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# INSULIN ACTS AS A SOMATOMEDIN ANALOG IN STIMULATING MYOBLAST GROWTH IN SERUM-FREE MEDIUM

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## SUMMARY

A serum-free medium that supports the proliferation of myoblasts (but not of fibroblasts) has been developed recently in this laboratory. It is composed of $10^{-6}$ *M* insulin, $10^{-7}$ *M* dexamethasone, and $10^{-5}$ *M* fetuin, and is designated medium MM-1. The latter two components gave optimal stimulation at or near "physiological" concentrations, but insulin was required at levels far in excess of those found in serum. Accordingly, we have now investigated the possibility that insulin acts as a weak analog of the somatomedins, as has been suggested in other systems. We found that maximal growth rates were observed when $10^{-6}$ *M* insulin was replaced by 0.5 to 1.0 μg/ml multiplication stimulating activity (MSA), indicating that insulin serves a somatomedinlike function of MM-1. We also investigated the possibility that a contaminant of fetuin is responsible for its action in MM-1 but found no evidence to support this suggestion. We conclude that MM-1 is suitable for the study of muscle cell growth and differentiation under rather well-defined conditions, and that insulin probably is serving as a somatomedin analog in this medium.

*Key words:* myoblasts; multiplication stimulating activity; serum-free medium; somatomedin; insulin; fetuin.

## INTRODUCTION

A serum-free medium MM-1 for the growth of muscle cells in culture was described recently by Florini and Roberts (1). It consists of a relatively simple, inexpensive mixture of commercially available components, and we expect it to prove useful for studies on myoblast growth and differentiation. As reported previously (1), this simple mixture of $10^{-5}$ *M* fetuin, $10^{-6}$ *M* insulin, and $10^{-7}$ *M* dexamethasone suffices to support near-maximal rates of proliferation by rat myoblasts (primary cultures of Yaffe's L6 line) but not by fibroblasts.

The concentration dependence and glucocorticoid specificity reported initially (1) demonstrate that dexamethasone's role can be attributed to its action as a glucocorticoid. But insulin is not so clearly functioning in a "natural" manner, and results of other investigators suggest that a contaminant of fetuin may be responsible for some or all of its effects. Insulin is required in MM-1 at levels several orders of magnitude greater than those normally found in blood, and the possibility existed that it could be acting as a weak analog of the somatomedins (2). Accordingly, the current study was undertaken to investigate these possibilities. This was done by substituting Temin's MSA for insulin in MM-1, by attempting to detect the presence of a more active impurity in the insulin preparations used in our experiments, and by fractionation of (and substitution for) fetuin. The results indicate that most (if not all) of the action of insulin in MM-1 can be attributed to its somatomedinlike effects. We have been unable to find any positive indications that a contaminant of fetuin is responsible for its activity in this system, and several observations suggest that fetuin itself is the active material.

## MATERIALS AND METHODS

*Materials.* Unless specified otherwise, all tissue culture supplies were purchased from GIBCO (Grand Island, NY). Bovine insulin, dexamethasone, bovine serum albumin (RIA grade), bacitracin, and the A and B chains of bovine insulin were from Sigma Chemical Co. (St. Louis, MO), and fetuin prepared by the Deutsch (3) procedure was from GIBCO. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) were

from Collaborative Research (Waltham, MA). Temin's MSA was prepared from Buffalo rat liver cell (BRL) conditioned medium through the Sephadex G-75 column step as described by Moses et al. (4); material corresponding to their Peak II was used in the studies described here.

*Cell cultures.* In general, cell culture and medium preparation techniques are as detailed in the previous paper (1). To avoid complications resulting from variable inactivation of Spiro fetuin during the dialysis that is essential to remove $Zn^{++}$ and $Ba^{++}$ ions, fetuin prepared by the Deutsch method (3) was used in the experiments described here.

Clones of the L6 myoblast line first isolated by Yaffe (5) were used in all of these experiments. For most of the studies on cell proliferation, L6-C5 (a rapidly growing, slowly fusing clone) cultures were established and maintained essentially as described previously (1). Details of individual experimental cultures are presented in the legends for the tables and figures. Cells were counted in a Coulter Model ZB1 as detailed by Florini and Roberts (1). Unless stated otherwise, all results are presented as means ± SE of data from triplicate cultures.

## RESULTS

*Effects of MM-1 components on myoblast proliferation.* This study was initiated by reinvestigating the effects of MM-1 components on the proliferation of the L6-C5 clone of Yaffe's L6 line. As summarized in Fig. 1, the results were generally similar to those reported earlier (1) with the parent L6 line. The individual components of MM-1 had relatively little effect on the proliferation of this clone of myoblasts, although $10^{-6}$ *M* insulin did allow a 67% increase over zero time and medium-only control values. Various combinations of MM-1 components gave increases in cell number close to the maximum attained in 10% fetal bovine serum (FBS) (higher serum concentrations did not support more rapid proliferation of these cells). It should be noted that these results were obtained with fetuin prepared by the Deutsch (3) procedure; similar preparations of purified fetuin were inactive in the embryonic teratocarcinoma cells studied by Rizzino and Sato (6).

*Role of insulin in MM-1.* The previous study of MM-1 (1) had shown that at least $10^{-7}$ *M* insulin was required for maximal myoblast proliferation in MM-1. This is about 1,000 times normal circulating levels of insulin and much higher than the levels required for activity of growth factors such as EGF, so the possibility existed that a contaminant of insulin might be responsible for its action in MM-1. To investigate this question, we treated commercial insulin by gel filtration on Sephadex G-50 in 1 *M* acetic acid as described by Davoren (7); this gave a preparation that exhibited a single band on sodium dodecyl sulfate (SDS) gel electrophoresis. Our experience in the purification of MSA indicates that contaminating somatomedinlike agents would have been removed from insulin by this procedure; the G-50 elution profile did not reveal the presence of any detectable protein contaminant in the insulin used in these studies. If the activity of insulin is due to such a contaminant, it must be very similar to insulin or very tightly bound to insulin. In the same experiment we examined the possibility that a portion of the insulin molecule might be responsible for its activity in MM-1. The data (Table 1) show clearly that neither the A nor the B chain

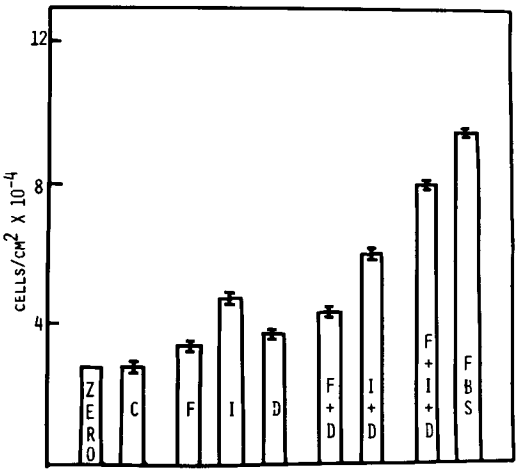


FIG. 1. Effect of MM-1 components on myoblast proliferation. The proliferation of L6-C5 myoblasts was determined essentially as described by Florini and Roberts (1) in cultures initially plated at $10^4$ cells/cm², established for 24 h by incubation in medium containing 5% horse serum, serum-starved for an additional 24 h, and then incubated in the indicated materials for 50 h before counting. The symbols denote the following components and concentrations (added to Ham's F12 medium): *C*, medium only; *F*, $10^{-5}$ *M* Deutsch fetuin; *I*, $10^{-6}$ *M* insulin; *D*, $10^{-7}$ *M* dexamethasone; and FBS, 10% (vol/vol) FBS. The *zero time bar* indicates the number of cells per culture after the 24-h serum starvation; i.e., when the specified components were added in fresh medium. *Vertical* lines depict SE for triplicate incubations.

(nor a mixture of the two) significantly stimulated myoblast proliferation when added to the other components of MM-1. Other experiments (data not shown) demonstrated that there was no difference in activity of commercial bovine, ovine, porcine, or equine insulins.

If the requirement for large quantities of insulin could not be attributed to a contaminant or partial activity of a fragment of the molecule, it was possible that the requirement for nonphysiological levels of the hormone could be attributed to its rapid degradation by cells or other components of the medium (8,9). Indeed, when MM-1 was incubated 48 h with L6 cells and then transferred to fresh serum-starved myoblasts, proliferation rates were depressed unless fresh insulin was added back to the exhausted medium (Table 2). In all cases in which fresh insulin was added, proliferation was significantly increased in response to that addition; there was no parallel effect of adding back fresh fetuin or dexamethasone, individually or together. However, it should be noted that substantial growth-promoting activity remained in MM-1 after a 48 h incubation (compare the first and last lines of Table 2). Growth rates in presumably unchanged MM-1 (incubated without cells at 4° C) were substantially greater than those in "conditioned" MM-1 with or without addition of fresh MM-1 components. Presumably this reflects depletion of nutrients from the F12 medium or the release of inhibitory materials during the initial incubation with L6 cells.

Further investigation of the stability of MM-1 components stored at 4° C confirmed the apparent stability suggested by the results presented in Table 2. After 1 wk at 4° C, MM-1 stimulated proliferation to an extent not significantly different from that observed in fresh MM-1; this stability is a substantial experimental convenience. However, there was some deterioration on further storage; after 2 wk the stimulation by MM-1 was decreased about 50%.

The data presented in Table 1 suggest that degradation of insulin may account — at least in part — for the requirement for high levels of this hormone in MM-1; the presence of an active insulin-metabolizing system in muscle is well known (10). However, an attempt at direct evaluation of this possibility was unsuccessful. When we added bacitracin at $10^{-3}$ *M* to inhibit insulin degradation (8), the shape of the insulin dose-response curve was unchanged (data not shown). It remains possible that bacitracin does not inhibit the insulin metabolizing system in muscle cells, and thus supraphysiological concentrations of the hormone are necessary to maintain it at adequate levels. However, it seems more likely to us that insulin might function as a weak analog of somatomedins, which we (11) regard as the principal endocrine stimulators of myoblast proliferation. Insulin at high concentrations may be cross-reacting with the somatomedin receptor, thus stimulating myoblast proliferation. [The recent demonstration (12) that insulin acts via the somatomedin receptor to stimulate fibroblast growth reinforces this view.]

To investigate this possibility, we measured the effect of insulin on myoblast proliferation in the presence or absence of MSA, a somatomedin analog we have used in several previous studies of this system (11,13–15). As shown in Fig. 2, at least 99% of the insulin could be replaced by MSA with no decrease in growth. Cell proliferation rates greater than those in MM-1 containing $10^{-7}$ *M* insulin were obtained when $10^{-9}$ *M* insulin and 0.5 μg/ml MSA were added to the usual

TABLE 1

EFFECT OF FRACTIONATED INSULIN ON MYOBLAST PROLIFERATION IN MM-1[a]

| Addition | Final Cell Number | |
|---|---|---|
| | $10^{-4}$ × Cells/cm² | Percent Control Value |
| None | 2.67 ± 0.29 | 65 |
| Dexamethasone and fetuin (control) | 4.08 ± 0.10 | (100) |
| Dexamethasone, fetuin, and insulin | 5.34 ± 0.08 | 131 |
| Dexamethasone, fetuin, and sephadex-treated insulin | 5.80 ± 0.36 | 142 |
| Dexamethasone, fetuin, and insulin-A chain | 4.36 ± 0.14 | 107 |
| Dexamethasone, fetuin and insulin-B chain | 4.30 ± 0.16 | 106 |
| Dexamethasone, fetuin and insulin-mixed A and B chains | 4.35 ± 0.05 | 107 |

[a] Myoblast proliferation was determined as described under Fig. 1 at 50 h after addition of the specified components: $10^{-7}$ *M* dexamethasone; $10^{-5}$ *M* fetuin; $10^{-6}$ *M* insulin. The A and B chains of insulin were added at $5 \times 10^{-7}$ *M* each.

TABLE 2

STABILITY OF MM-1 COMPONENTS INCUBATED WITH MYOBLASTS[a]

| Additions to MM-1 Incubated 48 h With Myoblasts | Final Cell Number | |
|---|---|---|
| | $10^{-4}$ × Cells/cm² | Percent Control Value |
| None (control) | 5.73 ± 0.19 | (100) |
| $10^{-7}$ *M* Dexamethasone | 5.42 ± 0.10 | 95 |
| $10^{-5}$ *M* Fetuin | 5.59 ± 0.39 | 98 |
| $10^{-6}$ *M* Insulin | 7.81 ± 0.43 | 136 |
| Dexamethasone and fetuin | 5.26 ± 0.06 | 92 |
| Dexamethasone and insulin | 7.54 ± 0.33 | 131 |
| Fetuin and insulin | 7.44 ± 0.02 | 130 |
| Fetuin, insulin and dexamethasone (complete MM-1) | 7.13 ± 0.56 | 124 |
| MM-1 Incubated 24 h at 4° C | 9.01 ± 0.50 | 157 |
| 10% FBS | 9.17 ± 0.18 | 160 |
| F12 medium only | 2.96 ± 0.15 | 52 |

[a] Yaffe's L6 myoblast cultures were prepared and incubated as described under Fig. 1. For all data except the last three lines of this Table, MM-1 was incubated 48 h with myoblast cultures as described under Methods. The medium was then removed, centrifuged, and mixed with appropriate volumes of 100-fold concentrated fresh components to give the final concentrations specified here. These mixtures were then added to serum-starved myoblasts prepared as described under Fig. 1 and the number of cells determined after 50 h.

quantities of dexamethasone and fetuin. It should be noted that insulin may not serve entirely as a somatomedin analog; there was a slight stimulation of proliferation when $10^{-9}$ *M* insulin was added to MM-1 containing MSA at a level that gives maximal effect, and the most rapid proliferation was obtained when insulin at $10^{-6}$ *M* was added *with* this concentration of MSA. In view of the well-established impairment of muscle growth in the absence of insulin in vivo (16), it seems probable that insulin plays a physiologically significant role in maintaining the metabolic functions without which the stimulation of growth by somatomedin could not occur. The relatively rapid growth observed in MM-1 in which insulin is completely replaced by MSA (the zero insulin concentration in Fig. 2) may be attributed to the insulinlike action of this somatomedin; King et al. (12) recently demonstrated the cross-reactivity of MSA with the insulin receptor of adipocytes.

*Role of fetuin in MM-1.* Although well within the "physiological" range (compared to its concentration in medium containing 10% FBS), the molar concentration of fetuin in MM-1 is even greater than that of insulin. This raises the possibility that its action may be attributable to the presence of a contaminant, as has been indicated in earlier studies of the actions of fetuin on cultured cells (7,17,18). There certainly are precedents for a highly active contaminant being found responsible for the apparent action of a hormone preparation; perhaps the most striking is that described by Nishikawa et al. (19). However, all our attempts to demonstrate that a contaminant is responsible for the actions of fetuin gave negative

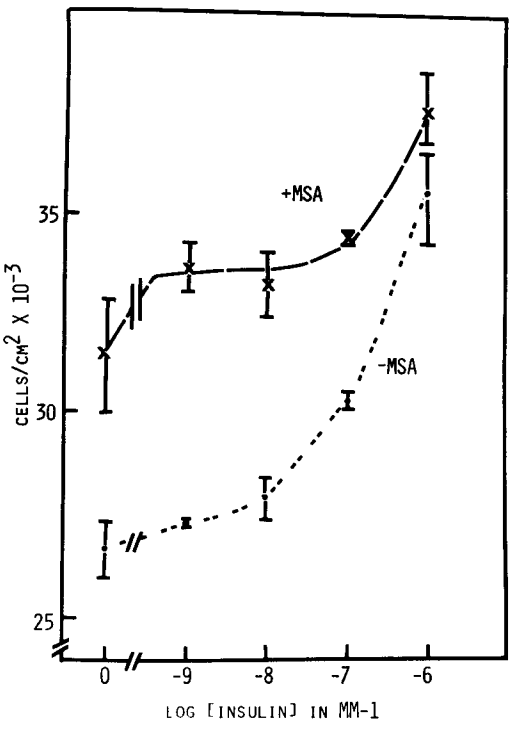


FIG. 2. Replacement of insulin by MSA in MM-1. Myoblast proliferation was measured as described under Fig. 1. In this experiment, insulin concentration was varied as specified in the presence or absence of MSA at 0.5 μg/ml as indicated in the Figure.

results. Ion exchange chromatography under the conditions used by Lieberman et al. (18) (and all other conditions we tried) gave no separation of the growth-promoting activity from the bulk fetuin protein. Similarly, attempts to inactivate fetuin by removal of its sialic acid residues with neuraminidase did not affect its growth-promoting activity. In general, these experiments with fetuin have been hampered seriously by the relative instability of the growth-promoting activity of fetuin, and by the absence of an established biological function of intact fetuin to which any observed changes in its activity in MM-1 might be compared. Overall we regard these experiments as rather inconclusive and accordingly do not present the results in detail.

We also attempted to demonstrate that fetuin might be replaced in MM-1 by some agent present as a minor contaminant in the fetuin used in the medium. However, replacement of fetuin by MSA, EGF, or FGF gave essentially negative results. This observation is consistent with the heat lability of the growth-promoting activity of fetuin (1); the growth factors are generally quite heat-stable. Reports (20,21) that fibronectin enhanced myoblast attachment and replaces fetuin in supporting the growth of mouse embryonal carcinoma cells (22) prompted us to test this material as a substitute for fetuin in MM-1. Here, too, the results were negative; although fibronectin gave the expected enhancement of myoblast attachment, it did not stimulate myoblast proliferation, whether added with or without insulin or dexamethasone. Thus we have found no evidence, either direct or indirect, that the action of fetuin in MM-1 can be attributed to a contaminant of commercial fetuin preparations.

## DISCUSSION

The results presented here, coupled with those in the initial description of MM-1 (1), provide a convenient basis for experiments on the growth of muscle cells in culture under rather closely defined conditions. It seems to us that MM-1 provides conditions usefully analogous to those in which myoblasts proliferate and differentiate in vivo. It contains a glucocorticoid at approximately physiological levels and a purified protein (fetuin) that gives maximal activity at concentrations well below those found in the fetal circulation. Even the supraphysiological levels of insulin required for myoblast proliferation and differentiation in MM-1 can be explained on the basis of the action of this hormone as a weak analog of the physiologically active but relatively unavailable somatomedins; the use of insulin in MM-1 is a pragmatic response to its relatively low cost and ready availability compared to somatomedins. It should be noted that the concentrations of MSA that fully replaced $10^{-6}$ *M* insulin in MM-1 are somewhat lower than those found by Moses et al. (23) in fetal and early post-natal rat blood.

Medium MM-1 offers the additional advantages of supporting the proliferation of rat myoblasts while allowing little fibroblast growth (1). However, it may not be universally useful without modification; in our experience (unpublished observations), chick myoblasts do not grow well in MM-1, and Linkhart et al. (24) found it necessary to add FGF to MM-1 to obtain satisfactory proliferation of mouse myoblasts. Nevertheless, it appears that MM-1 offers a number of advantages for study of the mechanisms and control of rat myoblast growth in culture.

Medium MM-1 may be equally useful for the study of myoblast differentiation. The stimulation of chick (25) and of L6 myoblast (26) fusion by high levels of insulin or by insulin and dexamethasone (27) has been reported, and we (28) found recently that L6 myoblast fusion (measured as percent nuclei in myotubes) is increased at least threefold in MM-1 (compared to serum-free controls). As in the experiments on proliferation reported here, we find that $10^{-6}$ *M* insulin can be replaced by physiological levels of MSA in stimulating myoblast differentiation.

We conclude that the MM-1:L6 system is an attractive one for the study of muscle cell growth and differentiation under precisely defined conditions.

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