Homologous Inhibition of Myoblast Fusion *in vitro*[1]

William E. Bishop[2] and Alice S. Bennett
Ball State University

Abstract

Effects of homologous extracts prepared from mature avian skeletal muscle on the development of isolated myoblasts from the thigh muscle of 11-12-day-old chick embryos were studied *in vitro*. Fusion of myoblasts occurred in a predictable manner under the culture conditions used in this study. Some factor(s) present in extracts of the homologous adult organ was able to partially inhibit this fusion. The inhibition occurred maximally between 12 and 24 hours after myoblasts were placed in the *in vitro* environment and was only partially reversible by the re-establishment of optimal culture conditions. The inhibitory factor(s) was long lived, non-dialyzable, heat labile, and subject to inactivation by proteolytic enzymes.

While investigating the nutritional requirements of isolated embryonic chick myoblasts *in vitro* it was noted that in some cultures which had been exposed to extracts prepared from mature avian skeletal muscle, the fusion of myoblasts and subsequent development of multinuclearity was inhibited. In other cultures the extent of fusion and multinuclear development exceeded that of controls (Bishop, unpublished results). A number of workers have documented the inhibition of differentiation of regeneration of body parts by specific extracts of those parts in a wide variety of invertebrate and vertebrate systems (2, 3, 7, 8, 11, 12). This investigation was an attempt to clarify the effect of homologous extracts on myogenesis *in vitro*. The level of muscle differentiation based on the expression of multinuclearity was quantitatively determined.

Materials and Methods

Materials: Biochemicals for tissue culture media were purchased from Grand Island Biological Company and crude collagen from Worthington Biochemical Corporation. Fertilized egges of White Leghorn stock were obtained from Hy-Line Chicks of Indiana, Tipton, Indiana. Eggs and tissue cultures were incubated at 37°C in a General Electric Model 805 incubator. Sterile conditions were maintained throughout the culturing procedure.

Preparation of culture medium and plates: Collagen coated petri plates were prepared by washing glass petri plates in a collagen solution (50 mg crude collagen in 75 ml 0.1M sodium chloride and 75 ml 0.5M sodium acetate solution, pH 4.5), drying under ultraviolet light, and rinsing twice with sterile Minimum Essential Medium. Complete

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[2] Present address: Department of Bionucleonics, Purdue University, WestLafayette, Indiana.

growth medium consisted of 80 parts Minimum Essential Medium (Eagle's formula) prepared with Hank's salts, to which 25mM N'-2-hydroxyethyl-piperaxine-N'-ethanesulfonic acid (HEPES) buffer had been added; 15 parts horse serum; 4 parts chick embryo extract; and 1 part Antibiotic-Antimycotic Stock (100x) containing 10,000 units penicillin, 10,000 mcg streptomycin and 25 mcg fungizone per milliliter.

Chick embryo extract was prepared by passing decapitated 11-12-day-old embryos through a sterile 20 cc syringe, and diluting 1:1 with Hank's solution. This suspension was sonified for a total of 1.5 min in 15 sec pulses (Branson Sonifer, Model J-15A) and centrifuged at 1000 rpm for 10 min (International Refrigerated Centrifuge, Model B-20). The supernate was removed and sterilized by filtration through a fiberglass prefilter, a graded series of pre-washed membranes and finally a 0.45 Millipore filter.

Preparation of homologous extracts: Thigh muscle of healthy one- to three-day-old chickens was rinsed with sterile Hank's solution, minced with scissors, suspended in 5 ml Hank's solution per gram of tissue, and sonified for 1.5 min. The homogenate was extracted for one hour at 4°C, then centrifuged at 5,000 x g for 10 min to give the crude muscle extract solution. If not used immediately extracts were stored at —20°C. Extracts were diluted with Hank's solution. Five milliliter portions of muscle extract were dialyzed against one liter of Hank's solution at 4°C for 24 hours; other portions were heated on a boiling water bath for 30 min; and others were incubated with a 5% trypsin solution for 15 min at 37°C. One-half milliter of a given extract was added to each 2 ml aliquot of complete growth medium prior to sterile filtration.

Preparation of innoculum: Myoblast cell suspensions were prepared from the thigh muscle of 11-12-day-old chick embryos by a modification of Konigsberg's procedure (5). Pooled thigh muscle from three to six pairs of legs was dispersed in 5 ml 0.2% trypsin by gentle pipeting with a Pasteur pipet and incubated at 37°C for 15 min. The enzymatic dissociation was stopped by the addition of an equal volume of complete growth medium. The resulting cell suspension was passed through six layers of sterile cheesecloth in a Swinney type filter holder fitted to a 20 cc syringe. Cells were harvested by centrifugation and resuspended in 5 ml complete growth medium by passing the suspension in and out of a 2½ in, 20 gauge needle ten times. This cell suspension, with an additional 8-10 ml complete growth medium was plated in a 100 mm collagen coated petri plate and incubated at 37°C for 10 min. The final cell suspension was drawn off and diluted to final plating density with complete growth medium.

Culturing techniques: Myoblasts were maintained in high density culture. Collagen coated 60 mm petri plates were innoculated with 2.5 ml complete growth medium containing 1.6 x 10^6 cells. Two milliliters of the growth medium was replaced with fresh medium 24 hr after plating and then every 48 hr subsequently. Cultures were incubated at 37°C for not longer than 144 hr. Effects of variously treated homologous extracts on myoblast fusion were studied by incubating replicate cultures for 144 hr with complete growth medium containing a given extract. In one set of experiments, cultures were treated at

the time of plating with homologous extracts diluted 10^2 times. The extract containing medium was removed 12, 18, 24, 48, and 72 hours folowing plating. The cultures were washed twice with sterile distilled water, re-fed complete growth medium, and incubated for a total of 144 hr. All incubations were stopped by rinsing the cultures in distilled water and fixing them in cold formalin solution for a minimum of 12 hr.

Staining and counting techniques: Fixed cultures were rinsed in cold distilled water and stained with Harris Hematoxylin for 2-3 min. Following staining, plates were placed in cold running water for 10 min. Early in this period the plates were dipped in NH_4OH (2 or 3 drops/50 ml distilled water); followed by successive dips in 50%, 70% and 90% ethanol; two rinses in 100% ethanol for 2-3 min; a single rinse in 100% ethanol and xylene (1:1) for 2-3 minutes; and two final rinses in 100% xylene for 2-3 min each. Plates were air dried and stored under distilled water at 40°C.

Counts of nuclei were made on ten random fields per plate under a microscope equipped with phase optics. All stained nuclei with each field were scored as being within either single cells (mononucleate) or within fused cells (multinucleate). Only those cells containing three or more distinct nuclei were scored as fused. Percent fusion was determined by dividing the number of nuclei of fused cells by the total number of nuclei counted.

Results and Discussion

The cellular events associated with myogenesis under culture conditions described in this report (Figure 1) are similar to those reported by other workers (4, 6, 13). Between twelve and twenty-four hours there was a rapid increase in the rate of fusion, with 30% of the observed nuclei within fused cells at the end of 24 hours. Forty-eight hours after plating myoblast fusion was extensive and an increase in the size of the individual cells was apparent. At 144 hours between 75 and 85 percent of the total number of nuclei observed were within multinucleated cells.

There was no significant difference between the extent of myoblast fusion in cultures treated with dialyzed and nondialyzed extracts at any of the dilutions tested (Table 1). The effect of dilution, however, was pronounced. Extracts which were diluted 1:1,000 or 1:10,000 failed to exhibit any inhibitory activity. Cultures treated with extracts diluted 1:10 or 1:100 exhibited a significant reduction in the total extent of myoblast fusion. Cultures treated with extracts diluted 1:10 had a mean percent fusion value of 54%, a 28% reduction from the control mean of 82%. Cultures exposed to extracts diluted 1:100 had a mean percent fusion of 57%, a difference of 25% from the controls (Table 1).

Those cultures exposed to extracts treated with trypsin or heated in a boiling water bath, failed to exhibit any significant difference from control cultures in the total extent of myoblast fusion (Table 2). Inhibition of myoblast fusion did not differ significantly between cultures treated with a 24-hour-old extract and cultures treated with a 480-hour-old extract at either of the dilutions tested (Table 3). The results

of these experiments indicated that the inhibitory factor was either a protein or had to be associated with a protein in order to be active. It was also relatively stable and unaffected by freezing and thawing.

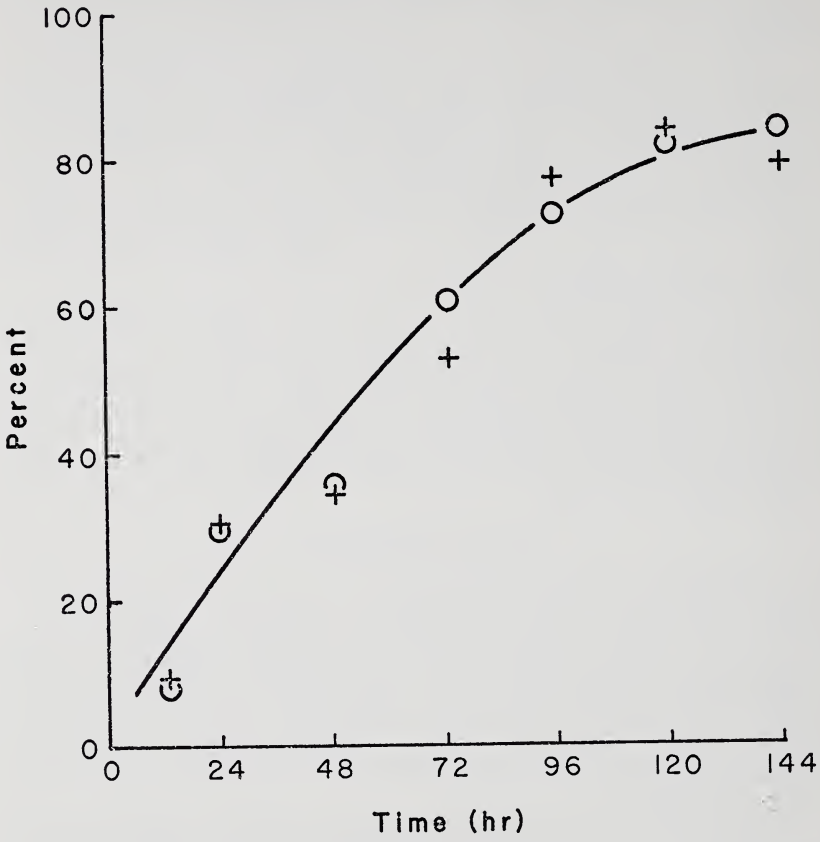

FIGURE 1. *Percentage of nuclei in fused cells. (+ and O represent two separate experiments.)*

Cultures exposed to homologous extracts during the first twelve hours following plating exhibited only a 4% reduction in the total extent of myoblast fusion at the end of 144 hours (Table 4). Cultures exposed to the extracts for 24 hours had a 22% reduction in the amount of total myoblast fusion. Inhibition could only be slightly increased by exposing the cultures for more than 24 hours (Table 4). The inhibitory factor(s) exerted its maximum effect between 12 and 24 hours of culture time. Since maximal inhibition occurred after removal of the extract at 24, 48 and 72 hours, inhibition of myoblast fusion was not dependent on the continual presence of the inhibitory factor(s) in the external *in vitro* environment. It is suggested that the factor(s) was incorporated into single myoblasts before fusion. Selectivity of

this incorporation, that is, the reason some myoblasts retained the capacity for fusion is unexplained.

Other workers have reported the inhibition of myogenesis *in vitro* by the thymidine analog, 5-bromodeoxyuridine (BUdR) (1); the chelating agent, methylene glycol tetraacetic acid (EGTA) (10); and

TABLE 1. *Effects of homologous extracts on myoblast fusion after 144 hours.*

Dilution	Number of nuclei in single cells[2]	Number of nuclei in fused cells[2]	Total nuclei counted[3]	% nuclei in fused cells
Control	106	488	594	82
10x a.	310	324	634	51
10x b.	281	352	633	56
10^2 a.	217	352	614	57
10^2 b.	271	348	618	56
10^3 a.	153	453	616	74
10^3 b.	152	454	607	75
10^4 a.	130	483	613	79
10^4 b.	141	479	620	77

[1] For each dilution, one set of cultures (a) were treated with extracts dialyzed against Hank's solution for twenty-four hours; the second set of cultures (b) were treated wih non-dialyzed extracts.

[2] Average of five replicate cultures for controls and three replicate cultures for experimentals.

TABLE 2. *Effect of heat and proteolytic enzymes on inhibitory activity of homologous extracts.*

Treatment	Number of nuclei in single cells	Number of nuclei in fused cells	Total nuclei counted	% of nuclei in fused cells
control[1]	113	468	581	81
heat[2]	147	440	586	75
trypsin[2]	131	457	588	78

[1] Average of six replicate cultures.

[2] Average of twelve replicate cultures. Extracts diluted 10^2x.

TABLE 3. *Effect of age of homologous extract on myoblast fusion.*

Age of Extract	Number of nuclei in single cells[1]	Number of nuclei in fused cells[1]	Total nuclei counted[1]	% nuclei in fused cells
Control	127	467	594	79
480 hours $(10x)^2$	303	320	623	51
24 hours $(10x)^2$	304	312	615	51
480 hours $(10^2)^2$	315	308	623	49
24 hours $(10^2)^2$	306	310	618	50

[1] Average of six replicate cultures.

[2] Dilution factor.

TABLE 4. *Effects of exposure time to homologous extraction myoblast fusion.*

Length exposure	Number of nuclei in single cells[1]	Number of nuclei in fused cells[1]	Total nuclei counted[1]	% nuclei in fused cells
Control (0 hr.)	121	467	586	80
12 hr.	145	466	611	76
18 hr.	217	402	619	65
24 hr.	252	353	605	58
48 hr.	281	353	633	56
72 hr.	290	349	638	55

[1] Average of twelve replicate cultures for controls, six replicate cultures at 1e, 18, 48, and 72 hours and 24 replicate cultures at 24 hours.

by regulation of the calcium concentration of the growth medium (9). In all cases the inhibition was complete and fully reversible. The results of this investigation suggest that inhibition of myogenesis *in vitro* by some substance(s) present in extracts of mature avian skeletal muscle is not complete and only partially reversible. The inhibitory factor(s) is apparently long lived, non-dialyzable, heat labile, and subject to inactivation by proteolytic enzymes.

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