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A SERUM-FREE MEDIUM THAT SUPPORTS THE GROWTH OF CULTURED SKELETAL MUSCLE SATELLITE CELLS

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SUMMARY

A serum-free medium has been devised that supports the proliferation and differentiation of primary cultures of rat skeletal muscle satellite cells for up to 4 d. The medium consists of a mixture of Dulbecco's modified Eagle's medium and MCDB-104 plus insulin, dexamethasone, pituitary fibroblast growth factor, Deutsch fetuin, and linoleic acid. In addition to promoting the formation of myotubes from satellite cells, a decrease in fibroblast contamination of these cultures was observed when cultures grown in serum-free medium were compared to cultures grown in serum-containing medium.

Key words: satellite cell; myogenesis; serum-free; fibroblast growth factor; insulin; fetuin.

INTRODUCTION

Eukaryotic cell culture techniques have proved to be powerful tools in the study of cell growth and differentiation, and the development of serum-free, defined media has enhanced our ability to precisely address questions of cell regulation (2). Unfortunately, serum-free medium conditions have not been developed for all cell types; this is especially true for cells in primary cultures.

In the course of our studies of skeletal muscle satellite cell proliferation and differentiation, the need for a serum-free medium has arisen. Serum-free media for L6 cells (7) and chick embryo breast muscle cells (5) have been described previously, but neither of these media adequately supports the growth and differentiation of myogenic cells from postnatal rat muscle. Starting with these two defined medium formulations, we have developed a serum-free medium that will support the proliferation and differentiation of rat skeletal muscle satellite cells.

MATERIALS AND METHODS

Materials. Medium, serum, penicillin, streptomycin, fungizone, and Deutsch fetuin were purchased from GIBCO (Grand Island, NY). Fibroblast growth factor (FGF), bovine serum albumin-linoleic acid, epidermal growth factor (EGF), platelet derived growth factor (PDGF), prostaglandin E_1, thyroid hormone, insulin, selenium, transferrin, and fibronectin were purchased from Collaborative Research, Inc. (Waltham, MA). Dexamethasone, biotin, β-hydroxybutyrate α_2-macroglobulin, and pronase were purchased from Sigma Chemical Co. (St. Louis, MO). Gentamicin was purchased from Schering Corp. (Union, NJ). Male Sprague-Dawley rats were obtained from the Division of Animal Resources, University of Arizona.

Satellite cell cultures. Primary cultures of rat skeletal muscle satellite cells were established according to Allen et al. (1) from 200 to 400-g Sprague-Dawley rats. Cells were ultimately seeded into 16-mm culture wells that had been previously coated with human fibronectin. Culture wells were coated with human fibronectin in Dulbecco's modified Eagle's medium for approximately 2 h, as suggested in the instructions from Collaborative Research (the supplier). Because final cell suspensions contain such large amounts of myofibrillar debris and fiber fragments, direct determination of cell concentration is extremely difficult. Greater repeatability has been achieved by plating cells on the basis of initial tissue weight; for example, a volume of cell suspension equivalent to 0.5 to 1.0 g of starting material is used in each 16-mm well. All cultures were maintained at 37° C in a humidified atmosphere of 95% air:5% carbon dioxide. From 0 to 48 h in culture, cells received 1.5 ml/well of DMEM containing 10% horse serum (HS), 100 U penicillin and streptomycin/ml, 3 μg fungizone/ml, and 20 μg gentamicin/ml. At the end of the initial 48-h period, cultures were rinsed three times with 2 ml of DMEM and fed 1.5 ml of the serum-free treatment medium/well. Medium was replaced at 24-h intervals.

After 4 d in treatment medium, cultures were fixed and stained with Giemsa. Ten random fields from each of three culture wells per treatment were counted to determine the total number of nuclei and the number of myotube nuclei per square millimeter.

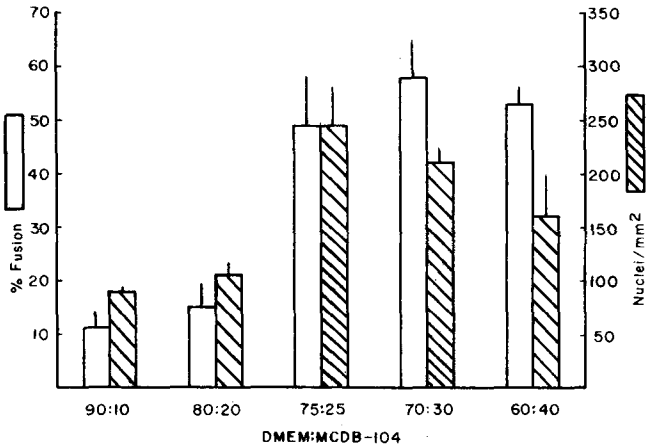

FIG. 1. Effect of mixtures of DMEM and MCDB-104 on fusion percentage and nuclei density. Cultures of satellite cells were maintained in serum-free medium from 48 to 144 h. Linoleic acid (1 μg/ml), Deutsch fetuin (0.5 mg/ml), dexamethasone (10^{-7} *M*), FGF (100 ng/ml), and insulin (10^{-6} *M*), were included. *Bars* represent means and standard errors for six wells/treatment.

RESULTS AND DISCUSSION

Based on previously reported defined medium formulations (5,7) and some preliminary experimentation, we initiated our studies using DMEM plus insulin (10^{-6} *M*), Deutsch fetuin (0.5 mg/ml), BSA-linoleic acid (1 ug linoleic acid/ml), human transferrin (5 ug/ml), dexamethasone (10^{-7} *M*), pituitary FGF (100 ng/ml), and selenium (3×10^{-8} *M*). Unlike the previous experiments, however, we used DMEM instead of F12 or MCDB-201; neither of these two basal media supported satellite cell growth, even though they contain a wider variety of individual constituents than DMEM. Furthermore, attempts to find an optimum combination of DMEM or one of these medium formulations or both resulted in the finding that mixtures of DMEM and MCDB-104, together, were able to promote greater satellite cell proliferation and more extensive fusion than either medium alone. As indicated in Fig. 1, different mixtures of DMEM and MCDB-104 varied in their ability to promote satellite cell proliferation and differentiation. Although cell number was not as great as in the DMEM-10% HS cultures, the percentage fusion in several treatments greatly exceeded the fusion found in cultures exposed to serum-containing medium. This was a consistent observation even when cultures in serum-containing medium were extremely dense and the persistence of unfused myogenic cells would not be favored. These results are consistent with the depression of fibroblast proliferation in serum-free medium as reported by Florini and Roberts (7). It is also possible that the serum-free medium is being conditioned more rapidly, thus leading to earlier onset of differentiation and fusion. In subsequent experiments we employed a 75:25 mixture of DMEM and MCDB-104, respectively.

Although the medium conditions employed in our preliminary experiments seemed adequate, the selection of additional media components was somewhat arbitrary and based on the work previously reported by other investigatiors. In an effort to determine the necessity of these supplemental media components we systematically deleted one component at a time from our tentative serum-free medium; results from these experiments (Fig. 2) revealed a critical requirement for linoleic acid, FGF, fetuin, and dexamethasone. As expected, based on previous reports (5–7), insulin was also required but its absence was not as devastating as the absence of the other constituents. Interestingly, removal of human transferrin did not have a detrimental effect, and in other experiments (data not presented) removal of transferrin actually resulted in an increase in satellite cell number and fusion percentage. In general, our results did not show a marked stimulation in growth upon the addition of transferrin as has been reported in avian muscle culture systems (8,9). In agreement with Florini and Roberts (7), we concluded that transferrin is not essential for our myogenic cell system, although human transferrin was a constituent of medium described by Dollenmeier et al. (5) and was shown to be a requirement for several cell lines by Bottenstein et al. (3). It should be noted, however, that $FeSO_4$ is included in the MCDB-104 medium, and this may alleviate the need for transferrin. It is also possible that transferrin could be a trace contaminant in the FGF or fetuin preparations; therefore, additional transferrin may not be required. Transferrin, however, was not able to alleviate the requirements for either FGF or fetuin (Fig. 2).

Our results are also consistent with the proposed requirement for fetuin and dexamethasone reported by Florini and Roberts (7). With regard to the fetuin requirement it should be emphasized that our culture dishes were coated with fibronectin, and fetuin was still required. Fetuin, therefore, is probably not functioning exclusively as a source of attachment factors. Salomon et al. (11) reported that the fetuin requirement of embryonal carcinoma cells and rat mammary epithelial cells in serum-free medium could be attributable to contamination of fetuin with a protein similar to α_2-macroglobulin and that the addition of α_2-macroglobulin could replace the requirement for fetuin. However, they used fetuin prepared by

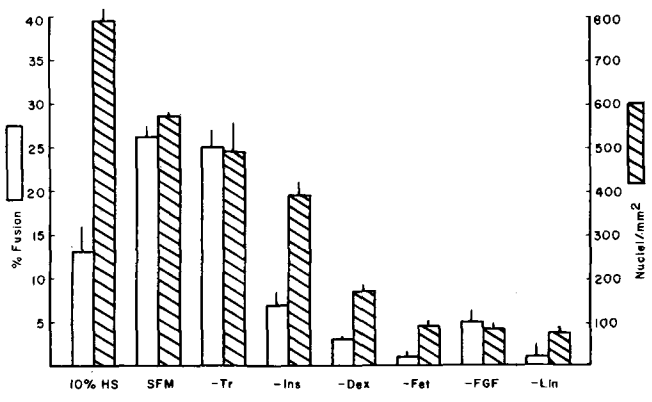

FIG. 2. Influence of deletion of individual serum-free medium (*SFM*) components on fusion percentage and nuclei density. The basal medium was a 75:25 mixture of DMEM and MCDB-104. The constituents deleted were transferrin (*Tr*), insulin (*Ins*), dexamethasone (*Dex*), Deutsch Fetuin (*Fet*), *FGF*, and linoleic acid (*Lin*). *Bars* represent means and standard errors for three wells/treatment.

the Pederson method (10), which is a relatively crude preparation. Further purification of this fetuin led to a loss of activity and the subsequent discovery of α_2-macroglobulin-like protein in the Pederson preparation. The work reported by Florini's laboratory (6,7) indicated that three different preparations of fetuin (4,10,13) supported the growth of L6 cells, and two of these preparations were of higher purity than the Pederson preparation. In addition, subsequent attempts to purify an active component from Spiro fetuin preparations (13) were unsuccessful. In their experiments the active component was heat labile, unlike many growth factors, and could not be replaced by epidermal growth factor, fibroblast growth factor, multiplication stimulating activity, or fibronectin. Note also that the concentration of fetuin used in their experiments is comparable to concentrations found in fetal bovine serum. In our experiments, the addition of α_2-macroglobulin to defined medium did not enhance cell proliferation or differentiation nor were we able to replace fetuin with α_2-macroglobulin. We cannot state with certainty, however, that contaminants in fetuin preparations are not responsible for the presumed action of fetuin in our system; clearly, a 0.1 to 0.001% contaminant would still be present in a nanogram per milliliter concentration range, an active range for some growth factors. Furthermore, it would be extremely difficult to detect this level of contamination in a protein preparation. We can only conclude that protein(s) present in the Deutsch fetuin preparations is required for satellite cell growth in the absence of serum.

In contrast to the Florini and Roberts medium formulation (7), our primary cultures required FGF and linoleic acid, findings more consistent with the results of Dollenmeier et al. (5). In addition to the constituents discussed above, we have also evaluated the influence of prostaglandin E_1, thyroid hormone, EGF, and PDGF. None of these components increased the growth and differentiation of satellite cells in culture (data not shown).

To further optimize our serum-free medium formulation, dose-response curves were constructed for linoleic acid, FGF, insulin, dexamethasone, and fetuin. All other supplements were maintained at the concentrations employed in previous experiments. Figure 3 *A-E* describe changes in fusion percentage and myotube nuclei number in response to variable concentrations of each component. Based on the dose-response data, the original formulation was not far from optimal for most constituents. The FGF was the possible exception because myotube nuclei continued to increase well beyond 100 ng/ml. This observation is comparable to the FGF effect observed in the chick myoblast-defined medium system (5) in that [^{3}H]thymidine incorporation had not reached a plateau at 300-ng level in their experiments either. Because one of the important applications of our defined medium is in the study of satellite cell proliferation, we decided to retain the 100 ng FGF/ml concentration so as not to maintain cells near their maximum proliferative rate. At 100 ng/ml, cell viability and a moderate rate of growth can be maintained, and increasing FGF concentrations increases the risk of adding significant levels of potential contaminants and increases the cost of medium, if commercial preparations are used. The FGF concentrations are relatively high compared to concentrations at which many polypeptide hormones and growth factors are active; this may be due to the purity of the FGF preparations or to the rapid degradation of FGF by these cultured cells. We found no compelling reason to alter fetuin concentrations, because the plateau seemed to be between 250 and 500 μg/ml.

With respect to insulin, dexamethasone, and linoleic acid, however, maximum myotube nuclei numbers were obtained at concentrations lower than those employed in our previous experiments. Consequently, each of these constituents was added to treatment media at one of two levels, the original concentration or the concentration derived from the dose-response curve that gave the maximal response. Furthermore, all eight possible combinations of these components were evaluated to guard against the possibility that alterations in the concentration of one constituent would change the response to a given concentration of another constituent. Results of this experiment are illustrated in Fig. 4, with nuclei density and fusion percentage displayed for each treatment. Treatment *A*

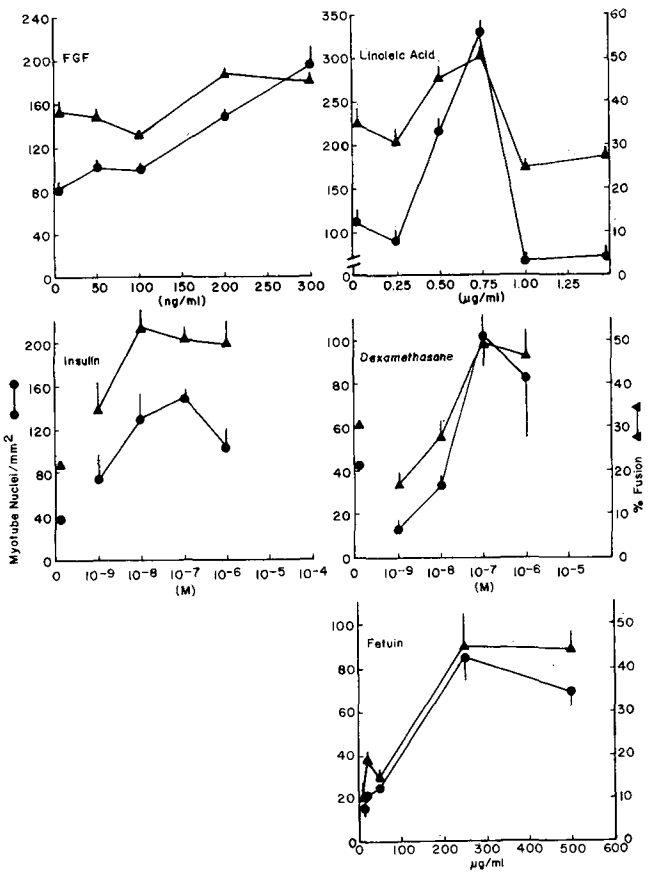

FIG. 3. Dose-response curves for FGF, linoleic acid, insulin, dexamethasone, and fetuin in serum-free medium. In each case serum-free medium components were maintained at previously described concentrations. Fusion percentage (▲_____▲) and myotube nuclei density (●_____●) are presented for each concentration employed in the experiments. *Points* represent means and standard errors for three wells/treatment.

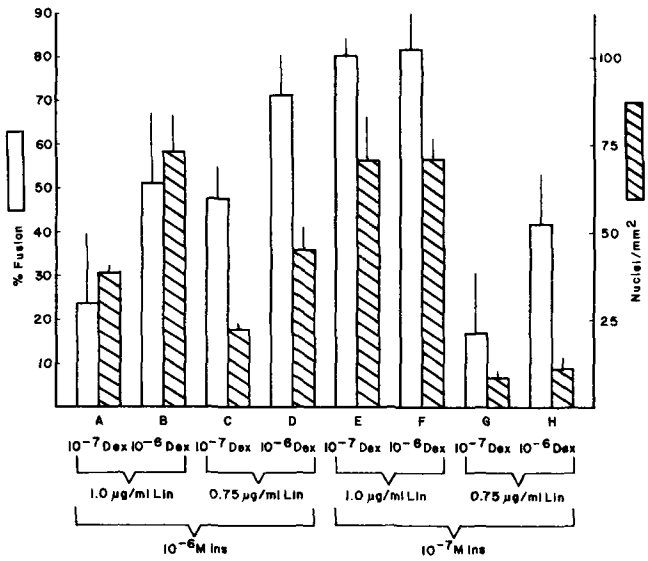

FIG. 4. Optimization of linoleic acid, dexamethasone, and insulin concentrations in serum-free medium based on the dose-response data for each component. Two concentrations of each component were employed, the concentration used in the original experiments and the concentrations giving a maximal response in the dose-response experiments. All possible combinations were examined, and the fusion percentage and nuclei density are displayed. *Bars* represent means and standard errors for three wells/treatment.

represents the original concentrations of linoleic acid, insulin, and dexamethasone. The three new concentrations are represented by treatment *H*; this combination produced a lower myotube nuclei density and fusion percentage than the original formulation. Other formulations, however, produced results that were far superior to the original (*A*) or treatment *H*; treatments *E* and *F*, in particular, have been shown to produce consistently superior results. Therefore, it seems that lowering insulin concentration to 10^{-7} *M* may be justified, but linoleic acid concentrations should be maintained at 1 μg/ml. The choice of dexamethasone concentration used in conjunction with 10^{-7}*M* insulin and 1 μg linoleic acid/ml may not be critical. Optimization of serum-free medium conditions resulted in an increase in myotube nuclei density and an increase in fusion percentage when compared with the original formulation and the DMEM-10% HS control cultures.

In addition to maintaining the proliferation and differentiation potential of satellite cells, the morphology of cells in these serum-free cultures seems to be similar to the morphology of satellite cells in serum-containing medium (Fig, 5). Myotubes are present by 3rd and 4th d in serum-free medium and the concentration of mononucleated cells, presumably fibroblasts or unfused myogenic cells, is less than in cultures receiving serum.

It is important to note that beyond Day 4 in serum-free medium cell viability decreases. Moreover, the usefulness of this medium may be compromised in some experiments because satellite cells must be initially plated in serum-containing medium, and best results are obtained when cultures are allowed to remain in serum-containing medium for the first 48 h. The reason for the initial serum requirement is not completely clear; serum may well be functioning to inactivate any proteinase remaining from the cell dissociation procedure or that might have leached out of the numerous muscle fiber fragments that are present during the first 24 h after plating. Alternatively, serum may be depositing essential components on the surface of the plastic (12). We have observed, however,

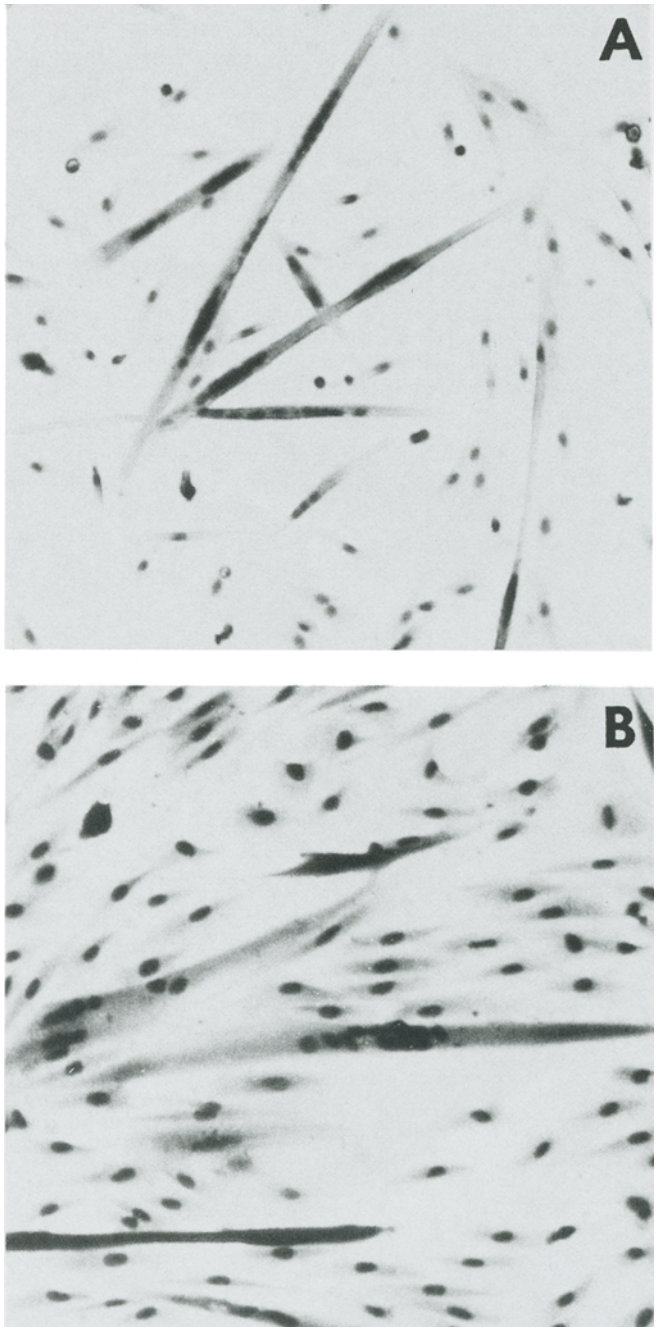

FIG. 5. Cultured myogenic cells in serum-free and serum-containing media: serum-free medium components were present in concentrations described in Fig. 4 treatment *F*. Satellite cells in serum-free medium (*A*); satellite cells in DMEM-10% HS (*B*). ×164.

that neonatal rat myogenic cells do not require as much time in serum-containing medium as satellite cells for acceptable growth. In all probability, we are still missing one or more critical components necessary to alleviate the serum requirement during the first 48 h and extend the period of cell growth in the absence of serum-containing medium. In spite of these limitations, 4 d in the presence of serum-free medium is adequate for many studies of proliferation and differentiation of satellite cells, and our serum-free medium formulation may serve as a basis for further development of serum-free conditions for the growth of primary cultures of mammalian myogenic cells.

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