Inhibition of Myoblast Differentiation In Vitro by a Protein Isolated from Liver Cell Medium

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ABSTRACT We have recently discovered that cells of Coon's Buffalo rat liver (BRL) line secrete a protein which is a potent inhibitor of skeletal myoblast differentiation in vitro. Using ion exchange and molecular exclusion chromatography, we have prepared this protein, which we designate "differentiation inhibitor" (DI), from the materials secreted by BRL cells maintained in serum-free medium. It is a relatively heat-stable protein which is inactivated by treatment with trypsin and mercaptoethanol and has an apparent molecular weight in the range 30,000–36,000. It exhibits no detectable mitogenic or lectin activity and differs from previously reported inhibitors of myoblast differentiation in several respects. It is active in all skeletal myoblast systems tested (Yaffe's L6 line as well as primary cultures of rat, chick, and Japanese quail myoblasts), and it blocks fusion, elevation of creatine kinase, and increased binding of α-bungarotoxin. Parallel fractionation of fetal bovine serum (FBS) and chick embryo extract (CEE) yields a peak of activity which similarly inhibits myoblast differentiation. We suggest that the differentiation inhibitor from BRL cells may correspond to the differentiation-inhibiting component(s) of FBS and CEE, and we call attention to the possibility that such a substance could play a role in embryonic growth of myoblasts and in satellite cell formation.

The mechanisms and control of muscle cell differentiation have been matters of active interest since the feasibility of measuring this process in cultured cells was demonstrated two decades ago. Two views have emerged: in essence, one states that the process is reversibly controlled by components of the medium without any requirement for DNA synthesis (1), and the other proposes that an essential event occurs during a "quantal mitosis" which gives at least one daughter cell irreversibly committed to differentiate (2). Subsequent papers have reported data interpreted as favoring one or the other view, but in many cases the interpretation has been clouded by the complex nature of uncharacterized medium components such as embryo extract (3). It seems clear that manipulation of the medium can substantially alter the time course of myoblast differentiation, but the point in the cell cycle at which myoblasts become irreversibly committed to differentiation rather than proliferation remains a matter of disagreement. This situation might be clarified if the processes involved were studied using defined medium components and cloned cells rather than the complex mixtures used in most of the published experiments.

A recent report from this laboratory (4) documents the stimulation by the somatomedins of differentiation in cloned L6 myoblasts grown in serum-free medium. Insulin at high concentrations (10^{-6} M) mimics this action of the somatomedins, probably by binding to the somatomedin receptor. During that study, we noted an unexpected inhibition of myoblast differentiation when cells were treated with crude preparations of MSA (multiplication stimulating activity, a member of the somatomedin family isolated from the medium of Buffalo rat liver (BRL) cells grown in serum-free medium). This inhibition did not occur with purified MSA and the inhibiting activity could be partially separated from MSA by molecular exclusion chromatography on Sephadex G-75. This paper is our initial report of the discovery and biological activity of this agent, which we have designated "differentiation inhibitor" (DI).

At present, the cell type specificity of DI action is a matter of considerable interest to us. Accordingly, we invite inquiries from investigators working with well-defined cell culture systems in which differentiation can be measured by morphological and/or biochemical criteria as in myoblasts.

MATERIALS AND METHODS

Materials

Tissue culture supplies were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). Insulin, bovine serum albumin (BSA, RIA grade), reagents for the creatine kinase assay, Dowex 50, and Sephadex G-

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 MAY 1982 395-401

75 were purchased from Sigma Chemical Co. (St. Louis, MO). All column equipment was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). BRL cells were obtained from Dr. Gary L. Smith (University of Nebraska). Cells cloned from Yaffe's L6 line (5) and selected for fusing (clone C1 and subclone K44) or nonfusing (clone C5) properties were used throughout this study. ^{125}I-labeled α-bungarotoxin was a generous gift from Dr. Thomas Podleski (Cornell University).

Preparation of the Differentiation Inhibitor

DI purification from BRL cell conditioned media follows the procedure used in several laboratories for MSA purification (6). In a typical preparation, a 1.5 l slurry of Dowex 50X8-100 (Na^+ form) is added to 4 l of BRL-cell-conditioned medium; the mixture is stirred at room temperature at least 30 min. The liquid is then removed by filtration through a large (160-cm inner diameter) sintered glass funnel. The resin is then washed three times with 2.5 l of 0.9% (wt/vol) NaCl and twice with 1.5 l of 0.1 M $NaHCO_3$ (adjusted to pH 9.0 with NaOH). The MSA-DI mixture is then eluted by two treatments with 700 ml of 0.1 M NH_4OH; the eluant is rapidly collected by vacuum filtration and immediately adjusted to pH 5.5 by addition of 5 N HCl.

This eluate (designated Dowex MSA) is reduced to ~200 ml by freeze-drying and dialyzed against 20 vol of 2% acetic acid followed by 20 vol of 1 M acetic acid. The dialyzed Dowex MSA is then lyophilized to dryness, taken up in 20 ml of 1 M acetic acid, and fractionated on a 5.0 × 80 cm column of Sephadex G-75 (in 1 M acetic acid). As shown in Fig. 1, the peak of DI follows the excluded volume and just precedes the broad peak which contains several subspecies of MSA activity. (In initial preparations, DI activity was localized in the excluded peak; this led to an erroneous initial indication that its molecular weight exceeded 80,000. However, we have found that it is tightly bound to the Cybacron Blue F3G-A chromophore in Blue Dextran; the results in Fig. 1 were obtained on a column run in the absence of Blue Dextran.) The DI-containing fractions were combined, frozen, and used for the experiments reported here. The purification procedure and its results are summarized in Table I.

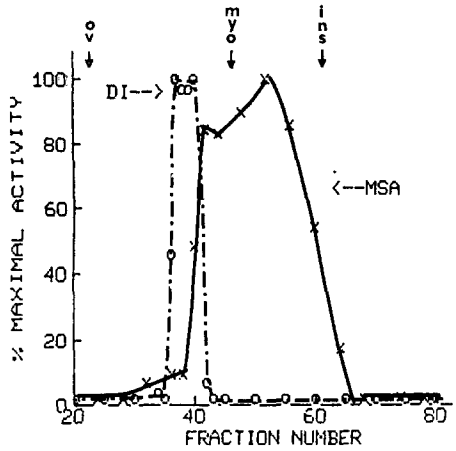

FIGURE 1 Fractionation of DI and MSA on Sephadex G-75. BRL-cell-conditioned medium was fractionated by batchwise absorption-elution on Dowex 50 as described under Methods. A 20-ml aliquot (containing 20-30 mg of Dowex MSA) was applied to a 5.0 × 80-cm column of Sephadex G-75-120 and fractionated in 1 M acetic acid at room temperature. Fractions (19 ml) were analyzed for protein, MSA, and DI activity as described under Materials and Methods. The elution peaks of molecular weight markers (*ov*, ovalbumin; *myo*, myoglobin; *ins*, insulin) used in calibrating the column are indicated at the top of the figure.

TABLE I

Partial Purification of the DI from BRL Conditioned Medium

Purification step	Total activity	Total protein	Specific activity	Purification factor	% Recovery
	U	*μg*	*U/mg*		
BRL conditioned medium	858	260,000	3.3	—	100
Dowex 50	660	20,000	33	10	76.9
Sephadex G-75	486	694	700	216	56.6

Cell Culture

Unless otherwise specified, L6 myoblast cultures were prepared by plating 5 × 10^4 cells/4 cm^2 well (in Costar 12-well plates) in 2 ml Dulbecco's modified Eagle's medium (DME) containing 2.5% horse serum and 0.5% chick embryo extract. After incubation for 24 h at 37°C in 95% air-5% CO_2, the cells were washed for 15 min with DME before the components studied were added in DME containing 0.5 mg/ml BSA. Quail and chick primary skeletal myoblasts were prepared as described by Konigsberg (7). Primary rat skeletal myoblasts were prepared by the method of Yaffe (8).

Assays of Myoblast Differentiation

Creatine kinase (CK) activity was the primary indicator of myoblast differentiation used in measuring activity of the DI. After the specified incubation periods, myoblast cultures were rinsed twice with phosphate-buffered saline (pH 7.2), covered with 100 μl of glycylglycine buffer (0.05 M, pH 6.8) and stored at −60°C. On the day the assays were done, cells were thawed, scraped from the dish with a rubber policeman, and vortexed. Aliquots (33 μl) were assayed for CK activity by the method of Shinberg et al. (9), and 50 μl of the remaining suspension was taken for analysis of DNA (10) and protein content (11).

In some experiments, fusion was also evaluated by determining the percent nuclei in myotubes; for this, the cultures studied were fixed at the specified times with methanol and stained with Wrights-Giemsa stain (12). Nuclei in random fields were counted until a total of 800–1,000 nuclei was reached.

α-Bungarotoxin binding was measured after washing the cell surfaces with Earle's balanced salt solution (BSS) containing BSA (2 mg/ml). For determination of nonspecific binding, 10^{-7} M unlabeled bungarotoxin was added to half the cultures and incubated at room temperature for 20 min. Solutions containing the unlabeled toxin were removed and all cultures then incubated with ^{125}I-α-bungarotoxin in 0.6 ml Earle's BSS containing BSA for 30 min at room temperature. Cell surfaces were washed three times with Earle's BSS containing BSA for 5 min each. The cells were solubilized with 0.8 ml of 0.5 N NaOH for 3 h and binding determined by counting in a Beckman Model 4000 gamma counter (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, IL). Specific binding was calculated in the customary manner by subtracting nonspecific from total binding for each experimental condition.

RESULTS

Effect of DI on Myoblast Differentiation

The concentration dependence of DI action on L6 myoblast differentiation is illustrated in Fig. 2. These effects of DI are not restricted to the L6 myoblast line; as shown in Table II, we have found similar inhibition in primary myoblasts cultured from rat and Japanese quail muscle (whether expressed on a per culture or per cell basis), as well as in chick myoblasts.

All tested aspects of myoblast differentiation were inhibited by DI; Fig. 2 documents the effects on fusion and creatine kinase elevation in L6 myoblasts. The effects of the inhibitor on α-bungarotoxin binding were also measured in experiments in which we assessed the potential usefulness of this system for routine assays during DI purification. The results of one such experiment are summarized in Table III; although they show a clear effect in inhibiting formation of acetylcholine receptors, the procedure had no advantages in sensitivity, convenience, speed, or economy over the determination of creatine kinase levels, so we chose the latter as our standard for DI determinations.

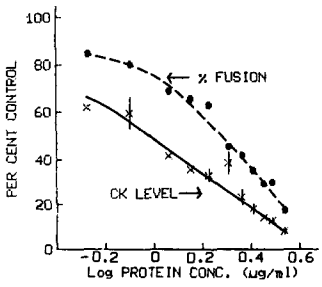

FIGURE 2 Effects of DI on myoblast fusion and elevation of CK. L6-K44 myoblasts were plated as described under Materials and Methods. 60 h after the addition of 10^{-6} M insulin and DI (at the specified concentrations) plates were processed and creatine kinase or fusion measured as specified under Materials and Methods.

TABLE II

Effects of DI on Rat and Quail Primary Myoblasts *

Conditions	CK Level			
Myoblast source Addition to medium	No DI	+DI	No DI	+DI
	U CK/culture		*U CK/mg DNA*	
Rat				
None	193 ± 3.6	92 ± 8.3	nd	nd
10^{-6} M Insulin	267 ± 8.3	121 ± 4.2	nd	nd
Quail				
None	77.1 ± 2.1	27.1 ± 4.8	28.9 ± 1.7	9.7 ± 1.0
10^{-6} M Insulin	145.8 ± 18.1	43.8 ± 6.3	36.4 ± 5.3	11.2 ± 2.0

nd, no data.

* Primary rat and quail skeletal myoblast cultures were prepared as described under Materials and Methods. Cells were plated at 1×10^4 cells/cm^2 in 8 cm^2 plates in DMEM containing 10% CEE and 15% horse serum. After 24 hours, the plates were rinsed with DMEM and fresh medium (containing 1% horse serum (for rat cells) or 0.2% horse serum (for quail cells) plus the specified additions in DMEM with 0.5 mg/ml BSA). Sephadex-purified Differentiation Inhibitor was added at a final concentration of 10 μg/ml. Samples were analyzed 60 hours after the medium change, and the data are means ± SEM of triplicate determinations.

TABLE III

Effect of Differentiation Inhibitor on Bungarotoxin Binding to Quail Muscle Cell Culture *

Addition	^{125}I-α-Bungarotoxin bound (cpm/mg protein)			
	Total	Nonspecific	Specific	% Control
None (control)	3,670 ± 90	1,250 ± 22	2,420 ± 93	100
DI	2,460 ± 148	1,460 ± 75	1,000 ± 166	41

* Quail myoblasts were prepared as described by Konigsberg (7) and plated at 5×10^4 cells/4 cm^2 well in DME containing 15% horse serum, 10% CEE, and 3.6 μg/ml DI in Costar 12-well plates. After 19 h, the cultures were washed with serum-free DME and then fresh medium comprised of 10^{-6} M insulin, 1% horse serum in DME:Ham's F-12 (1:1) (with and without DI as indicated) was added. After 48 h, binding of ^{125}I-α-bungarotoxin was measured as described under Materials and Methods. Data are means ± SEM of triplicate determinations.

Action of the DI was sustained for an extended period when fresh inhibitor was added at 24-h intervals (Fig. 3). Reversibility of DI action was indicated by the observation that 62% of nuclei were found in myotubes 48 h after the inhibitor was removed by gently washing the cell surfaces and adding fresh (inhibitor-free) medium at the end of the experiment illustrated in Fig. 3. Further evidence that the DI had no injurious effects on the cells is shown in Fig. 4; the morphology of myoblasts treated up to 72 h with DI was not detectably different from that of cells at the beginning of the treatment. The relatively long time before fusion was observed after DI removal suggests that it blocks an early event in the differentiation of myoblasts. This view is supported by the results of the experiment summarized in Fig. 5; the DI had little effect when added 24 h after insulin was added to stimulate myoblast differentiation, although there was no detectable fusion of L6 cells at the time the inhibitor was added.

Determination of the Mitogenic Activity of the DI

It has been reported that some (13) but not all (4) mitogens inhibit the differentiation of myoblasts. Thus it was of particular interest to determine the mitogenic activity of the DI. As shown in Table IV, the partially purified (i.e., MSA-free) DI used in these experiments had no detectable mitogenic activity either in the rapidly fusing K44 clone or the nonfusing C5

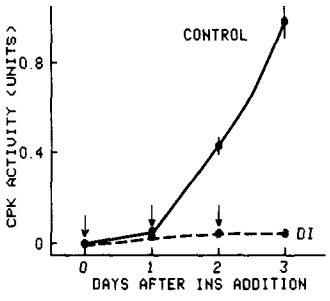

FIGURE 3 Persistence of the action of DI on myoblasts. L6-C1 myoblast cultures were prepared as described under Materials and Methods. At zero time, insulin (10^{-6} M) in the presence or absence of DI (10 μg/ml of Sephadex-purified material) was added to the cultures. Daily medium changes (arrows) were made with the addition of fresh insulin and DI. Points indicate means and bars represent SEM of triplicate determinations.

clone of L6 myoblasts. It should be noted that the Inhibitor preparation was added at 3.6 μg/ml, a concentration which gave >90% inhibition of myoblast differentiation in a parallel experiment (see Fig. 2). The DI exhibited no mitogenic activity when it was added alone or in the presence of mitogens (and fusion-stimulating agents) such as insulin and MSA. The latter are the conditions in which we routinely assay DI activity; indeed, in this experiment inhibition of fusion in the K44 cells incubated with DI and either MSA or insulin was observed microscopically at 48 h. In fact, the loss of countable myoblasts as they fused to form myotubes gave results which could be misinterpreted to indicate a mitogenic action of the DI if only the 48 h data for MSA and insulin in the K44 clone were considered. In these cases, the number of myoblasts counted decreased substantially as the cells fused between 24 and 48 h, thus giving higher cell counts in the cultures in which fusion was prevented by the DI. There was no such decrease in the nonfusing C5 clone under parallel conditions.

It should be noted (Table IV) that DI neither enhanced nor inhibited the growth-promoting actions of MSA or insulin; these observations are consistent with results of experiments which indicated that the inhibitor has no effect on the binding of ^{125}I-MSA to myoblasts (M. R. Benedict and J. R. Florini, unpublished observations) or the degradation of ^{125}I-insulin (D. Z. Ewton, unpublished observations).

In routine determinations of DNA (part of our standard DI assays), control and DI-treated cultures consistently exhibited DNA levels within 10–15% of each other; this was also the case in experiments with Japanese quail myoblasts (Table II). It should also be recalled that the DI can be detected in Sephadex

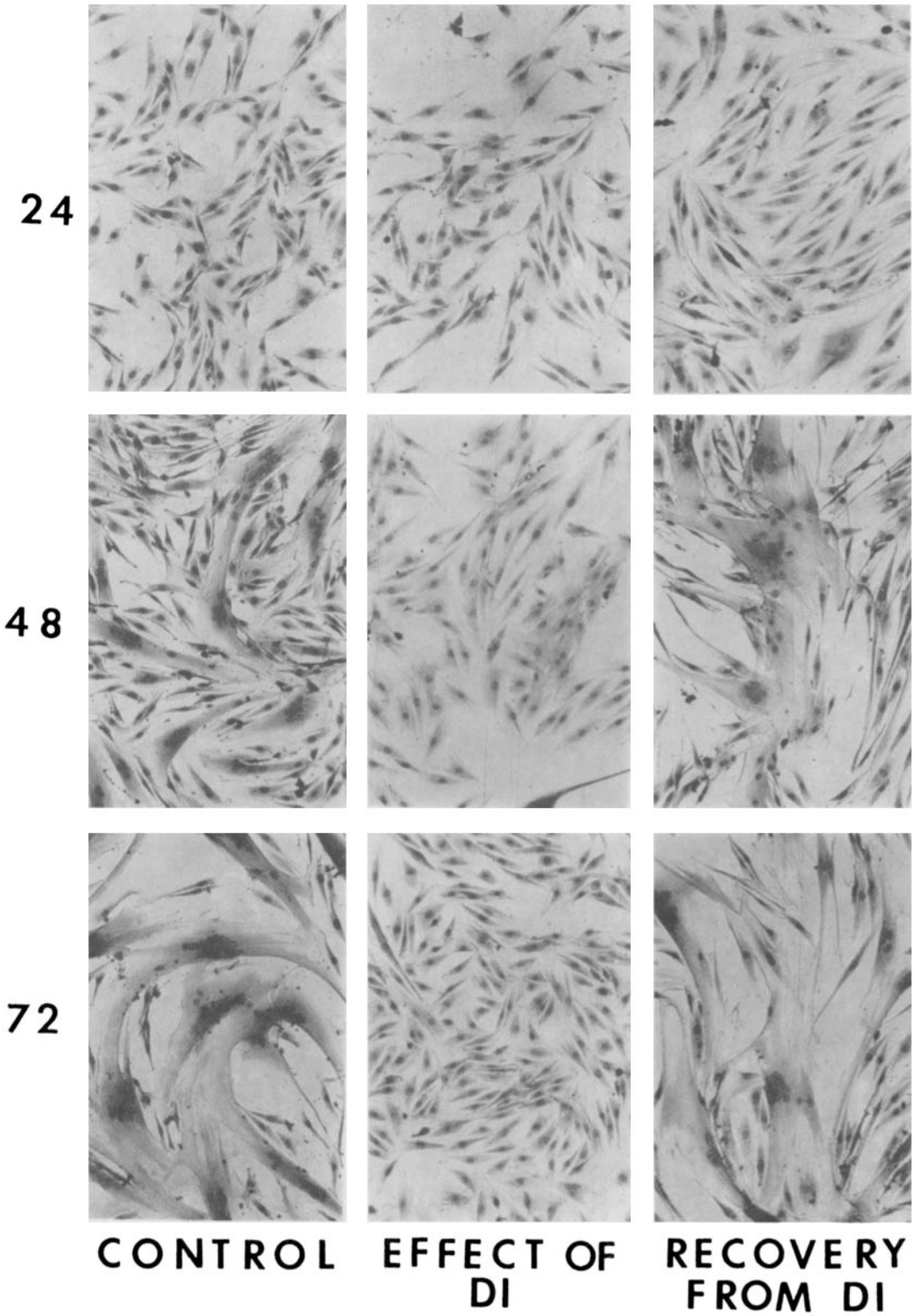

FIGURE 4 Morphology of L6 myoblasts before and after treatment with the DI. Cells were plated in 8-cm^2 plates as described under Materials and Methods. Insulin (10^{-6} M) in the presence and absence of DI was added at zero time (24 h after initial plating). At the specified times, plates were washed with phosphate-buffered saline, fixed in methanol, and then stained with Wright-Giemsa. For the *Control* and *Effect of DI* columns, these times (in hours) are measured from the initial addition of insulin. For the *Recovery from DI* column, the times are the interval after removal of DI and addition of fresh insulin to cultures which had been exposed to insulin and DI for 72 h; thus, these cultures were treated with insulin and DI for 72 h and then allowed to recover for an additional 24–72 h as indicated. × 80.

G-75 column fractions which are inactive in the ^{3}H-thymidine incorporation assay for MSA activity (Fig. 1). We conclude from this extensive set of observations that the DI is not a mitogen, at least for myoblasts and chick embryo fibroblasts under conditions and at concentrations at which it exhibits full activity in inhibiting fusion and elevation of CK in myoblasts.

Partial Characterization of the DI

Although free of detectable mitogenic activity, the DI is not yet pure. SDS gel electrophoresis and silver staining reveal the presence of at least five bands, but available quantities are not yet sufficient for rigorous evaluation of the purity and subunit structure of the DI. Nevertheless, some properties of the molecule can be established even with the impure preparations currently available. On the basis of its elution from Sephadex G-75, the DI has a molecular weight of 30,000–36,000; for this estimate, the column was calibrated using ovalbumin, myoglobin, and insulin as molecular weight standards (as shown in Fig. 1). DI activity is destroyed by treatment with trypsin or mercaptoethanol. It is relatively stable to heating; activity is not lost upon heating at temperatures up to 70°C for 10 min, although it is destroyed at 80°C and higher temperatures. It is quite stable in 1 M acetic acid at room temperature; these are the conditions in which the Sephadex G-75 column is run. Our most highly purified preparations exhibited no hemagglutination activity for erythrocytes at concentrations as high as 25 μg/ml under conditions (14) in which concanavalin A exhibited full activity. Purification and characterization of the DI molecule will be the subject of a subsequent publication.

Presence of a Similar Inhibitor in Fetal Bovine Serum and Chick Embryo Extract

High concentrations of fetal bovine serum (FBS) and/or chick embryo extract (CEE) are frequently used to suppress fusion during the growth of myoblast stock cultures. In initial experiments (S. C. Seifert and J. R. Florini, unpublished observations), we have found that fractionation of FBS and CEE by the procedure used for DI production yields a peak (in molecular exclusion chromatography on Sephadex G-75) of inhibitor activity very similar to that of the DI from BRL cells (Fig. 1). This material precedes (and slightly overlaps) a peak of mitogenic activity which presumably corresponds to the serum MSA described by Pierson and Temin (15) a decade ago. The relationship of the differentiation inhibiting components of FBS and CEE to the BRL cell DI is currently under investigation.

DISCUSSION

After our discovery of a differentiation-inhibiting material in BRL cell-conditioned medium, our initial experiments were devoted to evaluation of some possible trivial explanations for DI action and to comparisons with other agents reported to affect myoblast differentiation. From these, we reached the following series of conclusions.

The DI does not act by degrading insulin or MSA (which we use to stimulate differentiation in the serum-free medium in which these experiments are done); it blocks fusion in dense cultures incubated in the absence of these hormones. Furthermore, it does not affect the mitogenic (Table IV) or other (16, 17) actions of these hormones in myoblasts, does not degrade the iodinated hormones, and does not inhibit the binding of ^{125}I-MSA to myoblasts.

There are several reasons for concluding that it is not identical to the somatomedin-binding protein (which is also secreted by BRL cells (18)): (*a*) the action of DI is not overcome by large quantities of MSA or insulin (more than sufficient to saturate the binding protein); (*b*) it does not affect any actions of these hormones other than the stimulation of differentiation; (*c*) the binding protein does not bind insulin significantly (19) and thus would not affect the stimulation of differentiation by insulin, and (4) its apparent molecular weight is lower than that of the binding protein.

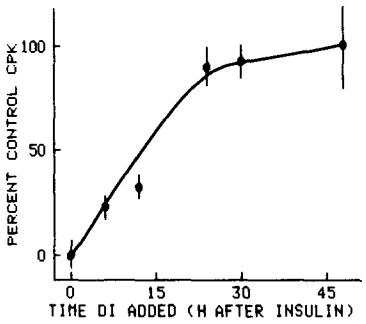

FIGURE 5 Effect of time of addition of DI on myoblast differentiation. L6-C1 myoblasts were plated as described under Materials and Methods. At zero time, the medium was changed to DME insulin (10^{-6} M); DI was added at the plotted times by adding 0.2 ml of a 100 μg/ml solution to 2 ml medium in each plate. All cultures were processed for CK assay 60 h after the addition of insulin. Data are presented as in Fig. 2.

TABLE IV

Determination of Mitogenic Activity of the Differentiation Inhibitor *

Clone Additions to medium	10^{-4} × cells/cm²			
	No DI	+ DI	No DI	+DI
	24 h		*48 h*	
K44				
None (control)	2.64 ± 0.06	2.74 ± 0.09	2.57 ± 0.20	2.33 ± 0.02
Insulin	4.60 ± 0.37	4.83 ± 0.35	3.28 ± 0.06‡	5.23 ± 0.12§
MSA	3.38 ± 0.16	3.29 ± 0.15	2.32 ± 0.06‡	5.09 ± 0.07§
Horse serum	4.49 ± 0.04	3.86 ± 0.08	8.65 ± 0.11	6.05 ± 0.05
C5				
None	6.09 ± 0.26	6.18 ± 0.25	5.11 ± 0.07	5.16 ± 0.10
Insulin	8.88 ± 0.53	8.56 ± 0.65	10.42 ± 0.54	10.18 ± 0.51
MSA	7.28 ± 0.09	7.40 ± 0.11	8.34 ± 0.37	9.45 ± 0.25
Horse serum	8.71 ± 0.22	8.37 ± 0.56	18.45 ± 0.37	16.38 ± 0.08

* Cells were plated at ~1.2 × 10^5 cells/4 cm² well in DMEM containing 2% horse serum and 0.4% CEE in Costar 12-well multiwell plates. Cultures were incubated at 37°C for 5 h to allow attachment of the cells, and then washed briefly with serum-free DMEM. At this time (designated zero time), sample K44 cultures contained 2.50 ± 0.05 × 10^4 cells/cm², and parallel C5 cultures contained 3.69 ± 0.06 × 10^4 cells/cm². The indicated compounds were then added in DMEM to give the following final concentrations: 10^{-6} M insulin, 1.0 μg/ml MSA, 5% (vol/vol) horse serum, and 3.6 μg/ml of partially purified DI (fre of MSA). At the specified time, cells were lifted by trypsinization and counted using a Model ZBI Coulter Counter. Data are means ± SEM of triplicate cultures.

‡ Extensive myotube formation was observed in these cultures, and in no others in this experiment.

§ Significantly ($P < 0.05$) larger than the corresponding 48-h culture incubated in the absence of the DI.

Finally, the DI does not appear to act by reducing available Ca^{++} below the level required for myoblast fusion; increasing the medium $CaCl_2$ by an additional 1.4 mM did not reverse the action of the DI. Furthermore, inhibition of fusion by partially purified DI (at 3.6 μg/ml) would require that each molecule of the protein (assuming a molecular weight of 30,000) bind nearly 10,000 Ca^{++} ions to reduce calcium from the 1.8 mM level in Dulbecco's medium to the 0.3 mM level which does not allow fusion in these cells.

With the exception of the inhibitor of fusion in CEE described by Slater (3), the DI does not appear to correspond to inhibitors of fusion previously described in the literature. Its lack of hemagglutinating activity, as well as its heat and acid stability, contrasts sharply with the lectins studied by several other groups (20–24). The lectin with properties closest to those we have observed is probably Podleski's "myonectin", but the DI is quite stable to exhaustive dialysis, is relatively heat-stable, and is not inactivated as a result of the loss of a small stabilizing molecule during gel filtration; all of these differ from myonectin (24; T. R. Podleski, personal communication). There are several differences in actions and/or stability between our DI and the fusion-inhibiting lectin described by MacBride and Przybylski (23); for example, the action of DI persists for at least 96 h when it is replaced daily, while chick cells overcome the effect of the lectin after a few days of daily renewal. The secretion of DI into the soluble portion of BRL cell medium is an additional indication that it is not a typical lectin; these compounds are usually associated with cell membranes or are bound to the culture dish surfaces. There are several additional reasons to conclude that the DI is not fibronectin or a closely related protein. Its solubility at neutral pH, lack of lectin activity, action on primary myoblasts as well as L6 cells, and ready reversibility are different from reported properties or actions of fibronectin in inhibiting muscle cell differentiation.

The complete lack of mitogenic activity of the DI contrasts sharply with fibroblast growth factor (FGF), which has been shown by Linkhart et al. (13) to inhibit differentiation in mouse myoblasts. The Differentiation Inhibitor exhibits some similarities to the acid-activated inhibitor to 3T3-adipocyte differentiation recently found in FBS by Kuri-Harcuch and Green (25), but BRL-cell-conditioned medium is fully active even without any acid treatment, and the activity of non-acidified FBS in blocking myoblast differentiation has been observed in many laboratories.

The physiological significance of the DI remains to be investigated. However, there are reasons to believe that it may play an important biological role. The BRL cell line was derived from liver cells of young rats (26) and it is best known for its secretion of a protein, MSA, which is found principally in the fetal circulation (6). This is consistent with our preliminary observation that the DI corresponds to the component of FBS and CEE which suppresses fusion of myoblasts in "enriched" media (1, 5). It is possible that inactivation of the DI, rather than degradation of mitogens, accounts for the accelerated fusion of myoblasts in "exhausted" medium observed by many investigators working with muscle cells.

We are particularly interested in the possibility that the DI could regulate fetal growth and development of muscle. If, as the behavior of muscle cells in culture suggests, myoblasts are capable of differentiation whenever sufficient cell density and appropriate cell environment coincide, then it is possible that the DI suppresses precocious fusion of myoblasts to form postmitotic myotubes until late in development, when adequate accumulation of myoblasts has occurred. (This view assumes that the levels or actions of DI decrease late in development.) In addition, localized high concentrations of DI could be responsible for the inclusion of undifferentiated myoblasts as satellite cells on the periphery of mature muscle fibers (27). Thus our discovery of the DI opens a number of possibilities for further investigation.

In summary, the results presented here demonstrate that BRL cells secrete a protein which exhibits potent activity in inhibiting the differentiation of myoblasts in culture. Action of the DI is not accompanied by any detectable effect on proliferation or viability of myoblasts, and differentiation proceeds normally upon removal of the inhibitor. The DI is active in inhibiting all measured aspects of myoblast differentiation (fusion, CK elevation, and α-bungarotoxin binding) in all myoblasts studied thus far; this includes muscle cells from rats, chickens, and Japanese quail, as well as Yaffe's L6 line of rat myoblasts. Thus it appears that the DI secreted by BRL cells may be an important tool for the study of muscle differentiation.

We are grateful to Linda MacArthur for the lectin assays and for technical assistance with other aspects of the work, and we thank Karen Turo, Susan Falen, and Mary Kay Killoran for maintenance of the BRL cell cultures and the initial steps in isolation of MSA and the DI from conditioned medium.

This work was supported by U. S. Public Health Service grant HL11551 and a grant from the Muscular Dystrophy Association.

Received for publication 19 October 1981, and in revised form 28 December 1981.

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