Establishment of Channel Catfish Satellite Cell Cultures

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Abstract

Little information exists on mechanisms of muscle growth in fish, particularly as it pertains to the role by which satellite cells mediate muscle growth. Consequently, our objective was to establish the conditions required for successful isolation and culture of satellite cells from channel catfish. Satellite cells were isolated from the muscle of 2-mo, 6-mo, 1-yr and 2 yr-old channel catfish. Muscle strips were minced in sterile phosphate buffered saline (PBS), twice exposed to a 0.1% trypsin solution for 30 min at 36.5 ± 1.0°C. The suspension was centrifuged at 300 x g for 10 min after which the supernatants were collected, pooled and centrifuged at 300 x g for 10 min. The supernatants were again collected and centrifuged at 700 x g for 10 min. The resulting pellet was then resuspended in HEPES-buffered Eagles Minimum Essential Medium (HMEM), supplemented with 10% fetal bovine serum (FBS) and pipetted over one part Percoll, five parts HMEM at 29.5 ± 0.5°C. Twenty-four hours after plating, satellite cells (SC) and fibroblast -like (FL) cells were easily recognized. Forty-eight hours after plating, cells began to reach confluence and formation of myotubes was observable. FL cells outnumbered SC cells in most experiments. Yield of SC cells per gram of digested tissue were 6.1 x 10^6 and 8.7 x 10^5 cells for 2-month and 1-year-old catfish, respectively. Coating of culture dishes with Matrigel did not enhance cell attachment ($P < .05$) but fish-derived collagen extract did. Using the Percoll/HMEM sedimentation procedure produced relative clean primary cultures. Replating of cells from 1-day-old cultures for the purpose of establishing secondary cultures was not successful. Increasing levels of FBS, IGF-I, FGF and TGF-β in the media resulted in a positive mitogenic effect ($P < .05$). The addition of IGF-I to medium increased ($P < .05$) differentiation as indicated by percentage fusion at 96 h while FGF and TGF-β reduced differentiation ($P < .05$). These data involving growth factors are indicative that receptors for these growth factors are present in catfish SC. Collectively, these data are consistent with available reports regarding the relative proportions of SC and FL cells in primary fish cultures, the poor proliferative activity of fish myogenic cells in culture, and the low plating efficiency of fish mesodermal cells. Because of the indeterminate growth displayed by fish, study of satellite cell proliferation and differentiation *in vitro* should provide unique opportunities to gain insight into the understanding of the regulation of skeletal muscle development.

Key words: catfish, muscle, satellite cells, growth factors, proliferation, differentiation.

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Farming of channel catfish *Ictalurus punctatus* is the most important fish industry in the United States [30, 32]. Although a significant research basis is available pertaining to nutrition [19, 26] and health management of channel catfish, there is very little data available on regulation of muscle cell growth in this species.

Depending on age and size of catfish, skeletal muscle mass accounts for 30-80% of the living weight of the fish, from which 40-60% is locomotion musculature. Catfish muscle is arranged in myomers (contractile units), occurring in two symmetrical bilateral series among the fish axial skeleton. Besides its role in propulsion, fish muscle is also important as an energy storage depot [4, 31]. As in mammals, differentiated muscle fibers in fish do not undergo division or fusion, and growth is limited to the

recruitment of new fibers, having the satellite cells as precursors [22, 31].

Muscle satellite cells have been described in several marine and freshwater fish species. The axolotl *Siredon mexicanus* [9], the rainbow trout [22], the shark *Galeus melastomus* [17], carp [16] and the Atlantic hagfish *Myxine glutinosa* L. [27]. Fish satellite cells are interposed between the plasma membrane and the basal lamina of the muscle fibers [27]. Morphologically, fish satellite cells seem to have a more complex form than the fusiform type widely described, bearing an ovoid nucleus 10-15 μm in length and 1-2 μm in diameter, a 0.1-0.3 μm cytoplasm layer around the nucleus, and elongated extensions of about 60 times the nuclear length [9]. Fish satellite cells possess highly heterochromatic nuclei, often deeply indented into the adjacent myofiber. They can have a cytoplasm of either scanty appearance with few organelles, or have abundant cytoplasm with a variety of cell organelles: prominent Golgi complexes near the nuclei, vesicles, numerous mitochondria, and free ribosomes or polysomes [19, 29].

Satellite cell differentiation, rates of muscle protein synthesis effected by SC-derived myonuclei and myofiber protein degradation are key regulation points of fish muscle growth. Those mechanisms are controlled by several factors. The genetic make-up of the fish, nutrition, peptide hormones, peptide growth factors, and level of activity are all involved in the regulation of fish muscle growth. The present work tested the hypotheses that: 1) SC are isolatable from catfish muscle; 2) SC respond to growth factors in a similar manner as that observed in mammalian SC cultures; and 3) addition of selected growth factors may significantly improve the viability and longevity of catfish SC *in vitro*.

Methods

Isolation of satellite cells from channel catfish

Fish were removed from tanks and maintained overnight in distilled water. Fish were immobilized by spinal cord ablation, killed by exsanguination, soaked in a 10% Nolvasan (Fort Dodge Laboratories, Inc.) for 10 minutes and then placed in sterile water on a stainless steel pan under the environment of a laminar flow hood. The skin mucus was removed by swabbing with 70% ethyl alcohol. Skin was then removed and lateral muscle mass excised, weighed and minced with scissors in a sterile, 50-mL centrifuge tube with a small amount of PBS. Minced tissue was centrifuged at 1,500 x g for 5 min, the supernatant was then discarded and 5.0 ml of 0.1% trypsin (Sigma Chemical Co.) solution was added per 1.0 g of minced tissue [3]. Tissue was incubated with the enzyme for 30 min at 35.5 ± 1.0°C with slight agitation every 10 min. The suspension was centrifuged at 300 x g for 10 min, supernatant collected and 10% Horse Serum (GIBCO) added. The incubation, centrifugation and collection procedures were repeated twice. Supernatants were then pooled and centrifuged at 300 x g for 10 min to sediment large tissue remnants. Retentates were discarded. Supernatants were centrifuged at 700 x g for 10 min [16, 24]. The resulting cell pellet was resuspended in HEPES Minimum Essential Medium (HMEM; Sigma Chemical Co.; pH 7.2; [33], supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co.). Unless otherwise noted, culture medium was supplemented with 10ml/L of Antibiotic-Antimicotic Solution 100X Hybrimax® (ABAM - 10,000 units of penicillin, 10 mg streptomycin, and 25 μg of amphotericin B per ml; Sigma Chemical Co.). Based on recommendations and findings of others [10, 11, 34], digested tissue was then plated over a layer of 20% Percoll (Colloidal PVP Silica, Pharmacia) suspension and incubated at 29.5 ± 0.5 C [9, 21, 23]. Unless otherwise noted, culture dishes and 24-well plates used in the experiments were uncoated Nunclon® brand (American Laboratory Supply). Six hours after plating, Percoll-containing medium was washed from the culture plates and observations on the attachment and differentiation of the cells were conducted using a Nikon inverted microscope under a 20X objective.

Plating efficiency values for satellite cells and fibroblasts

After 24 hours in culture, cells were fixed with 10% formalin [23], stained with Giemsa [10, 11], and the yields of satellite cells (SC) and fibroblast-like (FL) cells per gram of digested tissue were estimated. The number of SC and FL cells, and the percent composition of the attached cell population were calculated. Results were compared to the same parameters calculated for an established population of Brown Bullhead catfish (BB) cells seeded at 9.3 x 10^6 cells/cm^2.

Proliferation of satellite cells of channel catfish

Attempts were made to define the influence of fish age on the yield of myogenic cells in the primary cultures. Primary cultures were established with cells isolated from trunk muscle of 1.9 g, 2-month-old; 53.3 g, 1-year-old; 35 g, six-mo old; and 300 g, two-year old; channel catfish. Digested tissue was plated at 0.2 g/cm^2 per well in 24-well plates. After 24 hours in culture, medium was changed and cells were assigned to 4 treatments: HMEM supplemented with 2.5, 5.0, 10.0, and 15.0 % FBS. Additional experiments involved treatment of cells with IGF-I (50 and 100 ng/ml), bFGF (50 and 100 ng/ml) and TGF-β (1 and 5 ng/ml) for 48 h. Treatments were replicated three times, with one well representing one replicate. Cells were morphologically identified as satellite cells (SC) and fibroblast-like (FL) cells following published descriptions of others [6, 9, 13, 18], quantitated with microscopy and cultured for another 48 hours to assess proliferation. The number of SC, FL, and total number of cells per well were determined, and the influence of the various levels of FBS supplementation or growth factor treatment to the culture medium assessed. Observations on the proliferation of the cells were conducted using a Nikon inverted microscope under a 20X objective. Cells were counted using an objec-

tive-built counting grid with an area equal to 0.0025 mm^2. Ten random counting grid fields were counted per well.

Differentiation of satellite cells from channel catfish

To determine fusion characteristics of catfish SC, 10^4 cells per well were plated in 24 well plates and cultured in HMEM + 10% serum for 96 h. Cells were fixed in 100% methanol and stained with Giemsa at 24 h intervals post-plating. Total nuclei per well (TN) and total fused nuclei per well (TF) were determined. Percent fusion was calculated as follows: percent fusion (PF) = [total fused nuclei/total nuclei] x 100.

Statistical procedures

Data were subjected to Least Squares Analysis of variance by using General Linear Models procedures of SAS. When treatment means were protected by a significant F values, means were separated by Tukey's Multiple Range test [13, 28, 29]. Significance is reported at the 0.05 level of probability.

Results and Discussion

Substratum

Both satellite cells and fibroblast-like cells were isolated from channel catfish. The term satellite cells (SC) was applied to spindle shaped cells [7, 15] and the term fibroblast-like (FL) was used to identify the stellar-shaped cells [10]. Satellite cells and FL cells both attached to uncoated culture dishes. The coating of culture dishes with basement membrane Matrigel™ (Collaborative Research, Inc.) did not appreciably enhance cell attachment (data not shown). However, plating cells onto preparations of fish-derived collagen favored attachment of SC cells. When digested tissue suspension was initially pipetted over a layer of 20% Percoll/MEM, the resultant primary cultures contained much less tissue debris than cultures obtained from plating digested tissue directly into culture dishes. These data suggest that the use of the Percoll layer increases the likelihood that desired cells are included in subsequent test cultures. The residual presence of large numbers of FL on the culture dishes, however, show that this technique is not selective for SC.

Four to 6 hours after plating a large number of cells were attached to the culture plate. Some SC cells had already differentiated as evidenced by either spindle-shaped morphology, or fusion to form multinucleated myotubes. Thirty six to forty-eight hours after plating, SC seemed to be maximally differentiated as evidenced by large amounts of fused nuclei within myotubes. At this point, some plates were treated with 0.1% trypsin solution; and mononucleated cells were detached, resuspended and replated. Rarely could secondary cultures of sufficient robustness to effect/produce multinucleated myotubes be obtained under a variety of conditions. For example, the use of Matrigel™, the use of 5 or 10% FBS-supplemented DMEM, the extension of the attachment period to 12 or 24 hours, and the growth of cells at 25, 28 or 30°C could not produce myotubes. Further, catfish satellite cells did not survive at 37°C. The latter observation is consistent with the description of Hay et al. [14] that the bullhead-derived BB fibroblast cell line (available from ATCC) has a 1% replating efficiency, displays minimal temperature sensitivity and possesses extremely low handling tolerance. Koumans et al. [16] observed that following 17 hours of culture, carp myosatellite cells are attached and actively engaged in the differentiation processes of alignment and fusion. Powell et al. [24] reported cell confluence and formation of myotubes between 3 and 6 days in cultures of trout myosatellite cells. Formation of multinucleated myotubes in our studies most often occurred at 48 hours after plating. Differences in initial cell density and species-dependent temperatures [12, 33] may be important determinants of the differences seen between our data and that reported by others.

Age of Catfish

In an effort to address effects of initial plating density on subsequent performance of catfish cell cultures, as well as to determine the effects of fish age on SC's isolated, an experiment was conducted with cells isolated from 2-month to 2-year old catfish (Table 1.). Digested muscle tissue of 2-month-old channel catfish yielded 6.1 x 10^6 SC, which was approximately 18.7-fold greater than that from 2-year-old fish. Fibroblast-like cells typically outnumbered SC cells in all populations. The percentage of SC was higher (24.2%) in cultures derived from 2-month-old fish than in cultures from 1-year-old (15.1%) and 2-year-old (6.3%) fish. Our observations on isolation and culture of channel catfish satellite cells agrees with reports that suggest there is a decline in the frequency of satellite cells with an increase in maturity of both mammals and fish [1, 3, 7, 17].

Serum Influence

Our efforts to establish viable primary cultures with catfish-derived serum have been unsuccessful. Therefore, other sources of sera were explored and used in establishment of primary SC cultures. Initial data suggested that increasing the levels of FBS alone in the growth medium resulted in no significant enhancement of the proliferation of primary SC ($P > 0.05$). However, increased levels of FBS supplementation resulted in a cytotoxic effect ($P < 0.05$) on FL proliferation. Initial plating densities played a significant role on the final number of cells in all wells after supplementation with increased FBS levels ($P < 0.05$). While supplementing growth medium with 10% FBS is generally prescribed for many fish cell systems, our observation with channel catfish fibroblast cells and multiple lots and sources of FBS suggest a complete antagonism. Lee et al. [18] report that 10% FBS supplementation of the growth medium best stimulated the proliferation of trout RTG-2 fibroblastic cells. Our findings with channel catfish SC support the results of Lee et al. [18]. The low proliferative activity of catfish SC cells is likely due to the absence of factors which control cell growth, as is often more

Table 1. Cell yield and composition of satellite cells[a] (SC) and fibroblast-like (FL) cell populations isolated from catfish at different ages.

Cell Source	SC/g tissue plated	FL/g tissue plated	Ratio SC:FL (SC/FL X 100)	% SC cells of total cell number
2-mo old	6.1×10^6	19.1×10^6	32%	24.2
6-mo old	10.5×10^7	21.1×10^7	50%	33.2
1-yr old	8.7×10^5	4.9×10^6	18%	15.1
2-yr old	6.9×10^4	10.2×10^5	7%	6.3
BB line[b]	NA	NA	10%	9.1

[a]Based on morphometric criterion described previously [22, 25].
[b] Brown bullhead (BB) fibroblastic cell line (ATCC) were used as comparison controls.

evident when myoblasts and fibroblasts are cultured together [25]. Enhancing plating efficiency and inducing proliferative activity of SC of channel catfish remain areas needing further study.

Growth Factor Supplementation

Effects of different concentrations of FBS, IGF-I, bFGF and TGF-β supplementation of the growth medium on the proliferation of primary cultures of channel catfish cells are summarized in Tables 2 and 3. The increased proliferation by SC cells in response to IGF-I, FGF and TGF-β are consistent with observations of SC derived from mammalian muscle. These data suggest that receptors for these growth factors are present in catfish SC. Because our experiments dealt with introduction of a growth factor to the SC environment, observations of potential synergisms between exogenous and cell-derived growth factors were not possible. In the proliferation experiment, the greatest increases were observed with IGF-I at 100 ng/ml (46-fold greater than the control), followed by FGF (24-fold greater than controls) and TGF-β (10.5 fold greater than controls). The increases in SC numbers observed with the exposure to TGF-β are not consistent with reports that its mode of action is to prevent differentiation [2]. FGF has been proposed to act synergistically with a group of growth factors (IGF-I, TGF-β) in regulating growth and regenerative processes in muscle. Inhibitory growth factors like TGF-β, may indirectly allow satellite cell accumulation by preventing their differentiation into myotubes [2]. A parallel stimulation of proliferation by FGF may also result in satellite cell accumulation. Percentage of fused cells are also presented in Table 3. Under conditions employed, IGF-I promoted ($P < .05$) SC differentiation. This may in part be explained by the early effects of increasing cell numbers and, therefore, fusion was likely density dependent. In contrast, both FGF and TGF-β reduced SC differentiation by 9 to 13%, respectively. These later

Table 2. Effects of increasing percentage of FBS on proliferation of primary satellite cells (SC) and fibroblast-like (FL) cells isolated from channel catfish[a,b].

FBS (%)	*SC/well* Initial	Final	Increase (fold)	*FL/well* Initial	Final	Increase (fold)	*Total cells/well* Initial	Final	Increase (fold)
	($x10^6$)	($x10^6$)		($x10^6$)	($x10^6$)		($x10^6$)	($x10^6$)	
2.5	0.41	6.25	15.2[a]	0.63	4.0	6.4[c]	1.0	10.3	9.9[a]
5.0	0.21	7.75	36.9[b]	0.78	2.1	2.6[b]	1.0	9.8	9.9[a]
10.0	0.16	5.89	36.8[b]	0.60	1.5	2.4[b]	0.8	7.3	9.7[a]
15.0	0.08	6.11	76.4[c]	0.62	0.84	1.4[a]	0.7	7.0	9.9[a]
20.0	0.10	18.4	185.0[d]	0.70	0.81	1.2[a]	0.8	19.2	24.0[b]

[a]Differentiation at a 65% level was commonly observed after 48 h *in vitro*.
[b]Treatment means within columns with different superscripts differ ($P < .05$). Standard error of the least squares means are 1.5×10^3, 5×10^4 and 6×10^4 for the final SC, FL and total cells, respectively.

Table 3. Effects of addition of IGF-I, bFGF and TGF-β on the proliferation of primary satellite cells (SC) isolated from channel catfish[a,b].

GF	*SC/well* Final	Inc. (fold)	*FL/well* Final	Inc. (fold)	*total cells/well* Final	Inc. (fold)	*Percent* Fusion
	($x10^6$)		($x10^6$)		($x10^6$)		
Control							
0	5.20	13.1^a	2.0	5.0^b	7.2	18^a	68^b
IGF, ng/ml							
50	160.25	400.0^d	1.4	3.5^b	161.6	404^d	85^c
100	199.10	597.8^e	1.6	4.0^b	200.7	502^d	87^c
FGF, ng/ml							
50	40.5	101.2^c	1.8	4.5^b	42.3	106^c	55^a
100	125.7	314.2^d	1.6	4.0^b	127.3	318^d	60^{ab}
TGF, ng/ml							
0.1	25.9	64.8^b	0.5	1.2^a	26.4	66^b	57^a
1	55.1	137.7^c	0.5	1.2^a	55.6	139^c	59^a

[a]After plating at .4 X 10^6 cells/well, cells were fed medium containing 5% FBS supplemented with the growth factor for 48 h and then cells counted.

[b]Treatment means within columns within growth factor treatment with different superscripts differ ($P < .05$). Standard error of the least squares means are 1.5 X 10^3, 2 X 10^3 and 4 X 10^4 for the final SC, FL and total cells, respectively.

[c]Differentiation levels observed in parallel wells after 96 h of exposure to the treatments increased to 87% with IGF, which was maximum for all treatments. Standard error of the least squares mean for fusion was 3.2%.

observations deserve additional examination. Additional experiments will need to be performed to determine if the observed effects of these growth factors are partially mediated by improvement in the health of the cells. The second step for these growth regulators on altering SC activity in fish muscle growth, may be the stimulation of differentiation in the presence of IGF-I, the absence of TGF-β and the absence of FGF [2].

The work described here suggests that muscle satellite cells may be isolated from channel catfish and cultured *in vitro*. Furthermore, the proliferative activity of catfish-derived satellite cells depend on the initial number of total cells plated, and the environmental conditions (media type and type of serum supplementation) to which the cells are exposed. In addition, growth factor supplementation resulted in higher levels of proliferation of both SC and FL cells. Our results with catfish satellite cells encourage further research in this area. Because of the importance of the catfish industry to the United States as well as other countries, additional work needs to be performed in the area of catfish SC culture to gain information on the regulation of muscle growth in catfish.

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