

Substratum is an Important Determinant in Growth Factor Regulation of Trout-Derived Satellite Cells

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Abstract

The ability of trout-derived satellite cells to attach, proliferate and differentiate in response to different substrata, and their regulation by growth factors and/or hormones were evaluated *in vitro*. Of the three substrata examined, Matrigel (1:10) was the most efficient substratum, facilitating 70.6% ($p < 0.05$) attachment of satellite cells compared to fibronectin and pig-skin gelatin (57.7% and 17.3%, respectively). Basic fibroblast growth factor (bFGF), insulin-like growth factors I and II (IGF-I and II), epidermal growth factor (EGF), tri-iodothyronine (T_3), thyroxine (T_4), growth hormone and prolactin were examined for their ability to alter variables of satellite cell proliferation and differentiation. bFGF in combination with IGF-I and II, each at a concentration of 25 ng/ml, induced more proliferation of satellite cells plated on Matrigel, than fibronectin or pig-skin gelatin ($p < 0.05$). The combination of bFGF, IGF-I and IGF-II resulted in 39.6% fusion on Matrigel, as compared to 7.2% on fibronectin and 4.7% on pig-skin gelatin ($p < 0.05$). Alone, bFGF had no differentiative effect ($p > 0.05$). Subsequent addition of growth hormone and prolactin together to the defined treatment medium containing bFGF, IGF-I and II increased the rate of fusion to a maximum of 53.6% on Matrigel, 24.6% on fibronectin and 20.5% on pig-skin gelatin ($p < 0.05$). Plating density did not play any significant role in altering the plating efficiency or the final fusion percentages of satellite cells ($p > 0.05$). Consideration should be given to the process of cell attachment and proliferation in experiments attempting to maximize trout satellite cell differentiation *in vitro*.

Key words: trout, satellite cells, substratum, growth factors.

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Establishment and maintenance of cells, *in vitro*, are dependent upon complex interactions, not only from the soluble signals present in the conditioned medium of cells, but also from the extracellular matrix forming the substratum on which cells attach or reside [1-5, 9, 12, 20]. Serum, hormone or growth factor supplementation may compensate for a deficiency in the substratum [12]. However, long-term survival of cells (*e.g.*, greater than three weeks), depends upon both the substratum and soluble factors [5]. It has been proposed that growth factor/substratum interaction may be due to an alteration in growth factor potency, retention of either positive or negative growth factors in the vicinity of the cell, modification of growth factor diffusion properties, increase in growth factor stability, or

protection of growth factors from proteolytic degradation [6-8, 12, 14, 27, 32, 37].

Growth factors and extracellular matrix molecules may act in synergy to regulate processes such as cell differentiation [12, 13, 15, 26, 35]. For the purpose of this study, we postulated that trout muscle satellite cells, *in vitro*, are regulated by the complete components of the microenvironment that the cells are exposed to, including the substratum. The substrata selected for our studies were chosen because each is either a component of basal lamina associated with muscle fibers, an extracellular matrix component/adhesion molecule, or a factor postulated to influence myogenesis *in vitro* [14-21]. Our objective was to identify a substratum that complemented the growth effects (at-

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tachment, proliferation and/or fusion of trout-derived satellite cells) of the supplemented basal medium.

Materials and Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*), from 7-12 cm in length were obtained from Clear Spring Trout Company, Buhl, ID, and Blue Lakes Trout Company, Twin Falls, ID, and maintained in a living stream (Frigid Units, Inc., Toledo, Ohio) at 12°C. Trout were netted, killed by a sharp blow to the head [20], and the tail was immediately cut off to bleed the fish to reduce the number of red blood cells present in the cultures [20, 31].

Muscle isolation

Muscle tissue was isolated according to the procedure of Powell et al. [31]. Trout were scaled, externally sterilized by submersion in bleach solution (1:2000) for 1 min, rinsed in tap water and transferred to the environment of a laminar flow hood. The fish were then sprayed with 70% ethanol, followed by a rinse of sterile phosphate buffered saline (PBS; pH 7.08). After the skin was peeled away to expose the skeletal muscle, large strips of muscle tissue were excised and immediately placed in PBS.

Trout satellite cell cultures

Satellite cells were isolated according to the protocol of Dodson et al. [11]. In brief, the tissue was washed, transferred to a cell culture dish containing PBS and minced into small pieces. The tissue-PBS mixture was centrifuged at 1500 x g for 2 min, the supernatant was decanted and pronase (2 mg/ml; 1 ml/g) was added. After vortexing, the tissue homogenate-pronase solution was incubated for 1 hr at room temperature, and then centrifuged at 1500 x g for 6 min, retaining the tissue pellet. To this retentate, 30 ml of PBS was added, followed by vortexing for 30 seconds and centrifuging for 3 min at 400 x g. This last step was repeated three times. All of the supernatants were collected, pooled and centrifuged at 500 x g for 6 min. The resulting cell pellets were suspended in Leibovitz's L-15 medium, supplemented with 15% fetal bovine serum (FBS) and plated in precoated culture wells. Cultures were incubated at 20°C in a non-CO₂ environment [20, 31].

Substrata

Cell culture wells were coated with three substrata: Pig-skin gelatin (0.5 mg/well), bovine fibronectin (10 µg/well) and Matrigel (1:10 dilution; 200 µl/well) [12, 20]. Coated plates were incubated for 1 hr at room temperature. Before the cells were plated, excess substratum was removed, and the wells were gently rinsed with medium.

Defined medium

The defined medium was formulated as described previously [9] with one modification. Leibovitz's L-15 medium [20, 31], rather than McCoy's 5-A medium [9], was used as the basal medium due to the use of an incubation temperature of 20°C in a non-CO₂ environment [20]. The

defined medium was supplemented with 100 U/ml penicillin/streptomycin and 20 mg/ml gentamycin [9]. The treatment media were prepared by adding 25 ng/ml each of IGF-I and II (IGF treatments), and 10 ng/ml each of growth hormone (growth hormone treatment) and growth hormone and prolactin (growth hormone and prolactin treatment) to the defined medium. Note: 25 ng/ml bFGF was also a constituent of the basal medium as described [9].

Attachment assay

Satellite cells (1243 nuclei/mm²; 2.5 x 10⁵ cells/well) were dispersed into 24-well plates coated with the different substrata. After 24 hr, the cell cultures were washed with medium to remove debris, including red blood cells. Cultures were then fixed with methanol, stained with Giemsa and evaluated for nuclei attached per mm² [12].

Proliferation study

Satellite cells were suspended in Leibovitz's L-15 medium supplemented with 15% FBS and antibiotics. Equal numbers of cells (646 nuclei/mm²; 1.3 x 10⁵ cells/well) were then plated into 24-well plates coated with pig-skin gelatin, fibronectin or Matrigel. After a 48 hr attachment period, cultures were washed 3x to remove the serum and then supplemented with the defined treatment medium. Cultures were replenished with media every 24 hr for 10 days.

Fusion kinetics

Two experiments were performed to evaluate the fusion potential of primary trout-derived satellite cells, plated on different substrata and treated with the defined treatment medium. In the first experiment, satellite cells were plated at a density of 150 nuclei/mm² (3 x 10⁴ cells/well). After the attachment period, cultures were washed 3x with serum-free medium, and the defined treatment medium was then added. Cultures were fixed and stained after a period of 10 d, and fused nuclei were counted as previously described [12]. In the second experiment, satellite cells (50 nuclei/mm²; 1.0 x 10⁴ cells/well) were plated and allowed to attach on the three substrata. Cells were then allowed to proliferate in the defined treatment medium until the cell densities of each group reached approximately 150 nuclei/mm². At 120 hr (postplating) cell density was 156 nuclei/mm² on Matrigel and 147 nuclei/mm² on fibronectin. At 288 hr, cell density on pig-skin gelatin was 142 nuclei/mm². At these times, defined medium was added to all cultures. Ten days post-fusion, cultures were terminated and analyzed for myotube formation [12].

Differentiation of trout-derived satellite cells: MF20

Previous experiments verified that antibody MF20 reacts with myosin in trout satellite cell-derived myotubes, *in vitro* [20]. The immuno-cytochemistry was performed as follows: Cells on 24-well plates were washed 3x with L-15 medium and fixed 45 sec in ice-cold 70% ethanol:formalin:glacial acetic acid (20:2:1). After further rinsing 3x

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Growth and fusion of trout-derived satellite cells

Matrigel and fibronectin facilitated more cell proliferation than pig-skin gelatin, and Matrigel caused the best satellite cell differentiation as compared to fibronectin or pig-skin gelatin ($P < 0.05$; Figure 2A,B,C). Trout-derived satellite cells at a density of 646 nuclei/mm² (1.3×10^5 cells/well) were plated onto the different substrata and allowed to attach for a period of 48 hr. After the attachment period, the cells were washed with serum-free medium and then provided the defined treatment medium. To this basal defined medium, IGF-I and II were added and evaluated. Addition of both forms of IGF (together) induced the most proliferation when cells were plated on Matrigel or fibronectin, but not pig-skin gelatin ($P < 0.05$). The addition of growth hormone, or growth hormone and prolactin, to the treatment medium (that already included bFGF, IGF-I and IGF-II) did not appreciably improve the proliferative response of trout-derived cells ($P > 0.05$).

The addition of IGF-I and II, together with either growth hormone, or growth hormone and prolactin, did increase the number of fused nuclei on Matrigel (295 nuclei/mm²) compared to fibronectin (112 nuclei/mm²) and pig-skin gelatin (25 nuclei/mm²; $p < 0.05$; Figure 2B). We observed 39% fusion with the combined influence of Matrigel and the defined medium containing bFGF, and IGF-I and II. Fusion for the same combination of growth factors was 7.2% with fibronectin and 4.7% with pig-skin gelatin ($p < 0.05$; Figure 2C). The fusion percentage, obtained with the addition of bFGF, IGF-I and II, plus either growth hormone, or growth hormone and prolactin was higher if cells were on Matrigel ($> 50\%$ for both treatments), as compared to fibronectin ($\sim 25\%$ for the combined growth hormone and prolactin treatment) or pig-skin gelatin ($p < 0.05$; Figure 2C).

To verify that the differences observed in trout satellite cell differentiation were due to the interaction of substratum and growth factors/hormones and not due to initial cell density, cells were plated in serum-containing medium onto all three substrata at a density of 50 nuclei/mm² and allowed to proliferate until a cell density of 150 nu-

clei/mm² was reached (Figure 3A). After reaching this density (288 hr for pig-skin gelatin and 144 hr for both fibronectin and Matrigel), cultures were then exposed to the defined medium to promote fusion of satellite cells. Control cultures were plated at 150 nuclei/mm² and immediately exposed to the defined fusion medium. Results (Figure 3B) parallel those of Figure 2 and suggest that initial cell density on any substratum did not play a role in the final extent of cell differentiation (on that substratum). Alternatively, the interaction of medium factors and substratum type did influence differentiation; Matrigel facilitated 50.3% fusion, as compared to 20.1% for fibronectin and 17.0% for pig-skin gelatin ($p < 0.05$).

Satellite cell-derived myotube formation began approximately 4 d after cultures were exposed to the defined medium. These myotubes were maintained, *in vitro*, for over 10 d without detachment or decay (Figure 4). Trout-derived myotubes appeared thin, contained very little cytoplasm, and did not form extensive networks or sheets.

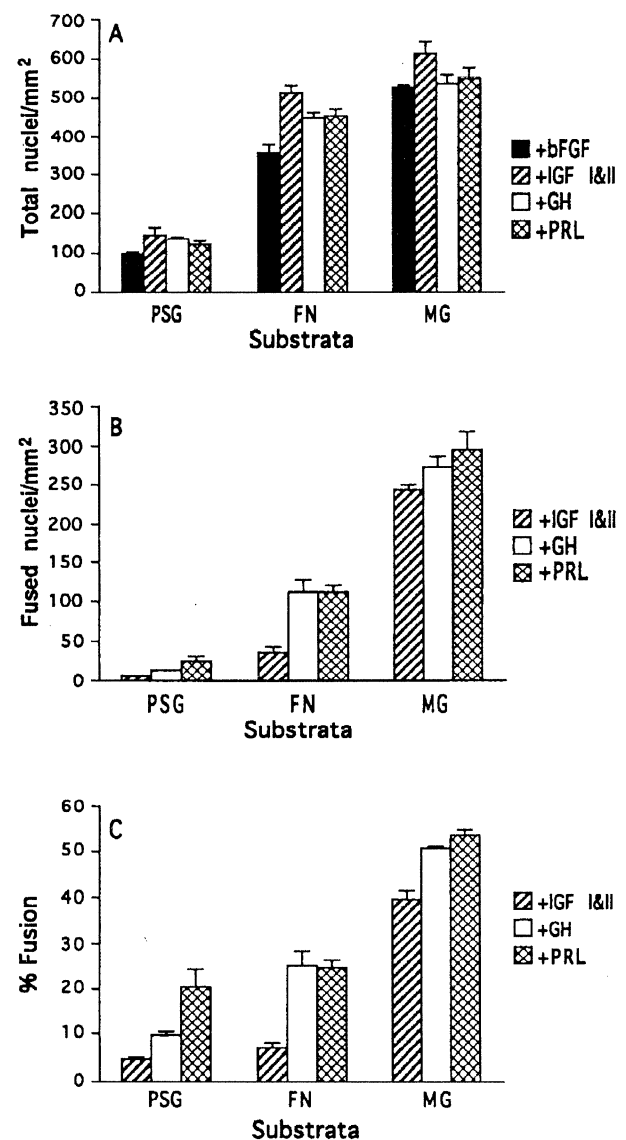


Figure 2. Influence of different substrata on trout-derived satellite cell proliferation and differentiation, as facilitated by growth factors and hormones. (A) Data represent the total cell numbers on the different substrata after being exposed to bFGF, IGF I and II, growth hormone and prolactin following a 10 d treatment period. (B and C) Data represent the fused nuclei and percent fusion, following the proliferative and differentiative periods. Percent fusion values for the three substrata at the end of the addition experiment were: Matrigel 54%, fibronectin 25% and pig-skin gelatin 20%. Each bar represents the Mean $\pm$ SEM ($p < 0.05$) of three culture wells per treatment group. Pig-skin gelatin (PSG), Fibronectin (FN) Matrigel (MG).

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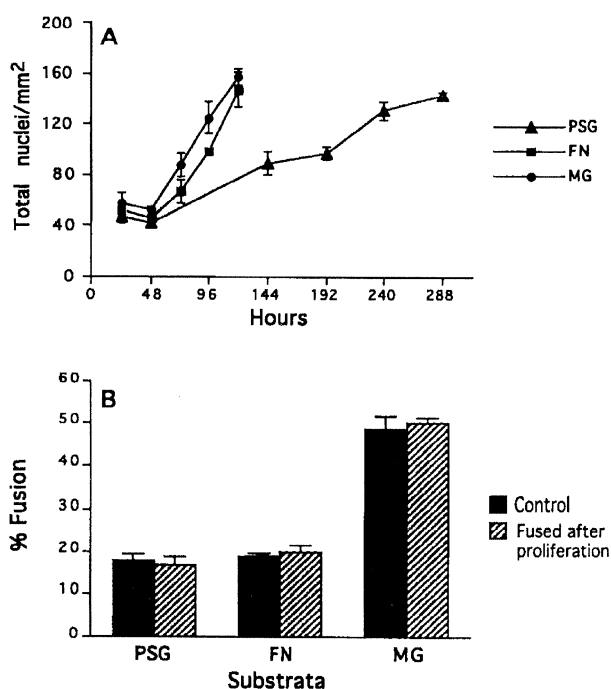


Figure 3. Effect of cell density on fusion of trout-derived satellite cells exposed to growth factors and hormones. (A) Data represent the proliferation dynamics of trout-derived satellite cells plated on different substrata. (B) Data represent the fusion levels. Each bar represents the Mean $\pm$ SEM ($p > 0.05$) of three culture wells per treatment group. Pig-skin gelatin (PSG), Fibronectin (FN) Matrigel

These tubes possess the ability to produce myosin, as seen by immunocytochemical staining, using anti-myosin antibody MF20, which detects both the fast and slow myosin isoform (Figure 5).

Discussion

The data presented confirm earlier work [20, 31] that satellite cells may be isolated from trout skeletal muscle and propagated *in vitro*. In addition, the results extend the previous observations by suggesting that the response of trout-derived satellite cells to growth factors may be modified, *in vitro*, depending on the substratum [12] used to culture the cells. For example, although many cells attached to both fibronectin and Matrigel, these data suggest that greater fusion of satellite cells occurred if Matrigel was used as the substratum coating. Also, even though initial plating densities resulted in significantly different numbers of attached cells, the plating efficiencies remained similar for like substratum.

Damsky et al. [6] reported that cell attachment is a dynamic process involving numerous cell-associated adherence molecules. Consequently, a cultured cell might attach and grow differently, based on the specific substratum selected to facilitate cell attachment [6, 23, 25-29, 31-35, 37-39]. Our data are consistent with these ideas.

From our results, it appears that the general order of substratum for facilitating attachment, proliferation and differentiation of trout-derived satellite cells was: diluted Matrigel > fibronectin > pig-skin gelatin. Undiluted Matrigel demonstrated only 35% attachment (data not shown).

In fish, satellite cells may be comprised of subpopulations [24], which may display different structural and functional capabilities [10, 24]. Therefore, if experiments are designed to examine effects of growth-regulating agents on the entire satellite cell lineage present in any given muscle at any one time, it seems that Matrigel would be the desired substratum to employ for cell attachment. Alternatively, pig-skin gelatin would likely not be the best substratum to use if the experimental goal was to maximize the likelihood of having the entire lineage of satellite cell subpopulations present in subsequent treatment designs. Pig-skin gelatin might be utilized, however, if the experimental design called for selective attachment of cells, such as in a cell cloning protocol. Since the satellite cell isolation regimen used for the present studies was not capable of selective cell removal, additional experiments are required prior to recommending use of discrete substrata for specific cell studies.

During our studies of trout-derived satellite cell proliferation and differentiation, the need for a serum-free medium became clear. The defined medium formulated for this study was a modification of the defined medium described earlier by our laboratory [9]. Defined medium components were previously shown to promote the healthy maintenance, but not growth, of mammalian satellite cells [9]. This trait of the basal defined medium allows for the addition of treatments (e.g., growth factors or hormones) to the medium, with the expectation that resulting observations are due to the treatment moiety [10]. Data derived from this study are consistent with the previous observations of the effect of the defined medium on trout-derived satellite cells.

Available defined media for mammalian satellite cells [1, 9], or media available for fish myogenic cell culture [28], are based on a bicarbonate buffer system and utilized at incubation temperatures in the upper 30°C range. Because trout are cold water fish species, we chose to use a non-bicarbonate buffer system that would specifically allow us to use an incubation temperature of 20°C [20, 31]. All cultured cells appeared healthy, robust and functional. Normal myogenic properties of attachment, proliferation, differentiation and myotube maturation were observed in all cell cultures. To our knowledge, this is the only fish satellite cell culture system that employs such medium and temperature conditions.

Eight growth factors and hormones, contained in the defined medium, were screened for ability to promote trout-derived satellite cell proliferation and differentiation. Consistent with previous work from our laboratory [9], 25 ng/ml bFGF was required in the basal defined treatment medium, to which other growth factors and hormones were

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added. Thyroid hormones and EGF, added to the defined basal medium and exposed to cultured cells, were determined to have no effect on trout-derived satellite cell proliferation and differentiation (data not shown). These negative data are similar to that reported for EGF and thyroid hormonal effects on rat-derived satellite cells [2]. Addition of IGF-I and IGF-II to the basal defined medium slightly increased total cell numbers and resulted in a small depression of final satellite cell fusion percentage. These

data demonstrate that the IGF's are active factors for trout-derived satellite cells. Alternatively, addition of growth hormone, or growth hormone and prolactin, to defined medium containing FGF and IGF-I and IGF-II, did not substantially increase any variable of satellite cell activity over that of the basal treatment medium. These data suggest that both growth hormone and prolactin may require a local mediator *in vivo* [2], if they are to actively exert an effect on trout-derived satellite cell activation,

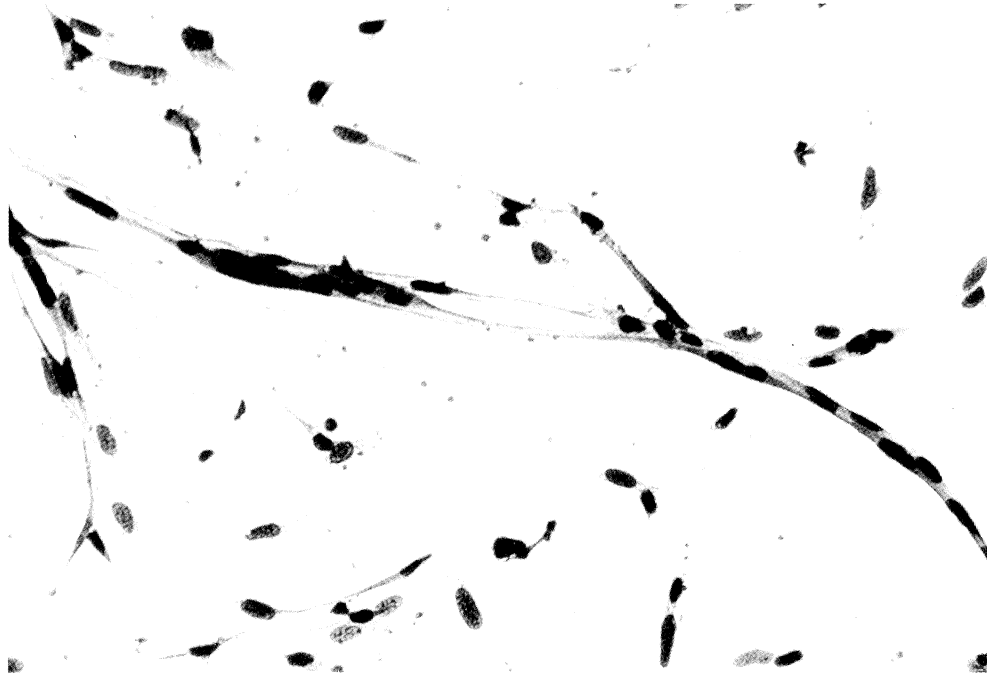


Figure 4. Trout satellite cell-derived myotube from d 10 treatment cultures, following exposure to defined treatment medium and a Matrigel substratum (x200).

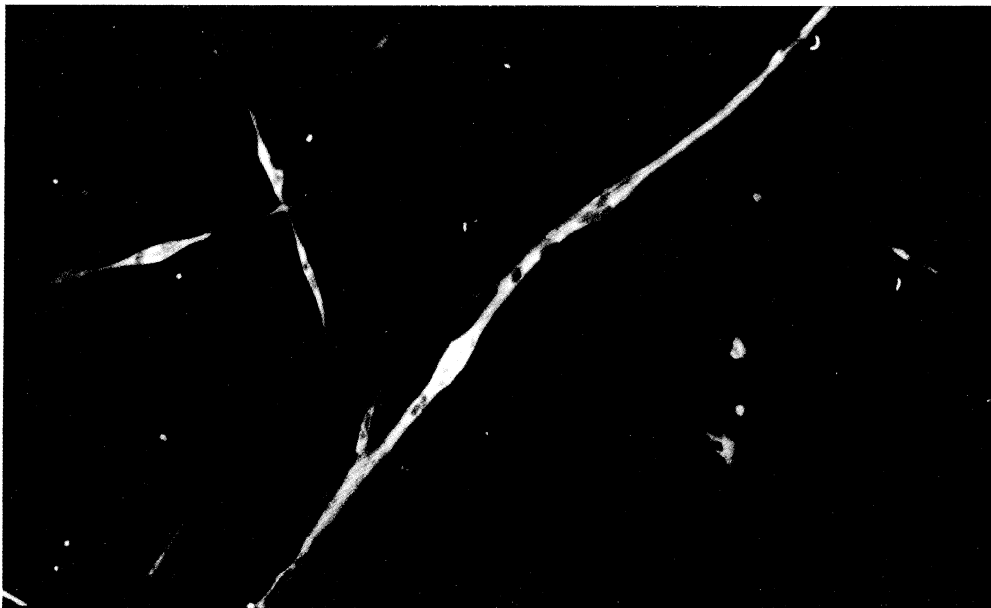


Figure 5. Trout satellite cell-derived myotube, following exposure to antibody MF20, antibody processing and fluorescence light. (x200).

proliferation or differentiation. A good candidate for the local mediator may include the IGF's. However, detailed studies with both IGF-I and II will lead to a better understanding of their role in fish muscle development.

Regardless of the type of growth factor exposed to trout-derived satellite cells, induction of trout-derived satellite cell activity was higher if the cells were plated onto the Matrigel substratum. Neither initial cell density nor time in culture altered the response of the cells to the treatment interaction. Therefore, under the conditions employed, a synergy apparently occurred between the substratum and trout-derived satellite cells, that was facilitated by growth factors. Macrobiotic agents that may have mediated the association between substratum and growth factors are the satellite cell-produced IGF binding proteins [10]. Therefore, these data should suggest caution about the extrapolation of data between experiments employing different physical conditions. Further, these data suggest that all environmental factors need to be included into discussions of mechanisms of growth factor action on cells *in vitro*.

In a previous communication, we described different morphologic profiles of cultured trout-derived satellite cells, when plated onto different substrata [20]. The observations of this work support the previous descriptions.

When exposed to different substrata, trout-derived satellite cells attached, proliferated and differentiated to different levels. Collectively, the relevance of extracellular matrix [6-8, 12-23, 25-27, 29-30, 32-39] to cell attachment, survival, growth, and differentiation is clear. In the present study the differences in the responses of trout-derived satellite cells to different substrata indicate that the chemical composition of the matrix, in combination with soluble factors (hormones, growth factors), may dictate whether the cells attach, proliferate or differentiate.

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