EFFECT OF FIBROBLAST GROWTH FACTOR ON THE DIVISION AND FUSION OF BOVINE MYOBLASTS

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ABSTRACT

The effect of fibroblast growth factor (FGF) on the rate of proliferation and fusion of bovine myoblast has been examined. Addition to the cultures of 0.1 μg-1 μg/ml of FGF stimulates the rate of proliferation and delays the fusion of primary cultures of bovine myoblasts cultured in 10% serum. Final cell densities reached in the presence of 0.1 μg/ml of FGF were fivefold higher than in controls; with 1 μg/ml, they were 10-fold higher. Increases in cell density were paralleled by increases in acetylcholine receptor sites as measured by the binding of ^{125}I-α-bungarotoxin. Both fusion and the appearance of acetylcholine receptor sites were delayed in the presence of FGF.

Growth hormone, insulin and testosterone, which have been reported to be mitogenic for rat and chick embryo myoblasts, did not have significant effects on DNA synthesis in bovine myoblasts when compared to the FGF. Conversely, FGF did not stimulate the proliferation of chick embryo myoblasts, indicating that it is not active in all vertebrate species.

The effect of fibroblast growth factor (FGF) on the rate of division and differentiation of myoblasts has been investigated. FGF is a mitogenic polypeptide isolated from brain (9) and pituitary (11). It is a mitogen for BALB/c 3T3 cells (8, 12–14), early passage fibroblasts (15), chondrocytes (11, 21), adrenal tumor cells (10), and glial cells (36). Subsequent studies have shown that FGF controls the proliferation of mesoderm-derived cells in tissue culture, while it does not affect ectoderm- or endoderm-derived cells (11). It seems that a cell's sensitivity to FGF is correlated with the cell's germ layer of origin rather than with its function. Therefore, the provisional name of FGF (given to FGF because of its effect on BALB/c 3T3 cells [8]) does not cover the full range of its activity. A more accurate name would be mesodermal growth factor. Recently, we have shown that FGF can induce proliferation of undifferentiated cells resembling those of a regeneration blastema in the stumps of amputated frog limbs (16).

Since the population of blastema cells is derived in part from muscle (18, 34), we have investigated the effect of FGF on the rate of proliferation and differentiation of primary cultures of myoblasts. Differentiation of myoblasts into myotubes can be easily followed in vitro by observing the morphology of the culture as well as by measuring the binding of neurotoxin to acetylcholine receptor sites which appear as myoblasts differentiate. Thus, with myoblast cultures, one can follow simultaneously the effects of FGF on both proliferation and differentiation.

MATERIALS AND METHODS

FGF was purified from bovine pituitary glands and brain as described previously (9, 11). It was greater than 95% pure on acrylamide gel electrophoresis. Dexamethasone was obtained from Sigma Chemical Co., St. Louis, Mo.; crystalline bovine serum albumin from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; cytochalasin B from Calbiochem, San Diego, Calif.; and benzoquinonium was a gift of Winthrop Laboratories, New York. Ovine growth hormone (preparation NIH-GH-S10) was a generous gift from the Endocrine Study Section, National Institutes of Health, Bethesda, Md. Sera and chick embryo extract were obtained from Grand Island Biological Co., Grand Island, N. Y., and trypsin from Nutritional Biochemicals Corp., Cleveland, Ohio. Tissue culture dishes were purchased from Falcon Plastics, Div. of BioQuest, Oxnard, Calif.

Myoblasts

Bovine Myoblasts

Primary cultures were established from myoblasts obtained by trypsinization of thigh muscle from 18- to 23-cm bovine fetuses (~3 mo gestation). The fetuses were obtained fresh from a local slaughterhouse (Talone Packing Co., Escondido, Calif.). The muscles were rinsed in phosphate-buffered saline, finely minced with scissors, and suspended in 50 ml of a 0.25% trypsin solution in Tris-buffered saline. The suspension was agitated at 37°C. After 20 min, the supernate was drawn off and mixed with horse serum to stop the trypsinization. Fresh trypsin solution was added to the remaining pellet, and the dissociation procedure was repeated for two additional 20-min periods. The supernates were then pooled, filtered through a Nitex cloth, and centrifuged at 400 *g* for 5 min. The pellet was suspended in 10 ml of medium A (medium 199, Dulbecco's modified Eagle's medium and fetal calf serum mixed in a ratio of 8:2:1 with 1% chick embryo extract). The suspension was distributed into three 15-cm dishes, and medium A was added to make a final vol of 35 ml per dish.

Selection of Myoblasts

PREPLATING: Since the attachment of myoblasts to the tissue culture plate is slower than that of the accompanying nonmyogenic cells, preplating was done to select for myoblasts (37). The cell suspension was left for 35 min on gelatin-coated dishes (30). The medium containing the unattached cells was then transferred twice to fresh gelatin-coated dishes for two additional 45-min periods. In our experience, this technique produces populations that consist of 60–80% myoblasts, but the yield of cells is low. To obtain more cells, we have used the cytochalasin B method as described by Sanger (31). We have found no difference in the subsequent growth and fusion behavior of myoblasts, whether selected by cytochalasin B or by preplating. Thus, the effect of FGF is not dependent on transformation of myoblasts by cytochalasin B.

CYTOCHALASIN B: After 24-h incubation at 37°C in a CO_2 incubator, the medium was removed, the cells were washed once, and 35 ml of medium A containing 1 μg/ml of cytochalasin B was added to each dish. After incubation for 24 h at 37°C, the cells which had rounded up and detached were presumed to be myoblasts (31). These rounded cells were shaken loose with gentle agitation and the medium containing the suspended cells was removed and centrifuged at 1,000 *g* for 5 min. The pellet was resuspended in medium B (medium 199 with Dulbecco's modified Eagle's medium and horse serum at a ratio of 8:2:1).

The cells were distributed into 35-mm gelatin-coated dishes (30) at 7.5×10^4 cells per dish. The dishes were then incubated at 37°C for the length of the experiment in a CO_2 incubator.

Two controls were made to ensure that the suspended cells were predominantly myoblasts. *(a)* When the cells were seeded at low density (100 cells per 6 cm dish), 80–90% of the colonies that developed formed myotubes (Fig. 1 and reference 17). *(b)* After extensive fusion had taken place in a dense primary culture of myoblasts, the remaining mononucleated cells were removed by trypsinization and replated. After several days of cell division, myotubes began to form. The extent of fusion in these secondary cultures was nearly as great as in the primary cultures, indicating that most of the mononucleated cells seen after fusion in primary cultures were myoblasts.

Fusion was observed at densities as low as 189 cells/mm^2. In the low density seeding experiments, fusion was observed with cell densities as low as 20 cells/mm^2. But under those conditions, 100 μg/ml of brain crude extract, pH 8.5, was added to the medium to promote differentiation (17).

Chick Myoblasts

Leg muscles of 11-day White Leghorn embryos were used. The technique used to prepare cell suspensions has been described elsewhere (22). Myoblasts were seeded at 1.5×10^5 cells per 35-mm gelatin-coated dish in 2 ml of medium 199 supplemented with 7.5% horse serum and 2.5% chick serum.

In contrast to bovine myoblasts, which could be selected successfully using either preplating or cytochalasin B, chick myoblasts were found to be sensitive to the cytochalasin B. In some cultures, chick cells did not survive the treatment, thus making that method unreliable in our hands. For that reason, preplating techniques were used routinely to select for myoblasts among chick cells. However, when selection of chick myoblasts with cytochalasin B was successful, results identical to those obtained with preplating were obtained.

DNA Assay

Determination of DNA content per culture was done as described by Burton (3). The assay was done in triplicate.

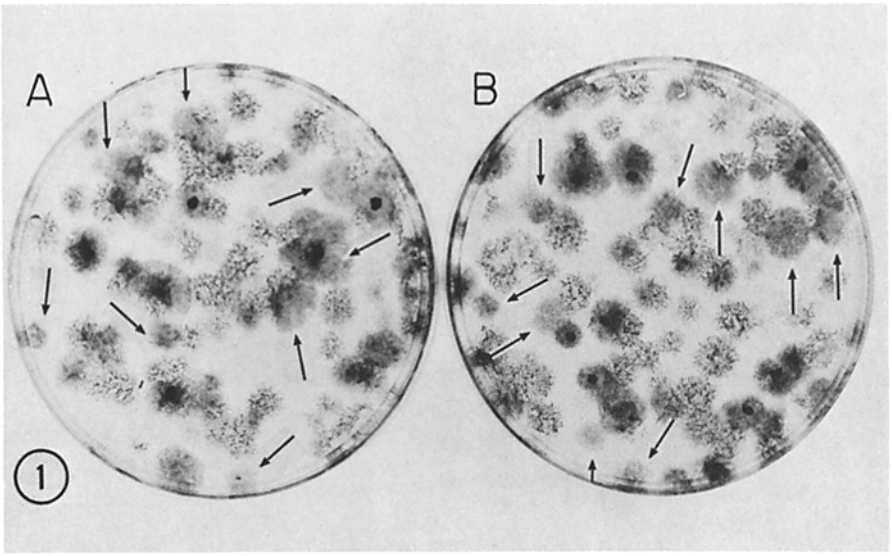

FIGURE 1 Clonal analysis of bovine myoblast populations after cytochalasin B selection procedure. 300 cells were plated in 6-cm dishes in 5 ml of 1:4 Dulbecco's modified Eagle's: medium 199 with 10% horse serum. Brain crude extract (100 μg/ml) were added 26 h later (17). After 7 days, colonies with extensive fusion could be seen. The medium was removed, and the cells were fixed with 10% formalin and stained with 0.1% crystal violet. Dishes A and B represent two separate experiments done after two separate dissociations. Two types of colonies could be seen in which fusion took place. One type was "spidery" in appearance, and fusion took place at very low cell density (20 cells/mm^2): these represent the majority of the colonies and were small in size since fusion prevented further cell division. Other colonies (black dots) had fusion taking place at high cell density (400 cells/mm^2), and fusion started in the center of the colonies. Colonies in which fusion was not taking place were recorded as fibroblast colonies and are indicated by the arrows. 80–90% of the colonies formed myotubes.

The rate of DNA synthesis per culture was measured daily by pulsing for 1 h with [^{3}H]thymidine (1 μCi per ml of medium). The pulse was terminated by removing the medium and adding 2 ml of 0.5 N NaOH per dish to lyse the cells. Determination of the amount of [^{3}H]thymidine incorporated into DNA in triplicate cultures was done as already described (8, 14).

To study the effect of various concentrations of FGF, insulin or serum on the rate of DNA syntheses in myoblast cultures, cells selected by the cytochalasin B method (31) were distributed at 1.5×10^5 cells per 35 mm dish in a medium composed of medium 199, Dulbecco's modified Eagle's medium, and horse serum in a ratio of 8:2:1. 24 h later, the medium was renewed and the horse serum concentration was lowered to 0.5%. Samples were added 12 h later, and the cells pulsed 12 h later with [^{3}H]thymidine, as already described (8), for 4 h. The cells were then lysed, and determination of the incorporation of [^{3}H]thymidine into DNA was done.

Neurotoxin Binding

α-Bungarotoxin was iodinated with ^{125}I, using chloramine T, to specific activities of $2\text{–}3.5 \times 10^{17}$ cpm/mol (24). Binding of ^{125}I-toxin was determined essentially as described by Patrick et al. (27). First, the medium was removed, then the cells were washed once with a balanced salt solution (BSS) containing 0.2% fetal calf serum, and finally covered with 1 ml of the same solution. To half of the plates, 10 μl of 10^{-2} M benzoquinonium was added. The plates were incubated at room temperature for 10 min with periodic shaking. 10 μl of a 10^{-6} M ^{125}I-α-bungarotoxin solution was then added to all plates followed by a 10-min incubation period. Then the medium was removed, the cells were rinsed three times, and the cells were removed from the surfaces of the dishes with a rubber policeman and collected by suction on an HEWP Millipore filter (0.45 μm). The filters were washed four times with BSS, dried, and counted in a scintillation counter. Specific binding was determined by subtracting the counts per minute recovered from dishes incubated with benzoquinonium plus ^{125}I-α-bungarotoxin from the counts per minute recovered from dishes incubated with ^{125}I-α-bungarotoxin alone. Every determination was done in triplicate. Nonspecific binding was 10% that of specific binding in cultures with extensive fusion.

RESULTS

Effect of FGF, Insulin and Serum on the Rate of DNA Synthesis in Myoblast Cultures

Myoblast cultures made partially resting by lowering the serum concentration to 0.5% responded to insulin, FGF, or serum with an increased rate of DNA synthesis (Fig. 2 A). FGF from brain was active at 0.005 μg/ml and was maximally active at 0.025 μg/ml. FGF from pituitary was active from 0.001 to 0.020 μg/ml. The maximal stimulation of DNA synthesis by brain FGF was greater than that produced by pituitary FGF, and was as great as that produced by 10% horse serum. While the effect of FGF on [^{3}H]thymidine incorporated could be most readily seen under conditions of serum starvation, it was also pronounced under conditions where serum was not limiting. Addition of FGF to cultures maintained in the presence of 10% serum gave a considerable increase in DNA content per culture (Figs. 3 and 4).

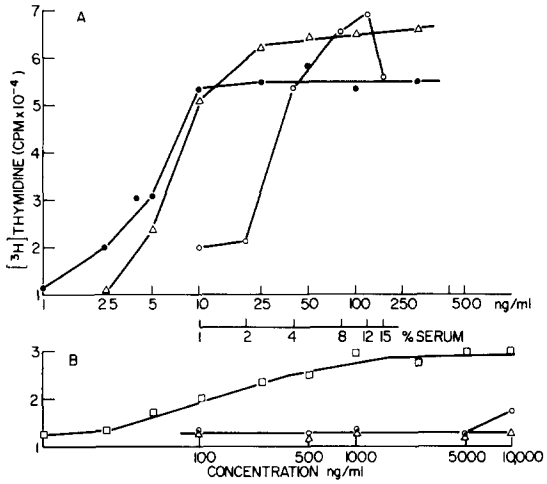

FIGURE 2 Effect of different agents on the initiation of DNA synthesis in myoblast cultures. (A) Comparative effect of increasing concentrations of brain FGF (△—△), pituitary FGF (●—●), and horse serum (○—○) on the initiation of DNA synthesis in partially resting populations of myoblasts. Cells selected by the cytochalasin B method (31) were distributed at 1.5 × 10^5 cells per 35 mm gelatin-coated dish in a medium composed of medium 199, Dulbecco's modified Eagle's medium and horse serum in a ratio of 8:2:1. 24 h later, the medium was renewed and the horse serum concentration was lowered to 0.5%. Samples were added 12 h later, and the cells pulsed 12 h later with [^{3}H]thymidine, as already described (8), for 4 h. The cells were then lysed and determination of the incorporation of [^{3}H]thymidine into DNA was done as already described (8, 14). (B) Comparative effect of increasing concentrations of testosterone (△—△), growth hormone (○—○), and insulin (□—□) on the initiation of DNA synthesis in partially resting populations of myoblasts. Cells were distributed as described in A. Determination of [^{3}H]thymidine incorporation was done as described in Materials and Methods and in A.

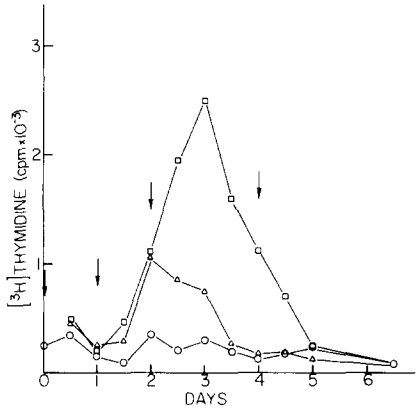

FIGURE 3 Rate of incorporation of [^{3}H]thymidine into DNA in myoblast cultures as a function of the time of incubation. Cells selected by the cytochalasin B method (31) were distributed at 7.5 × 10^4 cells per 35 mm gelatin-coated dish in a medium composed of medium 199, Dulbecco's modified Eagle's medium, and horse serum at a ratio of 9:2:1. The cultures were maintained in the presence of FGF, either 0.1 μg (△—△) or 1 μg (□—□) added on day 0, 1, 2, and 4 (arrows). Control cultures were maintained without any additions (○—○). The rate of DNA synthesis per culture was measured every 12 h by pulsing the cultures for 1 h with [^{3}H]thymidine (1 μCi per ml of medium). The pulse was terminated by removing the medium and adding 2 ml of 0.5 N NaOH per dish to lyse the cells. Determination of the amount of [^{3}H]thymidine incorporated into DNA in triplicate cultures was done as already described (8, 14).

The difference in biological activity between pituitary FGF and brain FGF could be due to small differences in structure. However, it should also be kept in mind that the 15–20% difference in specific activity between two mitogenic agents isolated from two different sources cannot really be considered as especially significant, and could reflect a higher degradation rate during the purification of the pituitary FGF as compared to brain FGF. The difference between the mitogenic effect of FGF and that of agents which have been considered mitogenic for myoblasts was highly significant, however.

Insulin concentrations of 1–2.5 μg/ml were re-

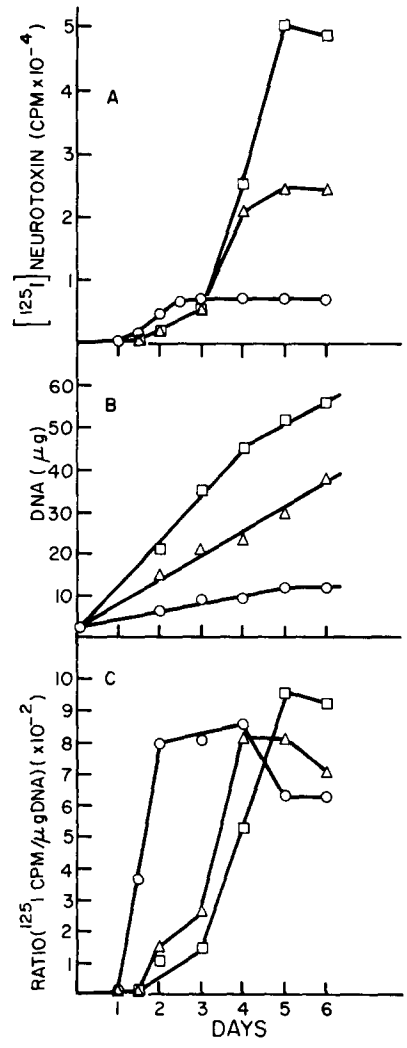

FIGURE 4 Comparison of the binding of [^{125}I]toxin and rate of DNA synthesis as a function of time in cultures of bovine myoblasts. Cells selected by the cytochalasin B method (31) were distributed at 7.5 × 10^4 cells per 35-mm gelatin-coated dishes in a medium composed of medium 199, Dulbecco's modified Eagle's medium and horse serum in a ratio of 8:2:1. Cells were maintained in the same medium for the length of the experiment, and FGF was added every other day at a final concentration of either 0.1 or 1 μg/ml. (A) Determination of the binding of [^{125}I]toxin as a function of time in myoblast cultures maintained in the presence of FGF (0.1 μg Δ—Δ or 1 μg □—□) or in its absence (○—○). The nonspecific binding amounted to 10% of the specific binding when fusion had taken place. Standard errors were within 10% of the mean. (B) Determination of DNA content as a function of time in myoblast cultures maintained either in the presence of FGF (0.1 μg/ml Δ—Δ and 1 μg/ml □—□), or in its absence (○—○). (C) Ratio of the binding of [^{125}I]toxin to the DNA content of the myoblast cultures maintained either in the presence of FGF (0.1 μg Δ—Δ and 1 μg □—□) or in its absence (○—○).

quired to produce a significant increase in the rate of DNA synthesis. That effect was 20% of that observed with an optimal concentration of added serum. Neither testosterone nor growth hormone had any effect alone, nor did they potentiate the effect of FGF or serum on DNA synthesis (Fig. 2 B).

Time-Course of DNA Synthesis in the Presence or Absence of FGF

The effect of FGF on the time-course of DNA synthesis in myoblast cultures is shown as a function of time in Fig. 3. In the absence of FGF, cultures maintained in 10% horse serum synthesized comparatively little DNA as shown by the small amounts of [^{3}H]thymidine incorporated during 1-h pulse labeling periods over the course of 6 days. In the presence of 0.1 μg/ml purified FGF, from either brain or pituitary, cultures showed a high level of DNA synthesis. The rate of [^{3}H]thymidine incorporation per culture reached a maximum 2 days after the FGF was added, remained constant until day 3, then decreased to a level similar to that of the controls by day 4. With 1 μg/ml of FGF, a high rate of [^{3}H]thymidine incorporation was sustained longer than with 0.1 μg/ml. The rate of incorporation per culture increased steadily until day 3, reflecting the increasing cell number. From day 3 to day 5 it diminished, and by day 5 the rate of DNA synthesis was similar to that of the controls. This indicates that the increase in cell number proceeds rapidly from day 0 to day 2 with 0.1 μg/ml of FGF and then at a decreasing rate from day 2 to day 4. With 1 μg/ml of FGF, the increase in cell number is greater and continues until day 5.

Comparison between the Binding of ^{125}I-α-bungarotoxin and Rate of DNA Synthesis as a Function of Time

The rate of appearance of acetylcholine receptor sites was monitored by measuring the binding of ^{125}I-α-bungarotoxin (Fig. 4). In controls, specific binding of toxin was noticeable at 24 h, and in-

creased until 60 h, when it reached a plateau. With 0.1 μg or 1 μg of brain or pituitary FGF per ml, the degree of binding during the first 72 h was lower than in controls, reflecting a lower rate of fusion in the presence of FGF. At 72 h, cultures in the presence of 0.1 μg/ml of FGF bound as much toxin as did controls. Binding increased to a maximum between days 4 and 5, reflecting a faster rate of fusion. Maximal binding to cultures maintained in the presence of 0.1 μg/ml of FGF was fivefold higher than in controls; with 1 μg/ml, it was 10-fold higher. This reflects an increased myoblast population.

The increase in DNA content of the cultures measured by colorimetry (Fig. 4 B) can be compared to the incorporation of [^{3}H]thymidine into DNA (Fig. 3) which reflects the rate of DNA synthesis. [^{3}H]Thymidine incorporation and colorimetry were done on separate sets of primary cultures. With 1 μg/ml of FGF, the rate of increase of DNA content slowed down by day 4 (Fig. 4B). This is in accordance with the results shown in Fig. 3, where between day 3 and 4 a decrease in the incorporation of [^{3}H]thymidine in the culture maintained with 1 μg/ml of FGF was observed. In contrast, with 0.1 μg/ml of FGF the rate of increase of DNA content did not change by day 4 although, as shown in Fig. 3 there was a slow down in the rate of DNA synthesis by day 4. This reflects the variation encountered from experiment to experiment.

Although the amounts of toxin bound per cell were nearly the same at the end of the experiment for all cultures, the time-course of appearance of toxin-binding sites was quite different. Whereas the ratio of toxin bound per cell reached a maximum on day 2 in controls, it reached a maximum on day 4 with 0.1 μg/ml FGF and on day 5 with 1 μg/ml FGF. When Fig. 4 C is compared with Fig. 3, it can be seen that, in all cases, the toxin binding per cell reached a maximum after the rate of DNA synthesis per culture dropped from its maximum, indicating that cells fusing into myotubes are removed from the pool of cells capable of synthesizing DNA.

Morphology of Myoblast Cultures Maintained in the Absence or in the Presence of FGF

When myoblasts were maintained in the absence of FGF, by day 1 short myotubes containing two to three nuclei were observed. Fusion progressed on day 2 with the formation of long anastomosing myotubes. By the third day, the cultures consisted primarily of anastomosing myotubes which could be seen contracting and surrounding shorter myotubes containing 5–10 nuclei per tube (Fig. 5). Single cells did not account for more than 20% of the total number of nuclei.

With FGF, at either 0.1 μg or 1 μg/ml, there was an obvious delay in fusion. By day 3, the cultures appeared the same as control cultures on day 1, but at a higher cell density. From day 3 to day 5, fusion progressed as in control cultures, ending with a mixture of long anastomosing myoblasts, shorter myotubes with 5–10 nuclei, and single myoblasts. The morphology of myoblast cultures maintained in the presence or absence of FGF until day 5 is shown in Fig. 5. Cell density in the presence of FGF was so great that it resulted in the packing of nuclei in multiple layers, and this precluded the construction of an accurate fusion kinetic curve. Representative views of experimental cultures shown in Fig. 5 clearly demonstrate the striking effect of FGF.

Effect of FGF on Chick Embryo Myoblasts

The effect of FGF on the proliferation of chick embryo myoblasts was investigated. At 1 μg/ml, FGF did not have any effect. In contrast, chick embryo extract stimulated the proliferation of chick myoblasts (Fig. 6). The lack of effect of FGF on the growth of myoblasts was confirmed by DNA and protein content measurements. No increase in DNA or protein content over controls was seen when the myoblasts were maintained in the presence of FGF.

DISCUSSION

These studies demonstrate that FGF is mitogenic for myoblasts, and is vastly more potent than insulin, growth hormone, or testosterone. While it has been reported by others that insulin and testosterone can be mitogenic for chick myoblasts (5) and for the L6 myogenic cell line (28, 25), respectively, the effects observed were small. With insulin, the DNA content of chick myoblast cultures increased twofold (5), while with testosterone, a 35% increase in labeled nuclei of L6 was observed (28). This is hardly comparable to the 10-fold increase in cell number observed with FGF. Also, while it has been reported that insulin promotes the initial cytological event in muscle development (i.e., the fusion of myoblasts to form multinucle-

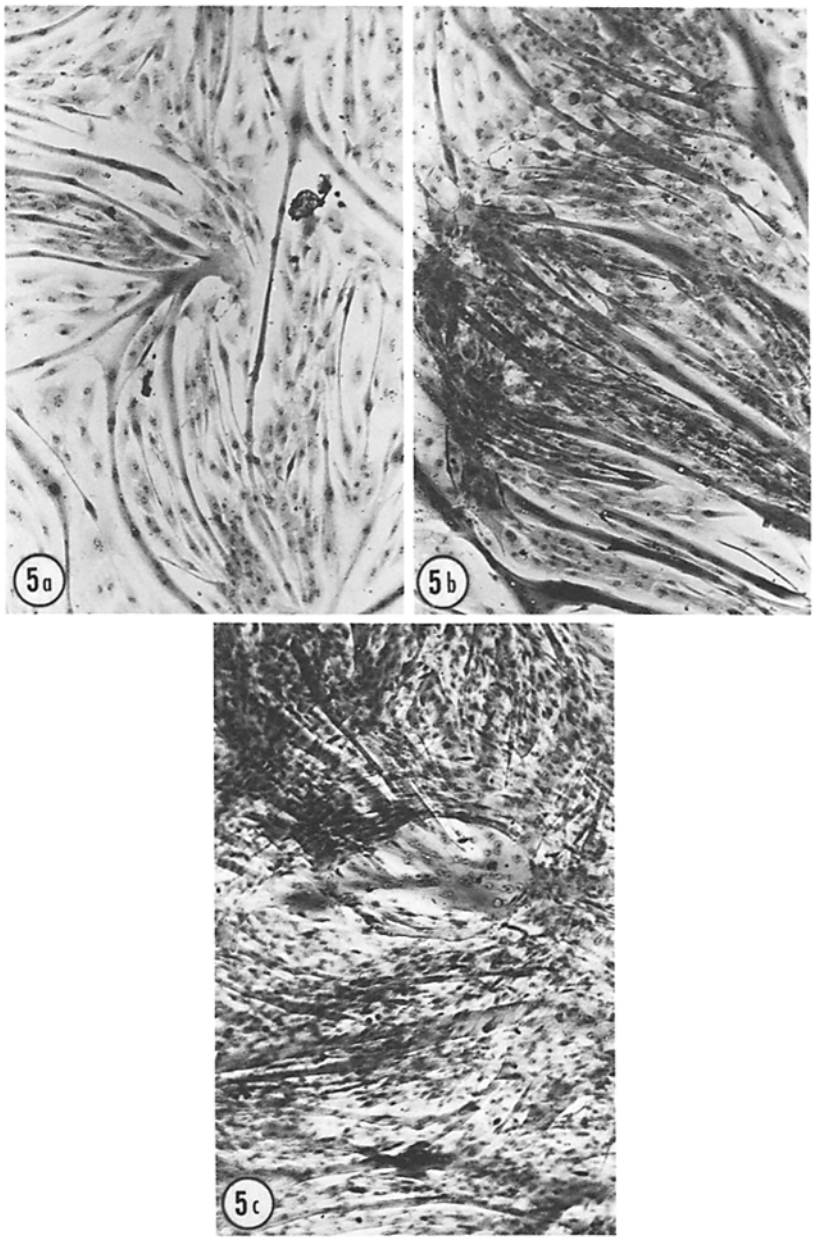

FIGURE 5 Morphological appearance of myoblast cultures maintained in the presence or absence of FGF. The cells were maintained during 5 days as described in Fig. 4. At day 5, the plates were washed and fixed for 1 h with 10% formalin pH 7.2 and then stained with 1.0% crystal violet. (A) control; (B) FGF 0.1 μg/ml; (C) FGF 1 μg/ml.

ated myotubes) in both chick myoblasts (5) and the L6 cell line (25), we have not observed such an effect of insulin using primary cultures of bovine myoblasts.

The response to FGF of myoblasts selected by either preplating or cytochalasin B was identical. The only difference was that with cytochalasin B the contamination with nonmyoblasts was less pronounced than with the preplating selection. The cytochalasin B treatment, therefore, did not

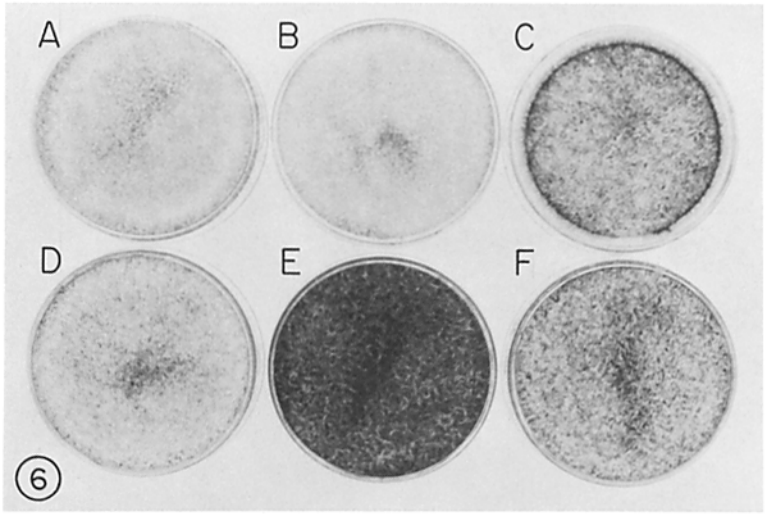

FIGURE 6 Comparison of the effect of FGF and chick embryo extract on cultures of bovine myoblasts and chick myoblasts after 5 days. Bovine myoblasts were distributed as described in Fig. 4. Chick myoblasts were distributed at an initial density of 1.5×10^5 cells per dish and were maintained in the presence of medium 199 containing 7.5% horse serum and 2.5% chick serum. The plates were fixed and stained as described in Fig. 5. (A) chick myoblasts control; (B) chick myoblasts with FGF (1 μg/ml); (C) chick myoblasts with 2% chick embryo extract; (D) bovine myoblast control; (E) bovine myoblast with FGF (1 μg/ml); (F) bovine myoblast with 2% chick embryo extract.

affect the response of the cells to FGF, since treated cells reacted to FGF in the same way.

One should note that 0.010–0.025 μg/ml of either brain or pituitary FGF was sufficient to maximally stimulate DNA synthesis (Fig. 2). In contrast, concentrations as high as 1 μg/ml of FGF were required to obtain a maximal effect on cell proliferation. This discrepancy may be explained by the difference between the cell density used in the assay for the initiation of DNA synthesis (156 nuclei/mm^2) and the final cell density reached in long-term cultures in the presence of FGF (2,360 nuclei/mm^2 with 1 μg/ml and 1,560 nuclei/mm^2 with 0.1 μg/ml). These higher cell densities might require a higher concentration of FGF for maximal stimulation, because the cells might release proteases into the medium which inactivate FGF (11). Other possibilities are that the threshold for stimulation by FGF is increased at high cell density or that the higher serum concentration used in the growth experiment as compared to that used in the DNA initiation experiment (10% vs. 0.5%) reduces the effective concentrations of FGF.

Our observation that at high cell density a higher concentration of mitogen is required is consistent with the observations of others (19, 20) who have shown that density-dependent regulation is due to a quantitative increase in the requirement for growth factors as cell density increases. It is not clear why crowded normal cells need a higher concentration of growth factors. It is known that growth factors are destroyed by the cells (20), and increasing the cell density probably increases the rate of destruction (19). In addition, it seems likely that cells could become less responsive to growth factors when their surface area and movement decrease as they become more crowded. There is much evidence that the surface area of the cells is important in density inhibition (4, 7, 32, 35, 38).

There are certain limitations to the mitogenic effect of FGF. It works most dramatically with cultures seeded at relatively high cell densities (10 cells/mm^2), and does not seem to have a pronounced mitogenic effect at cell densities less than 0.2 cells/mm^2. At very low cell density (1×10^{-3} cells/mm^2), another agent, different from FGF, is effective at promoting the division of myoblasts (17).

It has been our consistent experience with control cultures, as well as FGF-stimulated cultures, that specific binding of toxin started to increase when the myoblasts started to fuse in the culture. The final specific binding of toxin per culture in both control and FGF-treated cultures was proportional to the final DNA content per

culture, thus indicating that the average number of binding sites per cell (as indicated by toxin bound/μg DNA) was similar at the end of the experiment in all cultures. This demonstrates that the increased cell proliferation seen with FGF was due to an increased proliferation of myoblasts, and that the percentage of nonmyoblast cells, if any, was similar under all experimental conditions. It does not mean, however, that all the myoblasts obtained in the presence of FGF must fuse. It has been shown by Paterson and Prives (26) that arrested myoblasts prepared by growing the cells in low calcium medium in order to prevent fusion nevertheless elaborate acetylcholine receptors at rates similar to those of control cultures and reach comparable receptor levels in the absence of cell fusion. Normally, the kinetics of fusion can be followed simultaneously with the appearance of toxin-binding sites. However, as already explained, a quantitative fusion kinetic curve could not be done in cultures grown in FGF, since the cultures were composed of dense, multiple layers of myotubes in which distinguishing between fused and unfused myoblasts was quite difficult.

The specific binding of ^{125}I-α-bungarotoxin was 118 femtomoles per 10^6 nuclei in controls. This number agrees with the data of Buckingham et al. (1), who observed a binding of 100 femtomoles per 10^6 nuclei with primary cultures of bovine myoblasts, and with the data of Patrick et al. (27), who obtained a binding of 90 femtomoles per 10^6 nuclei for the L6 myoblast cell line.

Since it has been suggested that muscle cell fusion is independent of DNA synthesis in vitro, but depends upon a modification of the culture medium (2, 6, 22), an alternate possibility to explain the action of FGF could be that, with cultures maintained in its presence, conditioning of the medium will take place later than in control cultures. To test that hypothesis, conditioned medium obtained from control cultures of fused myoblasts was added to cultures of single myoblasts with and without FGF. In cultures with FGF, cell proliferation took place as already described and fusion was delayed. Since the medium was already conditioned for fusion, this further demonstrates that the primary effect of FGF is on cell proliferation, and not on a delay in the conditioning of the medium. Also, when the media were changed every other day, fusion took place on the same schedule as when the media were not changed. This further indicates that cell density, rather than conditioning of the media, was the determining factor for cell fusion.

The observation that FGF is mitogenic for mammalian myoblasts but not for chick myoblasts or chick fibroblasts (11) is important, because it points out that mammalian growth factors can have species specificity (even if it is not very restricted). Using chick embryo cells (either myoblasts or fibroblasts) as a model system to identify and study growth factors present in mammalian systems may lead to the selection of mitogenic agents which are insulin-related. It has been shown that insulin or insulin-related compounds such as multiplication stimulating activity (MSA) or nonsuppressible insulin-like activity (NSILA-S) are mitogenic agents for chick cells as well as mammalian cells (29, 33). Potent factors that are mitogenic only for mammalian cells may be overlooked when chick cells are used in the assay.

Myoblasts can be added to the list of mesoderm-derived cells such as fibroblasts, glial cells, chondrocytes, adrenal cortex cells, smooth muscle cells, and vascular endothelial cells, for which FGF is mitogenic. It is interesting to note in this context that FGF is not mitogenic for liver (23), thyroid (unpublished observation), or pancreas cells[1] which are of endodermal origin, or for epithelial cells from the epidermis[2] or anterior pituitary[3] which are of ectodermal origin.

Finally, the finding that FGF is mitogenic for myoblasts and chondrocytes (11, 21) is consistent with our observation that FGF can induce blastema-like regenerates on amputated limbs of adult frogs (16), since the cells of a regeneration blastema have been shown to be derived from muscle and cartilage (reviewed 18, 34).

Note Added in Proof: We have tested epidermal growth factor (EGF) on bovine myoblasts under the same conditions as described for FGF in this paper. EGF did not affect either cell division or fusion (unpublished observation). The lack of mitogenic activity of EGF on myoblasts is in contrast to human foreskin fibroblasts for which both EGF and FGF are mitogenic. The fact that fusion was not increased in the presence of EGF suggests that fusion is not enhanced by fibroblasts since EGF could be expected to stimulate the proliferation of any fibroblasts contaminating the culture.

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