultures, LIF had no effect on either the number of myotubes formed or the proportion of these with 4 or more nuclei whether grown on gelatin or Matrigel (Table 2). However, in BALB/c cultures, more myotubes formed in the presence of LIF in cultures grown on Matrigel (P < 0.05), and fewer myotubes formed with LIF in cultures grown on gelatin (P < 0.05).

**Effect of ECM Components on Myotube Formation and Desmin Expression**

In order to ascertain if any one ECM component was critical to myotube formation, cultures of BALB/c muscle (plated at 10⁶ cells/ml) and of H2-Kb(27) cells (plated at 10⁵ cells/ml) were prepared in wells precoated with gelatin, Matrigel, laminin, fibronectin, or collagen IV. These culture slides were fixed and stained for desmin after 4 days. Table 3 summarizes myotube formation by BALB/c cultures grown on various ECM components. Matrigel promoted the formation of larger and more frequent myotubes than was seen with either gelatin, laminin, fibronectin, or collagen IV alone (Figs. 4B and 4F versus 4A and 4E). None of laminin, collagen IV, fibronectin, nor gelatin promoted the formation of larger myotubes as well as did Matrigel, although fibronectin significantly enhanced the formation of smaller myotubes and laminin or collagen IV alone generally had similar effects to gelatin (collagen I). These ECM effects are reflected in their ratios of small to large myotubes (Table 3). The formation of mononuclear cells into aligned parallel arrangements was more marked and there were more desmin-positive mononuclear cells in cultures grown on Matrigel (Table 3). Myotubes were always desmin positive. Many of the mononuclear cells in these 4-day cultures were desmin positive, in contrast with mononuclear cells in 2 day cultures, although the proportion varied with the ECM component (Table 3).

The extent of desmin expression in these cultures was also quantitated using image analysis which included desmin present in myotubes as well as mononuclear cells. In this evaluation, the ratio per field of the total area of desmin-positive cytoplasm per total number of nuclei present was calculated. The statistical comparisons exactly matched those shown in Table 3 for the frequency of desmin-positive cells. The mean ratios (and their variances) were as follows: gelatin—0.0394 (0.0006), Matrigel—0.1051 (0.0006), laminin—0.0262 (0.00005), fibronectin—0.0634 (0.0028), collagen IV—0.0235 (0.0001).

Similar effects of ECM were seen in cultures of the pure myogenic H2-Kb(27) cells (Table 4) as more small myotubes were seen on gelatin than on any other matrix and larger myotubes were more frequent on Matrigel. Fibronectin and laminin had similar effects to Matrigel with more large myotubes being seen than on gelatin or collagen IV. This is clearly reflected in the ratio of small to large myotubes in Table 4. The extent of desmin expression in H2-Kb(27) cells was examined by image analysis (as above). The mean ratios (and
TABLE 2

Effect of LIF on Myotube Formation in Moderately Dense Cultures

<table>
<thead>
<tr>
<th></th>
<th>SJL/J (Mean number per field ±SE)</th>
<th>BALB/c (Mean number per field ±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myotubes (total)</td>
<td>With &gt;3 nuclei</td>
</tr>
<tr>
<td>Gelatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No LIF</td>
<td>36.8 ± 4.2</td>
<td>11.7 ± 2.0</td>
</tr>
<tr>
<td>LIF</td>
<td>34.5 ± 7.5</td>
<td>10.2 ± 1.2</td>
</tr>
<tr>
<td>Matrigel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No LIF</td>
<td>46.6 ± 3.3</td>
<td>14.6 ± 2.5</td>
</tr>
<tr>
<td>LIF</td>
<td>48.2 ± 1.9</td>
<td>19.6 ± 0.9</td>
</tr>
</tbody>
</table>

Note. The mean number myotubes per field ±SE and the mean number within 2–3 and 4 or more nuclei/myotube per field (net indexed) ±SE are shown.

their variances) were as follows: gelatin—0.2701 (0.0033), Matrigel—0.3959 (0.0054), laminin—0.4136 (0.0088), fibronectin—0.3028 (0.0063), collagen IV—0.2503 (0.0064), no ECM (N)—0.1983 (0.0113). The statistical comparison showed G < M; G < L; G < F; G ≈ C; G > N; M > F.

DISCUSSION

Response of SJL/J versus BALB/c Primary Muscle Cultures

Differences between primary muscle cultures from SJL/J and BALB/c mice have been reported previously with respect to a more rapid expression of the skeletal muscle-specific transcription regulators MyoD and myogenin [5]. As the time of onset of mpc replication is also earlier in regenerating muscle of SJL/J compared to BALB/c mice in vivo [24], it was the early events in the mpc response in the two strains that were of interest in the present study. Therefore, the primary cultures were challenged by growth factor at 24 h and the proliferation rates compared at 48 h. A greater mitogenic responsiveness to bFGF was seen in primary muscle cultures from SJL/J versus BALB/c mice but there was no difference in the response to the PDGFs and TGFβ1. All of these factors have been previously attributed a role in myogenesis [6–9]. Further differences between myogenic cells of these strains were confirmed by the striking finding of more frequent and larger myotubes formed in SJL/J cultures grown for 4 days on either gelatin (predominantly comprising collagen type I) or Matrigel (a cocktail of laminin, collagen type IV, heparan sulphate proteoglycans, entactin, bFGF, TGFβ1, and other unspecified cytokines), i.e., when the strains were compared on either a basal or a rich ECM environment the SJL/J mpc consistently showed superior myogenic potential in vitro.

Effect of Growth Factors

bFGF is a potent mitogen in vitro for mpc from mice [25–28], chickens [6, 29], rats [22, 30], and cows [31, 32] and is considered a competence factor which acts early in the cell cycle to move cells from G0 into G1 [8]. Our results showed bFGF to be a potent mitogen for all primary cultures but, since it was mitogenic for both 3T3 fibroblasts and C2C12 myoblasts, we cannot be certain which specific cell types were responding in the mixed population of the primary cultures.

Other studies in our laboratories have investigated the in vivo role of bFGF in the two strains. Immunofluorescence with bFGF antibodies showed a greater intensity of bFGF staining in cells of SJL/J compared with BALB/c regenerating muscles in vivo [33], although in this situation, it is also difficult to identify the mononuclear cells which are stained. Because of the importance of bFGF as a myoblast mitogen in vivo and as an angiogenic agent in vivo, we devised a major series of experiments to test whether exogenously administered bFGF could enhance the repair of injured BALB/c muscle in vivo. Administration of the bFGF (sometimes in combination with heparin), was by either direct injection or release from implanted polymers. Despite the considerable effort dedicated to this study, no effect of bFGF was seen on the regenerative response and the histological pattern was unaffected (Mitchell McGeeachie Grounds, submitted for publication). From that study, it was concluded that exogenous bFGF had no effect on muscle regeneration and that

FIG. 4. Photomicrographs of SJL/J (C, D, G, and H) and BALB/c (A, B, E, and F) primary muscle cultures grown on gelatin (A, C, E, and G) or Matrigel (B, D, F, and H) for 4 days, stained for desmin by immunoperoxidase, and nuclei lightly counterstained with hematoxylin; magnification A, B, C, D, ×64; E, F, G, H, ×160.
### TABLE 3

Myotube Formation in BALB/c Primary Cultures Plated on Various Extracellular Matrices

<table>
<thead>
<tr>
<th>Type of culture matrix</th>
<th>Mean value of 50 fields per well (± standard error)</th>
<th>Proportion of myotubes with 2–3 nuclei versus myotubes with &gt;4 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of myotubes with 2 to 3 nuclei</td>
<td>Number of myotubes with 4 to 6 nuclei</td>
</tr>
<tr>
<td>Gelatin</td>
<td>3.3 ± 0.23</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>Matrigel</td>
<td>4.2 ± 0.38</td>
<td>2.21 ± 0.31</td>
</tr>
<tr>
<td>Laminin</td>
<td>3.0 ± 0.24</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>4.67 ± 0.31</td>
<td>0.56 ± 0.11</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>2.94 ± 0.32</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>G &lt; M (P &lt; 0.04)</td>
<td>G &lt; M (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>G = L</td>
<td>G = L</td>
</tr>
<tr>
<td></td>
<td>G &lt; F (P &lt; 0.001)</td>
<td>G &lt; F (P &lt; 0.003)</td>
</tr>
<tr>
<td></td>
<td>G &gt; C (P &lt; 0.03)</td>
<td>G = C</td>
</tr>
<tr>
<td></td>
<td>M = F</td>
<td>M &gt; F (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

bFGF may not normally be a limiting factor during myogenesis of mature skeletal muscle. For these reasons, the tempting speculation that the greater proliferative response of SJL/J cells to bFGF seen in vitro compared with BALB/c may account, at least in part, for the greater number of mpc seen in regenerating muscles of this strain in vivo remains unproven.

The -BB and -AB isomers of PDGF are mitogenic for C2C12 mouse myoblasts [34], L6 cells [35], and primary chicken myoblasts [7] and, in our experiments, C2C12 cells also responded to the -BB and -AB but not the PDGF-AA isoform. Although PDGF A-chain mRNA is expressed by myoblasts, DNA synthesis is not stimulated by the PDGF-AA isoform in these cells; however, cultures of fibroblasts derived from chicken muscle have been shown to have higher binding of the PDGF-AA than of the -AB and -BB isoforms [7]. That our primary cultures comprised both myogenic and fibroblastic cells probably accounts for their response to all three isomers of PDGF. Previous work from our laboratories showed that replicating mpc, i.e., BrdU positive, also expressed desmin (which was readily detected using fluorescent probes) [5]. That few cells positive for both BrdU and desmin were detected by the peroxidase method used in this study probably reflects the greater sensitivity of fluorescent techniques [36]. The observations that (i) myotubes and (ii) desmin-positive mononuclear cells were less frequent in the wells containing bFGF or PDGF in relation to control wells are consistent with reports where inhibition of desmin expression

### TABLE 4

Myotube Formation in H-2Kb(27) Cell Cultures Plated on Various Extracellular Matrices

<table>
<thead>
<tr>
<th>Type of culture matrix</th>
<th>Mean value of 50 fields per well (± standard error)</th>
<th>Proportion of myotubes with 2–3 nuclei versus myotubes with &gt;4 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of myotubes with 2 to 3 nuclei</td>
<td>Number of myotubes with 4 to 6 nuclei</td>
</tr>
<tr>
<td>Gelatin</td>
<td>12.9 ± 1.0</td>
<td>3.53 ± 0.62</td>
</tr>
<tr>
<td>Matrigel</td>
<td>8.2 ± 0.6</td>
<td>7.64 ± 0.69</td>
</tr>
<tr>
<td>Laminin</td>
<td>10.0 ± 0.7</td>
<td>6.07 ± 0.88</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>9.9 ± 0.8</td>
<td>9.46 ± 0.69</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>10.2 ± 0.6</td>
<td>4.85 ± 0.62</td>
</tr>
<tr>
<td>No ECM</td>
<td>8.1 ± 0.8</td>
<td>3.27 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>G &gt; M (P &lt; 0.0001)</td>
<td>G &lt; M (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>G &gt; L (P &lt; 0.02)</td>
<td>G &lt; L (P &lt; 0.03)</td>
</tr>
<tr>
<td></td>
<td>G &gt; F (P &lt; 0.02)</td>
<td>G &lt; F (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>G &gt; C (P &lt; 0.03)</td>
<td>G = C</td>
</tr>
<tr>
<td></td>
<td>G &gt; N (P &lt; 0.0001)</td>
<td>G = N</td>
</tr>
<tr>
<td></td>
<td>M &lt; F (P = 0.05)</td>
<td>M &lt; F (P &lt; 0.05)</td>
</tr>
</tbody>
</table>
using anti-sense nucleotides abrogated myoblast fusion and myotube formation in C2C12 cells [37] and where FGF reversibly inhibited creatine phosphokinase gene expression in the fusion-incompetent myogenic BC3H1 cells [38].

Under our experimental conditions, TGFβ stimulated DNA synthesis in C2C12 myoblasts. This contrasts with other studies in C2C12 cells [39] and BC3H1 cells [27] where inhibition of myoblast differentiation by TGFβ; was not associated with stimulation of proliferation, as it is with bFGF [38, 40]. This discrepancy is possibly due to differences in the cell density, serum concentration, culture substratum (i.e., plastic or fibronectin-coated culture surface) and time in culture. In the primary mouse cultures of the present study TGFβ, did not affect proliferation. Similarly, other reports utilizing adult rat or bovine primary muscle cultures grown for 5 days on fibronectin [22, 31] or Matrigel [8] and assayed in defined medium, or embryonic chicken myoblast cultures grown on tissue culture plastic and assayed in 10% HS, 5% chicken serum [40], found that TGFβ did either not affect or inhibited proliferation. A common thread in discussions of TGFβ is the context dependence (i.e., what other factors are present) of its effects, especially in relation to modulation of extracellular matrix production [41]. Species differences in responsiveness have also been recorded [31].

The lack of proliferative response to LIF in 24-h mouse cultures is consistent with the latency in which LIF's proliferative effect on myoblasts is only detectable in cultures 5 days after exposure to LIF [42]. LIF is a pleiotropic growth factor which, in addition to its effects on hemopoietic cells [43], has been shown to influence myoblast proliferation with an unusually long latency of its affect [9]. LIF's structural identity with cholinergic neuronal differentiation factor [44] (isolated from cultured rat heart cells) which can direct the neurotransmitter phenotype expressed by rat sympathetic neurones in vitro and its reported effects on the differentiation of stromal cells [45] does not indicate why it should selectively influence myoblast formation by BALB/c mycultures but not on SJL/J in the present study.

**Effect of ECM Components**

Extracellular matrices function to provide physical support to a tissue and to maintain cellular viability. In skeletal muscle, the basement membrane, which forms an external lamina around each myofiber, is composed mainly of collagen type IV, the glycoprotein laminin, entactin, and heparan sulphate proteoglycans (HSPG). The interstitial ECM between the muscle fibers is composed mainly of type I collagen, fibronectin, and HSPG [46]. Each of these ECM components either binds growth factors, their proteolytic fragments show growth factor homology, or they are mitogenic themselves [17, 47] and so it might have seemed likely that the nature of the culture substratum would modulate the mitogenic response to exogenous growth factors [32]. However, the only difference seen in the proliferation of low-density primary BALB/c cultures plated on gelatin, Matrigel, entactin-free laminin, fibronectin, or collagen IV in response to low- or high-dose bFGF was the greater proliferation with the supramitogenic doses of bFGF seen with cultures grown on gelatin than in cultures grown on any other ECM. This could be interpreted as increased adsorption of bFGF by gelatin; however, the overall picture is that adsorption/inactivation of the soluble growth factor ligand or mitogenic activity of the ECM component itself did not influence the proliferative response in this assay system. This conclusion is further supported by the similar dose-response curves of moderately dense SJL/J cultures grown on Matrigel and gelatin in accord with previous work using embryonic chicken myoblasts [48].

The purpose of our proliferation studies was to compare the behavior of cells from the two strains and their relative responsiveness to various ECM components. It is difficult to compare the proliferation results in the present study to other myoblast studies in the literature because of variation in (i) the source of myogenic cells, either with respect to species, age of the animals used for primary cultures, or the use of immortalized myogenic cell lines; (ii) the culture conditions; and (iii) timing of measurements. For example, laminin applied to pollysine-coated plates is reported to be a selective mitogen for myogenic versus nonmyogenic cells from cultures of newborn rat hind limb, relative to collagens I or IV, but only after at least 36 h in culture with 10% HS [17], and these authors conclude that myogenic cells from different developmental stages (fetal, newborn, adult) may require and respond to different ECM’s. On the other hand, an increased rate of [3H]-thymidine uptake was seen only in the first 24 h of culture on laminin, versus fibronectin or collagen I, in MM14 myoblasts [16]; these culture plates were all precoated with collagen before laminin and fibronectin were added and the horse serum used was preabsorbed to be fibronectin-free. Furthermore, where primary and clonal cultures from fetal mouse skeletal muscle were used to compare the effects of laminin and fibronectin on myoblast differentiation, it was concluded that... . laminin stabilises the myogenic phenotype while fibronectin acts antagonistically to promote mpc proliferation” [11]. In summary, in the present work entactin-free laminin was used (entactin has a role in myotube adhesion [49]); the culture wells were plated only with the ECM component to be tested; the cultures were
from adult mouse skeletal muscle containing a proportion of nonmyogenic cells; and the relevant time course was 24–48 h of culture in either low serum with and without added bFGF or 10% entire horse serum.

In contrast with the minimal effect on mpc proliferation in the present study, the nature of the ECM did significantly affect myotube formation. It is not clear whether this is a result of enhancing mpc differentiation or of facilitating the fusion process. In moderately dense cultures there were consistently more myotubes on Matrigel than on gelatin in both strains of mice. There was also a marked difference between the strains with many more and larger myotubes being found in SJL/J compared to BALB/c particularly on gelatin. Similar results in response to Matrigel versus gelatin have been reported in cultures of adult rat satellite cells [12]. The individual contributions of the main components of Matrigel were assessed using (i) BALB/c primary muscle cultures (as it was BALB/c mpc which showed the greatest difference between gelatin and Matrigel) and (ii) the myogenic H-2K\(^a\)(27) cell line (which forms myotubes under nonpermissive culture conditions) with regard to the effect on numbers of desmin positive cells and the relative frequency and size of myotubes formed. None of the ECM components alone supported myotube formation as well as did Matrigel in the BALB/c cultures although fibronectin closely approximated Matrigel. Laminin and collagen IV had either a negative or no effect on myotube formation in these cultures in comparison with gelatin. In the H-2K\(^a\)(27) cultures, both fibronectin and laminin produced equivalent or better myotube formation to that seen with Matrigel. The correlation with the effect of fibronectin in primary cultures supports an important role for fibronectin in myotube formation. Other studies have also attributed a selective role in myoblast fusion to the fibronectin component of the basal lamina [50, 51]. It may be, again, that fibroblasts present in primary cultures modify the response of the mpc to fibronectin and to laminin and collagen IV [37, 52]. Desmin expression was also influenced by ECM in both primary and the pure myogenic H-2K\(^a\)(27) cultures. A difference between the two culture types was seen with laminin on which there was reduced desmin expression relative to gelatin in the primary cultures yet increased expression in the H-2K\(^a\)(27) cultures. This is no doubt related to the 10-fold greater frequency of desmin expression (per total number of nuclei) in H-2K\(^a\)(27) cultures which are pure myogenic cultures, relative to primary cultures which contain an unknown proportion of desmin-negative fibroblasts.

Culture density influenced the extent of myotube formation, in so far as very dense BALB/c cultures formed myotubes on gelatin at a similar frequency to very dense SJL/J cultures, even though on Matrigel, very dense BALB/c cultures still formed myotubes less frequently than did very dense SJL/J cultures. This relationship between culture density and ECM component is probably readily accounted for by paracrine stimulation of cultures by secretion of growth factors and ECM known to be produced by cultured mpc and fibroblasts. Paracrine stimulation of satellite cell proliferation has been reported [53] where conditioned medium from myotube cultures from young mice were mitogenic for satellite cells from old mice. Studies with bovine muscle cultures also showed that nonmyogenic fibroblast cells produce myotrophic (paracrine) factors which specifically increase mpc numbers and \(^3H\)thymidine uptake [52]. Thus, the denser cultures would probably have greater concentration of paracrine factors (myogenic or proliferative) [52] than less dense cultures and so enable BALB/c cultures to form myotubes at an equivalent rate to SJL/J. That SJL/J cultures still form more myotubes than BALB/c at high density on Matrigel further substantiates our previous findings of an intrinsic difference in the myogenic potential of SJL/J myoblasts.

SJL/J primary muscle cultures were more mitogenically responsive to bFGF than BALB/c in vitro. There was also a striking difference between these strains in their capacity to form myotubes and this was influenced by the nature of the ECM. These in vitro differences, particularly in myotube formation, correlate with the different capacity for muscle regeneration seen in vivo between SJL/J and BALB/c mice.

This work was made possible by the generous support of the National Health and Medical Research Council of Australia and by the Raine Medical Research Foundation of the University of Western Australia. We acknowledge the professional photographic work of the Medical Illustrations Unit of the University of Western Australia.

REFERENCES


Received October 24, 1994
Revised version received March 23, 1995