Initial Observations on the Growth Factor Regulation of Equine Satellite Cells *In Vitro*

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Abstract

The objective of this study was to determine the effects of several known growth regulators on equine muscle satellite cell proliferation and differentiation *in vitro*. Increasing concentrations of insulin-like growth factor I and fibroblast growth factor stimulated proliferation slightly above that induced by 2% horse serum alone. In time course studies, insulin-like growth factor I (at 72 hours in culture) and fibroblast growth factor (at 48 hours in culture) significantly stimulated equine satellite cell proliferation and differentiation ($P < .05$) 1.6 and 3 fold respectively above basal medium. When equine satellite cells were treated with 25 ng/ml of insulin-like growth factor I together with 25 ng/ml of fibroblast growth factor, insulin-like growth factor facilitated the effect of fibroblast growth factor ($P < .05$) on proliferation, but not ($P > .05$) differentiation. Transforming growth factor-β_1 and growth hormone inhibited both proliferation and differentiation of equine satellite cells. Epidermal growth factor, nerve growth factor and platelet derived growth factor had no effect on proliferation or differentiation of equine satellite cells. Results obtained in this study suggest that equine satellite cells may provide additional details of growth factor regulation of postnatal muscle satellite cells during growth and development.

Key words: horse, satellite cells, IGF-I, FGF, TGF-β.

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Internationally, the horse industry involves a large number of spectators supporting equine sport competitions including racing, showing, polo, rodeo, 3-day eventing, and many other equine sporting competitions. When accidents or tragic breakdowns occur in the heat of competition, there is high public exposure and emotional response. As a result, most horse-related research is focused on understanding the causes of career-ending injuries and developing preventative measures to avoid injuries. However, horses are unique livestock in that they are used for both recreation and meat purposes. The United States is the world's largest supplier of horse meat for human consumption [15], although the utilization of the horse as a food source is more extensive in certain European countries and Japan than in the United States. Information about measures that could promote increased feed efficiency would be beneficial to both producers of horses and consumers of horse meat. Therefore, definition of growth factor regulation of postnatal horse skeletal muscle growth may provide information about skeletal muscle adaptation to exercise, clinical treatment of muscle injury, and mechanisms involved in regulation of muscle composition.

Definition of growth regulators and their mechanism of action on muscle satellite cells from domestic animals remains a relatively new area of research. In laboratory animals, however, the response of muscle satellite cells to transforming growth factor-β_1 (TGF-β), insulin-like growth factor-I (IGF-I), and basic fibroblast growth factor (FGF) has been thoroughly studied [3].

Slight variations in response to various growth regulators have been found to exist between muscle satellite cells isolated from different species. Proliferation of primary cultures of rat satellite cells was inhibited by TGF-β [1]. Further, TGF-β inhibited proliferation of rat satellite cells

in the presence of both FGF and IGF-I [2]. In contrast, proliferation of bovine satellite cells due to FGF was further enhanced by the addition of TGF-β [10]. Proliferation of primary cultures of beef and pig satellite cells was depressed by TGF-β, while sheep satellite cells were not affected by TGF-β [13]. These studies demonstrate distinct species and/or developmental differences [12] in responses of satellite cells to growth factors/regulators. Satellite cells from horses have recently been isolated and grown *in vitro* [11]. Therefore, the objective of this study was to examine the effect of selected growth factors and hormones on the proliferation and differentiation of satellite cells derived from exercised and nonexercised horses.

Materials and Methods

Horses

Twelve Quarter Horse weanlings were randomly divided into two groups, exercised and nonexercised. The exercised horses were further divided into two groups consisting of either a 15-day or 30-day exercise protocol, and weanlings were exercised at a brisk trot (3 m/s) on a high speed treadmill. Muscle satellite cells were obtained from a surgical biopsy of the biceps femoris of all animals at the conclusion of the exercise protocols.

Materials

Insulin like growth factor-I (IGF-I) was purchased from Intergen (Purchase, NY). Transforming growth factor-β (TGF-β_1) was purchased from R & D Systems, Inc. (Minneapolis, MN). Bovine growth hormone (bGH; USDA-BGH-B1) was obtained from the USDA (Beltsville, MD). Nerve growth factor (NGF) was purchased from Becton Dickinson (Bedford, MA). All growth factors were rehydrated according to manufacturer's instructions. Penicillin/streptomycin, pig skin gelatin, trypsin and Giemsa were purchased from Sigma Chemical Company (St. Louis, MO). Gentamicin, Dulbecco's Modified Eagle's Medium (DMEM), and horse serum were purchased from GIBCO BRL (Grand Island, NY). Cultureware was purchased from Costar (Cambridge, MA).

Cell Culture Protocols

Equine satellite cell (ESC) cultures were established from the biceps femoris muscles of yearling horses as described by Greene and Raub [11]. ESC, stored in liquid nitrogen, were quickly thawed and suspended in DMEM supplemented with 10% horse serum and antibiotics. Cells were then plated in 15 cm dishes and maintained at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Cultures were provided new medium every other day until cells reached approximately 60% confluency. Unless noted otherwise, following trypsinization, rinsing and counting by hemocytometer, cells were plated at selected densities into 24 well plates coated with 0.5% pig skin gelatin in DMEM-10% horse serum. Plating efficiencies (PE) were determined at 24 hours to validate initial cell number at time of treatment. Following the treatment regimen, cultures were analyzed by counting the nuclei density of ten randomly selected fields per well [11].

Horse Serum Dose Response

Equine satellite cells originating from exercised weanlings were plated at a density of 30 nuclei per mm^2 in uncoated wells and treated with DMEM supplemented with 0, 1, 5, or 10% horse serum and antibiotics. Cells were provided fresh medium on daily intervals; then fixed, stained, and both mononucleated cells and myonuclei were quantitated at the end of 120 hours [11].

IGF-I/FGF Dose Response Protocols

This study was designed to establish a minimal level of serum-supplemented medium which would maintain the viability of ESC, and to determine the effects of IGF-I on proliferation and differentiation of ESC. IGF-I dose response experiments were conducted at densities of 1,500 and 2,500 cells/well, using DMEM containing 1.5% or 2% horse serum, respectively. IGF-I treatments (0, 0.25, 1.5, 2.5, 5, 15, 25, or 50 ng/ml) were applied every 24 hours. FGF dose response treatments (0, 0.5, 1.5, 2.5, 5, 15, 25, or 50 ng/ml) were applied to ESC cultures (original density: 5000 cells/well) every 24 hours. Cells were fixed, stained, and evaluated at 96 hours.

Effects of bGH, FGF, IGF-I, EGF, PDGF and NGF on ESC proliferation and differentiation

ESC from exercised and nonexercised horses were plated at 2,500 cells/well in 24 well culture plates. Culture medium was changed daily. ESC were treated with the following concentrations of hormones or growth factors: IGF-I, FGF, IGF-I/FGF combination, bGH, and EGF (25 ng/ml); NGF (10 ng/ml); and PDGF (2.25 μg/ml). Cells were fixed and stained at 24 hour intervals for 5 days.

Effects of TGF-β on ESC proliferation

ESC were plated at a density of 2,500 cells/well in 24-well plates. TGF-β (5 ng/ml) was provided daily for 5 days. Cell cultures were fixed, stained, and evaluated on days 1, 3, and 5.

Effects of TGF-β on ESC fusion

ESC were plated at 15,000 cells/well in 24 well plates. Following a 48 hour period to ensure ESC proliferation, fusion was induced by reducing horse serum concentration in the medium from 10% to 2%. In the treatment cultures, 5 ng/ml TGF-β was provided. Cultures were provided fresh medium daily. Cells were fixed, stained, and evaluated at 24, 72, and 120 hours.

Statistical Analysis

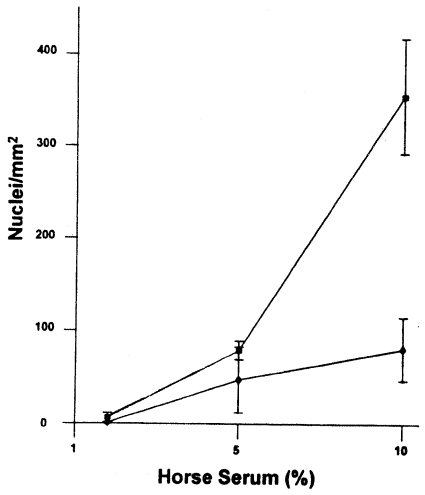
Data were subjected to one way analyses of variance to determine if differences due to treatment were evident. The student T-test was then used to separate treatment means at a significance level of ($P < .05$).

Results

There were no differences in either proliferation or differentiation of satellite cells obtained from within exercised or nonexercised weanling groups (P > .05). Therefore, all data derived from growth factor studies represents pooled data. Equine satellite cells exposed to 1, 5, or 10% horse serum exhibited a dose dependent increase in proliferation (Figure 1). At a concentration of 10% horse serum, the proliferation was significantly higher (P < .05) than cell cultures containing 1 and 5% horse serum.

In order to test the effect(s) of growth factors that have been shown to influence satellite cells from a variety of other species, it was next relevant to establish a minimum level of serum in the basal medium that could support ESC viability but not mask the effects of added treatment moieties. Numerous reports suggest a basal level of 1-3% serum is reasonable for such an objective [8]. For these preliminary studies, we selected 1.5 and 2% horse serum for the basal medium. ESC exhibited negligible proliferation or differentiation in cultures containing 1.5% horse serum, but IGF-I induced a slight increase in proliferation in 2% horse serum (Figure 2A). There was no significant effect of IGF-I on ESC fusion in the presence of either level of horse serum (Figure 2B). The 2% HS-supplemented basal medium was used for all remaining growth factor dose-response studies.

The effect of exposing ESC to 25 ng/ml of IGF-I at 24 hour intervals for 96 hours *in vitro* is shown in Figure 3A. There were no significant differences in proliferation at 24 or 48 hours. However, after 72 and 96 hours cells left their apparent quiescent state and began to proliferate. At the conclusion of the treatment period, there was a significant



igure 1. Influence of increasing levels of horse serum concentrations in medium on ESC activity. Cells were exposed to 1%, 5%, and 10% HS for 120 hours. Each point represents the mean of six culture wells adjusted for plating efficiency (■ total nuclei; ♦ fused nuclei).

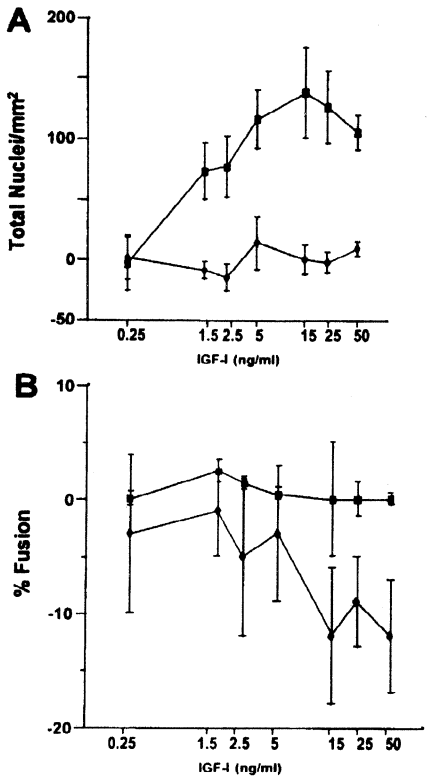


*Figure 2. (**A**) ESC proliferation and (**B**) ESC differentiation in response to varying concentrations of IGF-I. ESC were plated on substratum at 1500 nuclei/well. Wells were fixed and stained at 96 hours. The experiment was repeated, using a plating density of 2500 cells/well and increasing serum levels to 2% (♦ 1.5% HS; ■ 2% HS).*

increase in proliferation due to IGF-I as compared to horse serum alone (Figure 3A). Myotube nuclei density likely reflected the parallel increase in total nuclei (Figure 3B).

Other growth regulators were screened for effects on ESC varying concentrations of FGF resulted in a stimulation of proliferation (P < .05), but had little effect on differentiation (Figure 4). Additional effects of FGF (25 ng/ml) on proliferation and differentiation are shown in Figures 5A and 5B, respectively. Effects of exposure of ESC to FGF in combination with IGF-I are shown in Figure 6. Fused nuclei numbers were parallel to total nuclei density. There was a marked increase in proliferation at 48-96 hours when compared to the 2% horse serum treated cells (P < .05). When ESC were treated with EGF (25 ng/ml), PDGF (2.25 ug/ml), and NGF (10ng/ml), there were no significant effects on ESC proliferation or differentiation. Growth hormone (25ng/ml) inhibited proliferation, but did not affect ESC differentiation.

TGF-β has been shown to be an inhibitor of both satellite cell proliferation and differentiation. Figure 7 illustrates

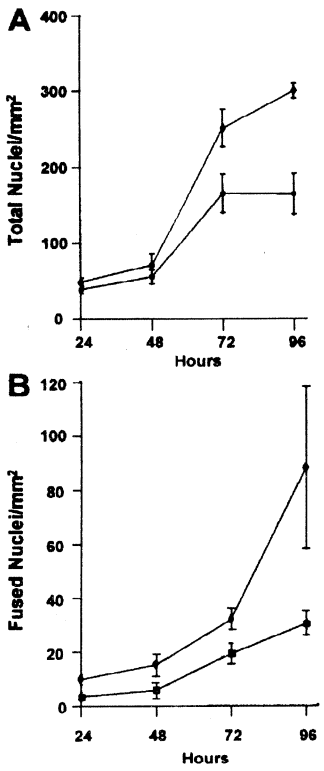


Figure 3. **(A)** *ESC proliferation and* **(B)** *differentiation in response to a consistent level of 25 ng/ml of IGF-I over 96 hours in vitro. Each point represents six culture wells adjusted for plating efficiency (* ■ *2% HS control;* ♦ *2% HS + IGF-I).*

the effects of exposing ESC cultures to TGF-β at 5 ng/ml for 120 hours. Proliferation of ESC was significantly inhibited at all time points examined ($P < .05$). Similarly, fusion also was inhibited in an experiment designed to evaluate the differentiation response of established and actively proliferating horse satellite cell cultures (Figure 8).

Discussion

The use of horses for satellite cell research provides an excellent cellular model for muscle changes due to intense exercise. Satellite cells play a role in the increases in skeletal muscle mass seen in response to exercise protocols [4]. The ease of training for both aerobic and anaerobic exercise protocols and the ability to collect large or multiple samples of muscle tissue make the horse a versatile candidate for exercise physiology experimentation. Further, the involvement of the satellite cell, or satellite cell-derived myonuclei in influencing variables of meat quality may prove significant for the horsemeat industry, as more efficient feed to lean muscle mass conversion would have a significant economic impact.

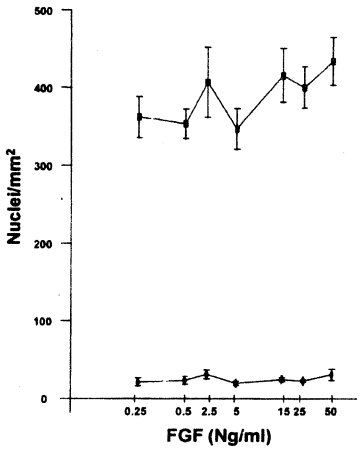


Figure 4. ESC response to varying concentrations of FGF. Each point represents six culture wells adjusted for plating efficiency values (■ *total nuclei;* ♦ *fused nuclei).*

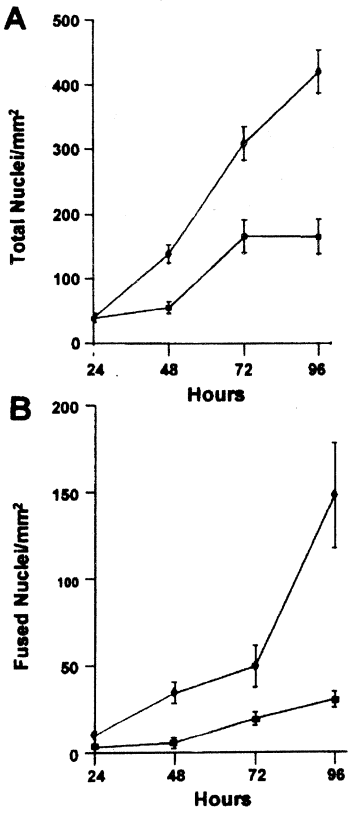


Figure 5. **(A)** *ESC proliferation and* **(B)** *differentiation response to FGF. Each point represents six culture wells, adjusted for plating efficiency (* ■ *2% HS control;* ♦ *2% HS + FGF).*

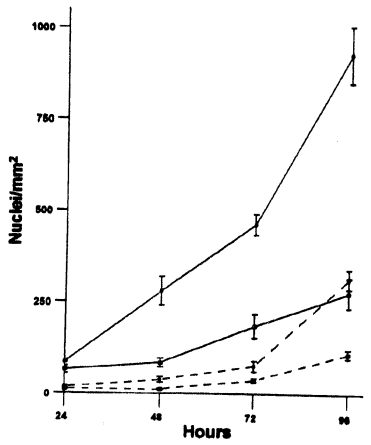


Figure 6. Effect of IGF-I + FGF on ESC over 96 hours in vitro. ESC were treated with IGF-I (25 ng/ml) + FGF (25 ng/ml) in DMEM-2% HS. Each point represents three culture wells (■ total nuclei-2% HS control; - ■ - fused nuclei-2% HS control; ♦ total nuclei-2% HS with IGF-I+FGF; -♦- fused nuclei-2% HS with IGF-I+FGF).

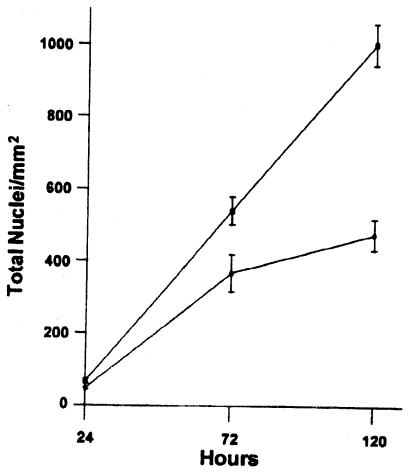


Figure 7. Response of ESC to TGF-β under conditions promoting proliferation. Cells were the fixed and stained every 24 hours for 96 hours (■ HS control; ♦ HS + TGF-β_1).

In addition to providing nuclei to muscle fibers during growth and development, satellite cells facilitate muscle repair [18] following myotrauma. Numerous factors influence cultured satellite cells from other species, including the medium [14] and the type of substratum used [16]. The serum dose response was performed to determine the minimal concentration of horse serum for support and maintenance of healthy cultures without large serum-induced effects on proliferation and differentiation. Since serum contains several mitogenic factors and binding proteins capable of influencing satellite cells, it was necessary to find a basal level of serum that would allow cell maintenance, but that would not completely mask the effects of growth factors. The medium supplemented with 2% horse serum maintained ESC with minimal effects on ESC proliferation and differentiation.

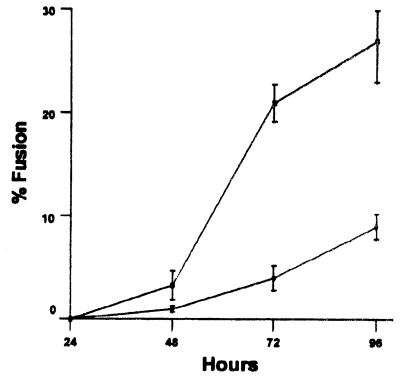


Figure 8. Response of ESC to TGF-β under conditions promoting differentiation (■ HS control; ♦ HS + TGF-β).

Florini and Magri [9] reported that IGF-I may influence myogenic cells in a biphasic manner. When the cell density is low, IGF-I influences proliferation. However, IGF-I stimulates differentiation in dense cell cultures. Cell density results with ESC are consistent with that model proposed by Florini and Magri. The general effects of IGF-I on ESC are similar to those reported for cattle [10]. Like cattle-derived satellite cells, when ESC were exposed to IGF-I alone, there was no significant effect on proliferation.

In satellite cell cultures derived from cattle, the effects of FGF on proliferation and differentiation were opposite to those induced by IGF-I. While the FGF dose-response experiment did demonstrate a stimulation of proliferation of ESC, there was not a consistent dose-dependent increase in cell number as seen in other species [10]. The time course data, however, were similar to that reported for satellite cells from other species. While ESC responded to FGF by increasing nuclei density rates with time, fusion of ESC appeared to occur in a density dependent manner. These observations are consistent to those demonstrated in rat [2], cattle [10], sheep [13], and pig [7]. Further, when cells were exposed to IGF-I in combination with FGF, there was an additive effect on proliferation.

Proliferation and differentiation of ESC was inhibited when they were exposed to TGF-β. These data are consistent with data from satellite cells from other species [1], with the exception being cattle [10]. When TGF-β was used in combination with FGF, there was an additive effect on proliferation in cattle.

In summary, data presented herein provide preliminary information on the effects of growth factors on ESC. The physiological effects and magnitude of response to the tested factors appear to be somewhat different than responses seen by satellite cells from other species. However, conclusive data from a more defined culture system is required prior to definitively addressing species or cell differences. Furthermore, the isolation of clones to create homogenous ESC populations may allow clarification of specific growth factor interactions and their effects on ESC. Collectively, it will be of interest to further study ESC *in vitro* to elucidate the role satellite cells play in the horse.

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