ISOLATION AND CHARACTERIZATION OF CANINE SATELLITE CELLS

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SUMMARY

Satellite cells were isolated from biopsies of the biceps femoris of adult dogs. Virtually all cells expressed muscle-specific proteins. Proliferation of satellite cells increased as the concentration of fetal calf serum (FCS) was increased from 1 to 10% of the basal medium. The addition of mitogenic growth factors resulted in greater proliferation than that of cells cultured in basal medium alone. Maximum proliferation was obtained when fibroblast growth factor-basic (FGF2) was added to the medium, but differences existed between sources or types. Proliferation did not plateau when the concentration of recombinant human FGF2 was 75 ng/ml but reached maximum levels when 50 ng/ml of bovine FGF2 or 10 ng/ml of growth hormone or insulin-like growth factor-1 were added to the medium. Proliferation of satellite cells decreased when more than 5 ng/ml of transforming growth factor-β was included in the medium. Exposure of canine satellite cells to chemically defined media induced greater fusion of total nuclei (ODM—34%; 4F, IGF-CF, and SPG—23%) than exposure to other treatments, such as basal medium plus 2 mg/ml of 1-β-D-arabinofuranosylcytosine, 5% chick embryo extract, 1% horse serum (average 9% fused nuclei), or 1% FCS (2% fused nuclei). Actin, myosin, desmin, neural cell adhesion molecule, MyoD1, and myogenin were expressed by canine satellite cells, but expression of major histocompatibility complex class II antigen was not detected. Reverse transcriptase-polymerase chain reaction detected expression of messenger ribonucleic acid for interleukin-6 (IL-6), IL-15, and leukemia inhibitory factor by canine satellite cells. Collectively, these data suggest that isolated canine satellite cells display properties of other types of myogenic cells and may be useful for further study of the regulation of postnatal myogenesis.

Key words: dog; muscle; cytokines; growth factors; myogenesis.

INTRODUCTION

Satellite cells are myogenic precursor cells located between the sarcolemma and the basal lamina of the mature myofiber (Mauro, 1961) that are present in skeletal muscle of all mammals (Schultz and McCormick, 1994). Although normally quiescent in adult muscle, satellite cells are responsible for both muscle cell hyperplasia and hypertrophy. Satellite cells become activated in response to stress or trauma to muscle tissue and proliferate, differentiate, and fuse to form myotubes (Schultz and McCormick, 1994).

Repar to damaged muscle is a complex and highly orchestrated event modulated by various growth factors, hormones, and cytokines. Growth factor regulation of satellite cell activity has been extensively documented in rats and mice, as well as other mammalian species, including cattle, sheep, chickens, turkeys, pigs, fish, and horses (reviewed by Burton et al., 2000). The most extensively studied factors related to satellite cell regulation include epidermal growth factor (EGF), basic fibroblast growth factor (FGF2), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). Growth factors may affect proliferation and differentiation. Satellite cell proliferation is generally enhanced by FGF2, HGF, IGF-1, and PDGF (Allen and Rankin, 1996; Bornemann et al., 1999, 2001). Transforming growth factor-β (TGF-β) is also proliferative to satellite cells (Austin and Burgess, 1991; Austin et al., 1992), and growth hormone (GH) is an important regulator of muscle growth and regeneration and enhances satellite cell proliferation in some species (Halevy et al., 1996). Satellite cell differentiation is generally inhibited by FGF2 and TGF-β and enhanced by IGF-1 (Allen and Rankin, 1990; Husmann et al., 1996; Bornemann et al., 1999, 2001). Cytokines are key factors in activating the immune system. Leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and IL-15 appear to play important roles in muscle regeneration, and muscle researchers have discovered that these cytokines can also have a profound effect on satellite cells. Rat myoblast proliferation is stimulated both in vitro and in vivo by LIF (Kurek et al., 1996, 1997). Interleukin-6 is secreted by activated monocytes and stimulates proliferation of human satellite cells (Cantini et al., 1995). Injured muscle expressed IL-6 messenger ribonucleic acid (mRNA) (Kami and Senba, 1998), and human myoblasts secreted IL-6 in vitro constitutively and after treatment with cytokines (Bar-tocci et al., 1994). Interleukin-15 is highly expressed in skeletal muscle (Quinn et al., 1995) and increases the accumulation of muscle-specific contractile proteins in differentiated myoblasts (Quinn et al., 1995, 1997).

There is very little information available regarding the charac-
Satellite cells were isolated by differential centrifugation and the resulting cell pellet was subsequently resuspended in Dulbecco modified Eagle medium (DMEM; GIBCO, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), penicillin (100 U/ml; Sigma-Aldrich, St. Louis, MO), and streptomycin (0.1 mg/ml; Sigma–Aldrich). Canine satellite cells were transferred to a tissue culture flask that was coated with 0.1% pig skin gelatin (PSG; Sigma–Aldrich) and incubated at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Cells isolated from the adult mongrel dogs were used for proliferation, fusion, immunostaining, and Western blot assays and were not allowed to exceed six passages. Virtually all cells expressed muscle-specific proteins.

### MATERIALS AND METHODS

**Sample collection and cell culture.** Sterile muscle tissue biopsies of the biceps femoris were obtained from adult mongrel dogs and immediately placed in sterile phosphate-buffered saline (PBS) for transport back to the tissue culture laboratory. The muscle tissue was minced with scissors, transplanted in sterile phosphate-buffered saline (PBS) for transport back to the tissue culture laboratory, and immediately used as a model for the study of Duchenne's muscular dystrophy because of similarities in both clinical and pathological findings (Nonaka, 1998). A more complete understanding of myogenesis in canine satellite cells may eventually lead to successful myoblast transplantation as treatment for myopathies. Therefore, the objectives of this study were (1) to successfully isolate satellite cells from canine muscle and culture the cells in vitro, (2) to characterize the proliferative response of canine satellite cells to growth factors, (3) to examine differentiation and fusion of canine satellite cells when cultured in defined media, (4) to characterize expression of myogenic proteins by canine satellite cells, and (5) to determine whether mRNA for LIF, IL-6, and IL-15 are expressed by canine myogenic proteins by canine satellite cells, and (5) to determine characteristics of canine satellite cells. The dystrophic dog is commonly used as a model for the study of Duchenne's muscular dystrophy because of similarities in both clinical and pathological findings (Nonaka, 1998). A more complete understanding of myogenesis in canine satellite cells may eventually lead to successful myoblast transplantation as treatment for myopathies. Therefore, the objectives of this study were (1) to successfully isolate satellite cells from canine muscle and culture the cells in vitro, (2) to characterize the proliferative response of canine satellite cells to growth factors, (3) to examine differentiation and fusion of canine satellite cells when cultured in defined media, (4) to characterize expression of myogenic proteins by canine satellite cells, and (5) to determine whether mRNA for LIF, IL-6, and IL-15 are expressed by canine myogenic proteins by canine satellite cells.

### TABLE 1

<table>
<thead>
<tr>
<th>Component</th>
<th>ITI</th>
<th>ITI–CF</th>
<th>SFG</th>
<th>4F</th>
<th>ODM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium</td>
<td>DMEM–F12 (1:1)</td>
<td>DMEM–F12 (1:1)</td>
<td>DMEM–F12 (3:1)</td>
<td>DMEM–F12 (1:1)</td>
<td>DMEM</td>
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<tr>
<td>rhFGF2</td>
<td>20 ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EGF*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>500 nM</td>
<td>850 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>0.2 nM</td>
<td>0.2 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>10 µg/ml</td>
<td>10 µg/ml</td>
<td>2 µg/ml</td>
<td>10 µg/ml</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Biotin</td>
<td>33 µM</td>
<td>1 µM</td>
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<td>1.22 µM</td>
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<tr>
<td>HEPES*</td>
<td>15 mM</td>
<td>300 µg/ml</td>
<td>2.5 µg/ml</td>
<td></td>
<td>10 nM</td>
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<tr>
<td>Fetuin</td>
<td>17 µM</td>
<td>17 µM</td>
<td></td>
<td></td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>Fibrinectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pantethenate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>50 ng/ml</td>
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<td></td>
<td></td>
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<tr>
<td>Bovine serum</td>
<td></td>
<td></td>
<td></td>
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<td>500 µg/ml</td>
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<tr>
<td>L-Glutamine</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Selenium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.8 mg/ml</td>
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<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Linoleic acid</td>
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<td></td>
<td></td>
<td></td>
<td>10 µg/ml</td>
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<tr>
<td>Calcium chloride</td>
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<td></td>
<td></td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Bactopeptone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>600 mg/L</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
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<tr>
<td>Gentamycin</td>
<td>30 µg/ml</td>
<td>50 µg/ml</td>
<td>50 µg/ml</td>
<td>50 µg/ml</td>
<td>50 µg/ml</td>
</tr>
</tbody>
</table>

* Deslex et al. (1986).  
* Vierck et al. (1996).  
* Schmidt et al. (1990).  
* Vierck et al. (1995).  
* rhFGF2: recombinant human fibroblast growth factor-basic.  
* EGF: epidermal growth factor.  
* HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

**Compositional of Defined Media**

- **Basal medium**: DMEM–F12 (1:1)
- **rhFGF2**: 20 ng/ml
- **EGF**: 0.8 nM
- **Insulin**: 1 nM
- **Triiodothyronine**: 1800 nM
- **Transferrin**: 5 µg/ml
- **Biotin**: 1.22 µM
- **Fetuin**: 10 nM
- **Fibrinectin**: 500 µg/ml
- **Pantethenate**: 2 µg/ml
- **Dexamethasone**: 0.1 µM
- **Bovine serum**: 500 µg/ml
- **L-Glutamine**: 3.8 mg/ml
- **Selenium**: 10 µg/ml
- **Vitamin C**: 10 µg/ml
- **Calcium chloride**: 0.5 mM
- **Bactopeptone**: 600 mg/L
- **Penicillin**: 100 U/ml
- **Streptomycin**: 0.1 mg/ml
- **Gentamycin**: 50 µg/ml

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- **Basal medium**: DMEM–F12 (1:1)
- **rhFGF2**: 20 ng/ml
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- **Triiodothyronine**: 1800 nM
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- **Selenium**: 10 µg/ml
- **Vitamin C**: 10 µg/ml
- **Calcium chloride**: 0.5 mM
- **Bactopeptone**: 600 mg/L
- **Penicillin**: 100 U/ml
- **Streptomycin**: 0.1 mg/ml
- **Gentamycin**: 50 µg/ml

**Sample collection and cell culture.** Sterile muscle tissue biopsies of the biceps femoris were obtained from adult mongrel dogs and immediately placed in sterile phosphate-buffered saline (PBS) for transport back to the tissue culture laboratory. The muscle tissue was minced with scissors, transferred to a sterile centrifuge tube, and incubated with pronase (1 mg/ml) at 37°C for 1 h. Satellite cells were isolated by differential centrifugation and the resulting cell pellet was subsequently resuspended in Dulbecco modified Eagle medium (DMEM; GIBCO, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), penicillin (100 U/ml; Sigma–Aldrich, St. Louis, MO), and streptomycin (0.1 mg/ml; Sigma–Aldrich). Canine satellite cells were transferred to a tissue culture flask that was coated with 0.1% pig skin gelatin (PSG; Sigma–Aldrich) and incubated at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Cells isolated from the adult mongrel dogs were used for proliferation, fusion, immunostaining, and Western blot assays and were not allowed to exceed six passages. Virtually all cells expressed muscle-specific proteins.

**Proliferation.** A methylene blue staining assay was used to estimate proliferation of canine satellite cells. Briefly, 2 x 10⁶ satellite cells/ml DMEM + 10% FCS was plated in PSG-coated, 96-well, flat-bottom microtiter plates and allowed to adhere for 24 h in a humidified atmosphere that contained 5% CO₂. The medium was subsequently removed, and cells were washed with PBS. To determine the effect of the level of sera in the medium on proliferation, cells were incubated in DMEM that contained 1, 3, 5, or 10% FCS. The mitogenic effects of growth factors were determined by incubating triplicate wells of satellite cells in DMEM that contained 1, 3, or 5% FCS plus 10, 25, 50, or 75 ng/ml recombinant human FGF2 (rhFGF2; R&D Systems, Inc., Minneapolis, MN), IGF-1 (Intergen, Purchase, NY), bovine FGF2, purified from bovine brain (bFGF2; R&D Systems, Inc., Minneapolis, MN), IGF-1 (Intergen), or GH (Sigma–Aldrich) or 0.1, 1, 5, or 10 ng/ml of TGF-α (Intergen). After 96 h, treatment medium was removed and cells were fixed with a solution of 3.7% formaldehyde in PBS. Methylene blue (1% in 0.01 M borate buffer, pH 8.3) was added to the wells, and the plates were incubated for 30 min at room temperature. Cell-bound dye was released with the addition of elution solution (one part 95% ethanol–one part 0.1 M HCl, v/v) and quantitated by absorbance at 620 nm.

**Fusion in defined medium.** The ability of canine satellite cells to differentiate and fuse to form myotubes was examined using five chemically defined media (Table 1) that have been reported to induce differentiation of satellite cells from other species: ITI (Deslex et al., 1986), ITI–CF (Vierck et al., 1996), ODM (Vierck et al., 1995), 4F (Serraro and Mills, 1987), and SFG (Schmidt et al., 1990). Cells were plated in PSG-coated 96-well microtiter plates at a density of 1 x 10⁶ cells/well in DMEM + 10% FCS and incubated at 37°C for 24 h in a humidified atmosphere that contained 5% CO₂. After this attachment period, media was removed and 100 µl of fresh DMEM +