Improved Serum-free Defined Medium for Proliferation and Differentiation of Chick Primary Myogenic Cells

Authors: Shiozuka, Masataka, and Kimura, Ichiro

Source: Zoological Science, 17(2) : 201-207

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.201

ZOOLOGICAL SCIENCE **17**: 201–207 (2000)

Improved Serum-free Defined Medium for Proliferation and Differentiation of Chick Primary Myogenic Cells

Masataka Shiozuka and Ichiro Kimura*

Department of Basic Human Sciences, School of Human Sciences, Waseda University, Saitama 359-1192, Japan

ABSTRACT—The development of serum-free and chemically defined media remains an important mission in the in vitro research of cells. The present study describes an improved serum-free medium that supports the proliferation and differentiation of chick embryonic primary skeletal myogenic cells, a cell type which has long been utilized for the study of cell differentiation.

We show that serum can be replaced with transferrin, insulin, serum albumin, and fibroblast growth factor-2 as supplements to Dulbecco's modified Eagle minimum essential medium, with no loss in the ability of the medium to support proliferation and differentiation of myogenic cells. This medium has several additional advantages over serum-supplemented medium in that it also suppresses the proliferation of contaminating fibroblasts, and may allow the sensitive evaluation of the effects of various humoral factors on myogenic cells. We believe this medium will prove useful to the primary culture of chick myogenic cells.

INTRODUCTION

In 1963, Konigsberg achieved a breakthrough in the study of cell differentiation by successfully culturing chick primary myoblasts and demonstrating induction of myotube differentiation (Konigsberg, 1963). Since then, myogenic cell culture systems of primary cells (embryonic myoblasts and satellite cells) and established cell lines have been among the most useful in vitro models for studying cell differentiation. Among the many kinds of myogenic cells in use, avian primary skeletal muscle cells have been shown to be particularly useful as an experimental system.

Typically, studies of in vitro avian myogenesis have been carried out in complex media containing undefined components, such as serum and chick embryo extract (EE) (Konigsberg, 1963). These components, which include hormones, growth factors, and other proteins, are critical for cell survival, proliferation, and differentiation in culture (Barnes and Sato, 1980). However, the development of a completely defined culture medium for avian primary myogenic cells with a potency comparable to serum-containing media is a long-standing goal.

Advances in our understanding of the specific in vitro culture requirements of different types of myogenic cells have led to the development of various serum-free media. These media have been shown to allow the myogenic differentiation of various primary and established cell types. However, the completely defined media developed for culturing chick primary cells have been found to be less than optimal in their potency and composition. For example, in the course of our studies on the regulation of proliferation and differentiation by humoral factors, we tried to culture chick embryonic myogenic cells in the medium reported by Dollenmeier *et al*. (1981), but were not able to obtain satisfactory results. Another medium reported by Pietrzkowski *et al*. (1988) contains an unnatural substance, Dextran T-500, and myotube formation seems to be rather unsatisfactory. The medium reported by Lyles *et al*. (1992) includes Matrigel, which may contain undefined components. Recently, Link and Nishi (1997) have reported a medium which was of a very simple composition and appeared to be quite potent. However, their medium has not been fully characterized with respect to its properties, such as relative potency compared to serum-supplemented media. In addition, many of the serum-free media for myogenic cells have been formulated hitherto as differentiation media.

Thus, it is clear that there is room for further improvement in the composition of serum-free media for primary chick myogenic cells. The declining cost and increasing purity of bioactive substances such as growth factors also provide opportunities to develop new formulations. The aim of our present study was to develop an improved serum-free medium for proliferation and differentiation of chick primary myogenic cells, based on previously reported formulations and on our own experience in culturing these cells. Our objectives were to formulate a medium that is simple and relatively inexpensive to prepare, and that is comparable to a serum-containing medium in its ability to support cell proliferation and differentiation. Here, we describe the formulation and activity

* Corresponding author: Tel. +81-42-947-6728;
FAX. +81-42-947-6728.
E-mail: kimura1@human.waseda.ac.jp

of an improved serum-free medium.

MATERIALS AND METHODS

Reagents

Human recombinant fibroblast growth factor (FGF)-2, chick transferrin (conalbumin, iron-complex type II from egg white) and bovine pancreas insulin were obtained from Sigma (St. Louis, U.S.A.). Eagle's minimum essential medium (MEM), Dulbecco's modified minimum essential medium (DMEM), Hanks medium, and PBS were from Nissui (Tokyo, Japan). Horse serum was purchased from Kojinbio (Tokyo, Japan), and chick serum (CS) from Gibco (New York, U.S.A.). Bovine serum albumin (fraction V: BSA) was obtained from Sanko (Tokyo, Japan). Human activin A was a generous gift from Dr.Etoh (Ajinomoto, Kawasaki, Japan) and monoclonal antibody MF20 (Bader *et al*., 1982) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). EE was prepared according to the procedure of Konigsberg (1979).

Cell culture

Primary myogenic cells were obtained from the breast muscle of 11-day chick embryos and cultured according to the procedure of Kimura *et al*.(1982). Cells were plated onto gelatin-coated 35-mm dishes (Falcon 3001, U.S.A.) at an initial density of 3×10^5 in 1.5 ml of culture medium and cultured at 37°C in a humidified 5% CO_2 atmosphere. Standard serum-containing basal culture medium (BCM) contained 85% MEM and 15% horse serum. Media consisting of 95%BCM+5%EE (BCM/EE), 98%BCM+2%CS (BCM/CS), or BCM+30 μg/ml Tf (BCM/Tf) were also employed. If not specified otherwise, cells were precultured in BCM/EE for 24 hr, then dishes were washed twice with Hank's medium, the media replaced with the test media, and then the cells cultured for a further 3 days. After the initial replacement of BCM/EE media with test media there was no further media replenishment. In some experiments, cells were grown without medium change from the beginning to the end of cultivation for 4 days. Finally, the cells were photographed with a Diaphot-TMD phase contrast microscope (Nikon; Tokyo, Japan) and harvested for creatine kinase (CK) assay. In some cases, the cells were immunochemically analyzed.

Immunocytochemistry

After a 4 day-culture, the cells were fixed in 10% formalin for 10 min at room temperature and then washed in PBS prior to antibody staining. Fixed cells were treated for 30 min with methanol at –20°C, blocked for 30 m with PBS supplemented with 10% horse serum at room temperature, washed extensively with PBS and then stained with the monoclonal antibody MF20 against sarcomeric muscle myosin and detected using a Texas Red-coupled secondary antibody.

Creatine kinase assay

Creatine kinase (CK) activity, which accumulates in the myotubes and is used as a biochemical marker of myogenic differentiation, was analyzed as described by Shiozuka *et al*. (1997) with the aid of a diagnostic kit (Iatrofine CK rate: IATRON;Tokyo, Japan) and autoanalyzer (Shimadzu CL-7100; Kyoto, Japan). CK activity (mU/dish) was expressed as the average of the results from three dishes.

RESULTS

Formulation of serum-free media for chick primary myogenic cells

Based on previous studies on serum-free media for myogenic cells and our own experiences, we began the present study using a medium composed of DMEM plus transferrin and insulin.

We chose DMEM as the basal synthetic medium after having tested DMEM, Ham's F12, Medium199, NCTC135, RPMI1640, Opti-MEM, MEM, BME media and their mixtures, all of which have often been used to formulate serum-free media for animal cells. We found that DMEM exceeded these others in its ability to support myogenic differentiation (data not shown). Among these others, Medium 199 was also found to be nearly as satisfactory as DMEM. Transferrin and insulin were chosen because many previous studies have shown that they are generally indispensable in serum-free media for a variety of animal cell types, including myogenic cells. Therefore, these substances were included in our prospective serum-free medium from the beginning of the study. The concentrations of insulin (10 μg/ml) and transferrin (30 μg/ml) were determined with reference to the formulations reported to date and to our previous studies (Ii *et al*., 1982).

Figs. 1-a, b, c show the results of cultures maintained in DMEM alone (D), DMEM+transferrin (DT), and DMEM +transferrin+insulin (DTI), respectively. Myoblasts did not proliferate or differentiate at all in DMEM alone. Although the addition of transferrin to DMEM promoted cell proliferation to a minor extent, no distinct myotubes were formed. When cultivated in DTI medium, myotubes were formed, although not well-developed, confirming that insulin is highly supporting for proliferation and differentiation of myogenic cells.

Next, BSA was chosen as the first candidate ingredient to be added to the DTI medium because BSA is believed to help stabilize bioactive substances such as growth factors and to allow their steady supply to the cells. Additionally, BSA is present in many products from commercial sources, such as growth factors. Therefore, adding BSA to the culture media would abrogate any effects of the BSA present in these products that could confuse the results. BSA was found to be highly effective in supporting myogenesis (Fig. 1-d): myotube formation was more marked in DTI+BSA medium (DTIB) than in DTI medium. The effect of BSA was dose-dependent and reached a plateau at about 1 mg/ml (data not presented).

Next we tested the myogenesis-supporting activity of FGF-2, a growth factor that is known to be a highly potent mitogen for myoblasts (Florini and Margi, 1989; Kimura *et al*., 1989). FGF-2 has been shown to delay the withdrawal of myoblasts from the mitotic cycle, and when exhausted, myoblasts undergo result profound myogenesis. As expected, we found that FGF-2 (10 ng/ml) markedly improved the myogenesis-supporting activity of DTIB medium (Fig. 1-e). This result was also verified immunocytochemically (Fig. 1-f) and biochemically (Fig. 1-g). The effect of FGF-2 is dose-dependent (data not shown, see Kimura *et al*., 1987), and we chose the concentration of 10 ng/ml in the present study.

We compared DTIBF to serum-supplemented media over 4 days with cells that had not been precultured in BCM/EE. Fig. 4 shows that DTIBF (CK activity: 192 mU/dish) is less potent than BCM/CS, but superior to BCM/Tf. This means that DTIBF may be used for cultures without any need for prior growth in serum.

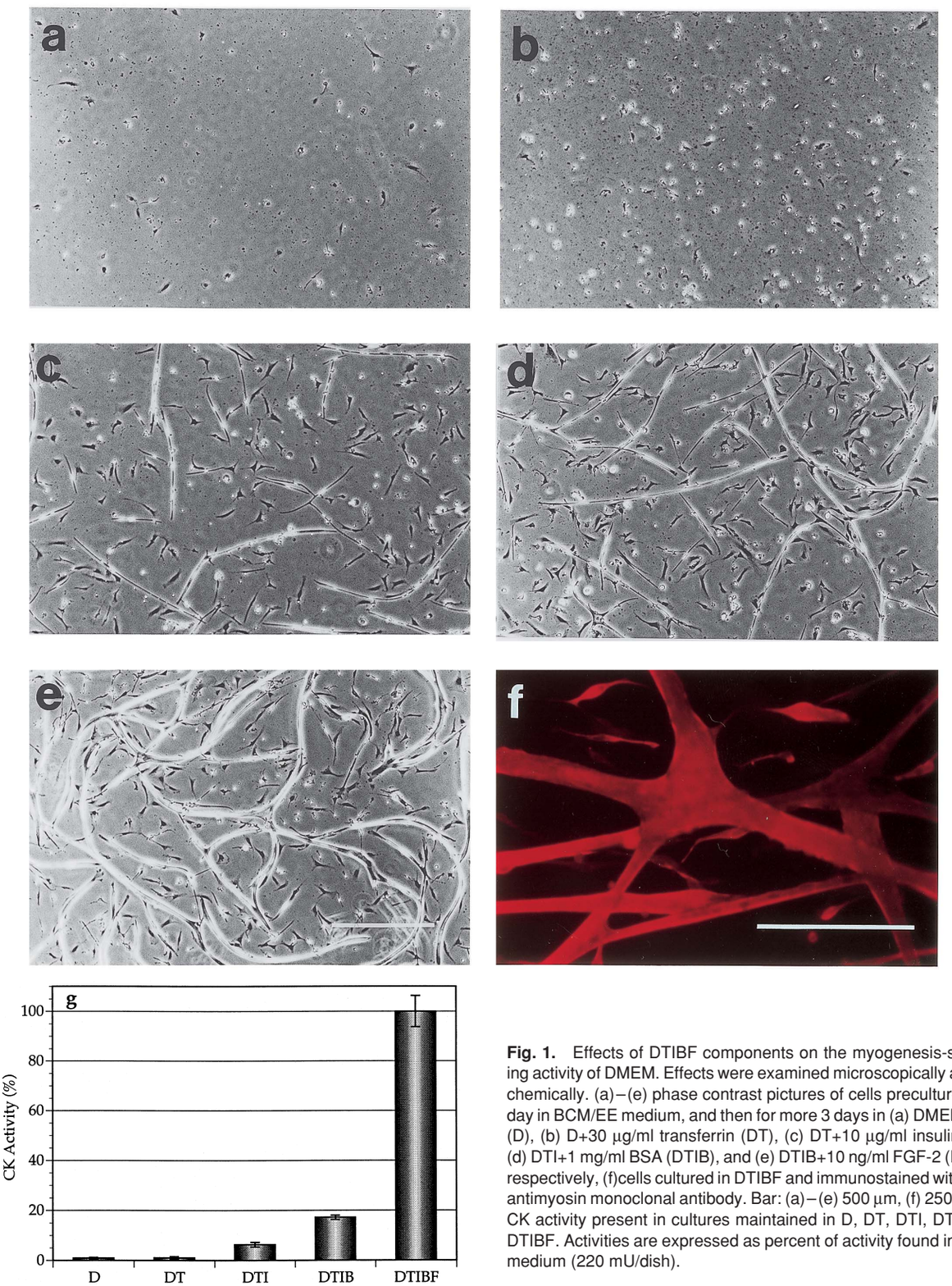

Fig. 1. Effects of DTIBF components on the myogenesis-supporting activity of DMEM. Effects were examined microscopically and biochemically. (a)–(e) phase contrast pictures of cells precultured for 1 day in BCM/EE medium, and then for more 3 days in (a) DMEM alone (D), (b) D+30 μg/ml transferrin (DT), (c) DT+10 μg/ml insulin (DTI), (d) DTI+1 mg/ml BSA (DTIB), and (e) DTIB+10 ng/ml FGF-2 (DTIBF), respectively, (f)cells cultured in DTIBF and immunostained with MF20 antimyosin monoclonal antibody. Bar: (a)–(e) 500 μm, (f) 250 μm. (g) CK activity present in cultures maintained in D, DT, DTI, DTIB, and DTIBF. Activities are expressed as percent of activity found in DTIBF medium (220 mU/dish).

Effect of removing individual ingredients from DTIBF

To confirm the requirements for each supplemental component, we examined the effect of removing individual components from DTIBF medium. As shown in Fig. 2, removal of any one of the four ingredients from DTIBF markedly reduced its activity. The CK activities for DIBF (DMEM+insulin+BSA

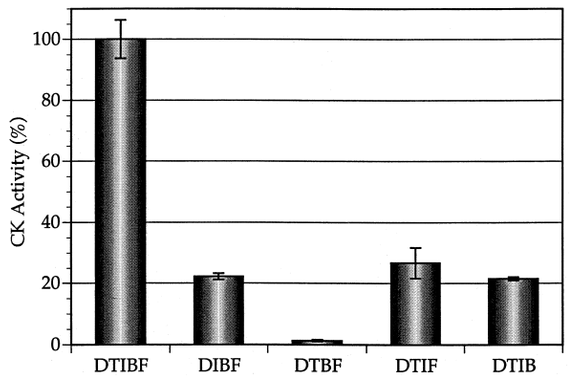

Fig. 2. Effects of removal of each ingredient from DTIBF. CK activities were determined from cultures maintained in DTBF (DTIBF minus transferrin), DTBF (minus insulin), DTIF (minus BSA), and DTIB (minus FGF-2), respectively. CK activities were expressed as percent of activity found in DTIBF medium.

+FGF-2, and so forth.), DTBF, DTIF and DTIB were 22%, 1%, 27%, and 22% of that of DTIBF, respectively. This result confirmed that each ingredient, viz. transferrin, insulin, BSA, and FGF-2, are indispensable.

Comparison of the myogenesis-supporting activities of DTIBF and serum-containing media

DTIBF medium was compared with serum-containing media with regard to its ability to support myogenesis (Fig. 3). DTIBF medium was shown to have less myogenesis-supporting activity than BCM/EE, but more than BCM/Tf and BCM/CS, which have generally been used for the culture of avian myogenic cells. Fig. 3-e shows the CK activities of cells cultured in DTIBF, BCM/Tf and BCM/CS. The result demonstrates that DTIBF has a potency higher than BCM/Tf, and is thus superior as a culture medium. In addition, DTIBF medium

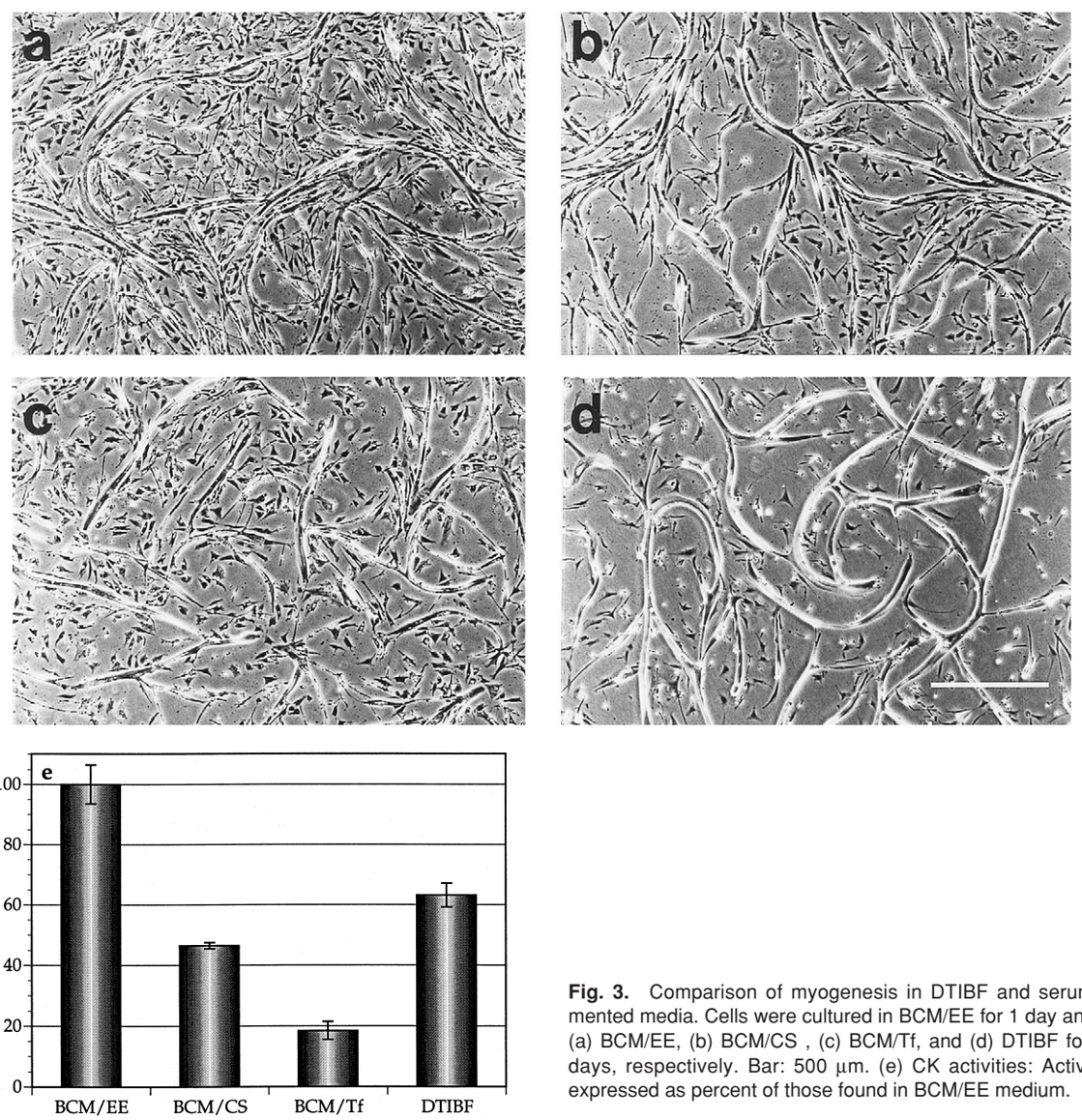

Fig. 3. Comparison of myogenesis in DTIBF and serum-supplemented media. Cells were cultured in BCM/EE for 1 day and then in (a) BCM/EE, (b) BCM/CS , (c) BCM/Tf, and (d) DTIBF for more 3 days, respectively. Bar: 500 μm. (e) CK activities: Activities are expressed as percent of those found in BCM/EE medium.

seemed to markedly suppress the proliferation of contaminating fibroblasts compared to serum-containing media (Fig. 3).

The effect of activin A on myogenic differentiation in DTIBF

In order to examine the utility of DTIBF medium in investigational studies, we examined the ability of activin A to inhibit the myogenesis of avian myogenic cells cultured in DTIBF versus serum-containing medium (Shiozuka *et al*., 1997; Link and Nishi 1997; Shiozuka and Kimura, 1999). As shown in Fig. 5, the dose-dependent inhibition of myogenic differentiation by activin A was more profound in DTIBF than in BCM/Tf: CK accumulation at 10 and 100 ng/ml activin A was 52 and 29% of the controls in BCM/Tf and 21 and 12% in DTIBF, respectively.

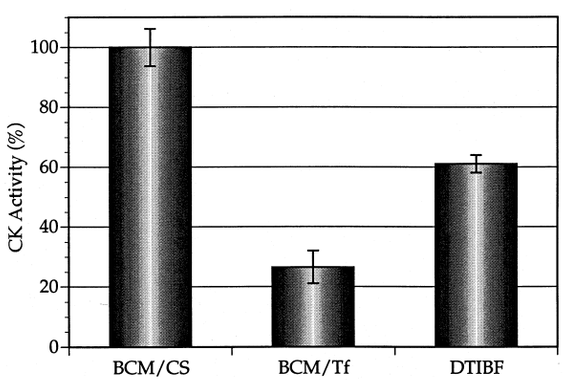

Fig. 4. Comparison of CK activities in cells cultured in DTIBF and serum-supplemented media (BCM/CS and BCM/Tf) without medium change. Cells were cultured for 4 days. Activities are expressed as percent of those found in BCM/CS medium.

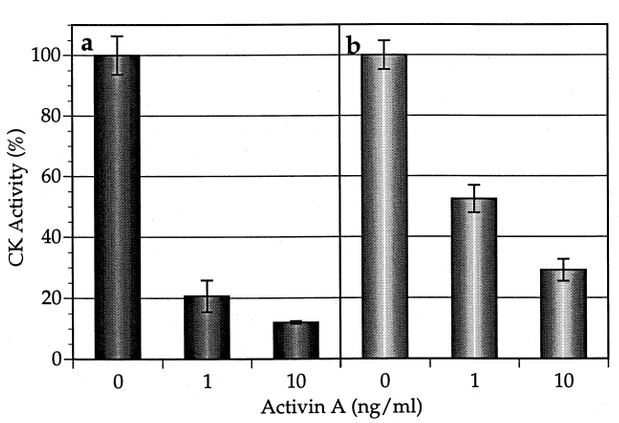

Fig. 5. Effects of activin A on myogenic differentiation in (a) DTIBF and (b) serum-containing medium (BCM/Tf). Cells were cultured for 4 days without medium change. Activin was added at the start of cultivation. Activities are expressed as a percent of the respective control values.

DISCUSSION

The aim of the present study was to develop a chemically defined, simple and economical medium system suitable for the in vitro study of chick primary myogenic cells. Although several serum-free media formulae have been reported, they each have problems in their composition and/or potency. The present medium formulation has fulfilled our criteria fairly well for a medium with a potency comparable to serum-supplemented media.

Serum (and/or embryo extract)-containing media are unquestionably beneficial for the in vitro study of myogenesis, since they allow the proliferation of myogenic cells and their differentiation into multinucleated myotubes. However, serum-supplemented systems suffer from some inevitable general disadvantages, including: (1) possible existence of unidentified components which may interact in an uncharacterized manner with other medium ingredients, test substances, and cells; (2) significant difficulties in isolating and characterizing materials secreted by cells into culture medium; (3) risk of contamination with mycoplasmas, viruses, antibodies, and cytototoxins; (4) batch-to-batch variations in sera (and embryo extract) and the resultant necessity of time-consuming tests to preselect suitable batches; (5) encouragement of the growth of co-existing unwanted cells.

Cell proliferation and differentiation in vitro are critically influenced by humoral factors, such as growth factors, hormones, and nutrients (Barnes and Sato, 1980). The selection of medium ingredients in the present study was based on both previous studies with chemically defined media for various types of animal cells, and our own experiences. We endeavored to avoid impure or unphysiological ingredients, such as Matrigel (Lyles *et al*., 1992), Dextran (Pietrzkowski *et al*., 1988), veratridine (Lyles *et al*., 1993), etc. which have been employed for some serum-free culture media for avian primary myogenic cells. On the same account, we also did not employ constituents such as fetuin (Allen *et al*., 1985), protamine (Nie and Ham, 1991), etc., which were used as constituents of serum-free media for mammalian myogenic cells.

We initially selected DMEM as the basal synthetic medium, as this medium has very often been successfully utilized for serum-free media for animal cells. It is reasonable that DMEM would be particularly suited as a basal synthetic medium, as it is composed of a higher concentration of amino acids, vitamins, etc., compared with other synthetic medium such as MEM. Furthermore, it is noteworthy that DMEM contains ferric iron, which can function as a ligand for transferrin. It has been established that transferrin (or iron) is indispensable for the proliferation and differentiation of animal cells in general (Barnes and Sato, 1980; Ozawa, 1989). Transferrin binds with iron to the cell surface transferrin receptor, is endocytosed into the cells where it donates the iron, and is then recycled intact back into the culture medium as inert iron-free apotype forms (Kimura *et al*., 1985). If iron is not supplied to the medium, all the transferrin molecules in the culture medium eventually become iron-free apotypes and thus non-

bioactive. If ferric iron is present in the culture medium, apotransferrin is converted back to the reusable bioactive holotransferrin (Kimura *et al*., 1985). Therefore, iron-containing media is superior as a synthetic media to iron-free media, such as the serum-free medium reported by Link and Nishi (1997) which utilizes L15 as its base.

Another consideration with using transferrin is species- or class-specificity. Dollenmeier *et al*. (1981) and Vallette *et al*. (1986) have reported the use of human transferrin as an ingredient in their serum-free media for chick cells, but Dollenmeier *et al*. observed that human transferrin exerted no marked promoting activity on growth. This could be due to the specificity of transferrin activity (Ozawa, 1989). It has been recognized that the bioactivity of transferrin is class-specific, i.e. mammalian transferrins have no bioactivity on avian cells and vice versa. Thus, although mammalian serum provides a high concentration of transferrin in synthetic media such as BCM (used in the present study), this media cannot sustain the survival, proliferation and differentiation of avian cells unless supplemented with avian transferrin as an iron donor. In this regard, the myogenesis-promoting activity of DTIBF in the present study was markedly, but not completely reduced by the depletion of transferrin (Fig. 2). The residual activity could be due to the existence of ferric iron in DMEM, since ferric ion is able to function as a substitute, albeit an inefficient one, for iron-bound transferrin (Saito *et al*.,1982). In the present study, we intentionally did not change the media during cultivation in DTIBF, since recycled and iron-free apotransferrin molecules must be converted to bioactive ones by binding to iron in the culture medium.

The effect of BSA on growth was so marked that we were concerned about possible contamination with some unidentified bioactive factor(s). BSA was not contained in the serum-free medium devised by Dollenmeier *et al*. (1981). We speculate, therefore, that addition of BSA itself is highly beneficial to chick primary myogenic cells. In this connection, we checked the effect of AlbuMAX (Gibco) and also found that it was markedly active in supporting myogenesis (data not shown). However, we did not use this ingredient in our medium, since AlbuMAX (lipid-rich BSA) contains unidentified lipids and other substances.

FGF-2 is indispensable for DTIBF medium. FGF-2 is a potent mitogen for myoblasts, and culture media containing a high concentration of this factor can function as a growth medium. Higher concentrations of FGF-2 result in greater myoblast proliferation and more prolonged delay in withdrawal from the mitotic cycle. In the present study, we did not change or replenish the DTIBF medium during myoblast cultivation. Therefore, DTIBF functioned as a growth medium at the early phase and then as a differentiation medium at the later phase of cultivation. After a 1-day preincubation in BCM/EE medium followed by medium change to DTIBF, myoblasts begin to fuse about 2 days in DTIBF medium. Dollenmeier *et al*. (1981) used FGF at a high concentration (300ng/ml) in their serum-free medium. This is conceivably because the chromatographically prepared FGF used in their study may have been relatively impure compared to the more recently available recombinant preparations.

In addition to transferrin, insulin, BSA, and FGF-2, we tried to introduce other substances to render DTIBF more potent. We evaluated such components as extracellular matrix substances and growth factors that have been certified to have myogenesis-supporting ability (Florini and Margi, 1989). Among them, fibronectin and insulin-like growth factors (IGF-1 and -2) potentiated the activity of DTIBF, but only slightly (data not shown). Thus, there is still room for further improvement of DTIBF.

For the precise analysis of the effect of humoral factors on cells in vitro, serum-free and chemically defined media are essential to avoid potential interactions of test substances with serum components. In the present study we were able to observe the inhibition of myogenic differentiation by activin A more clearly in DTIBF medium than in serum-containing media. We presume that this was because there was no interference with activin A activity in DTIBF due to serum component(s). Link and Nishi (1997) have also reported similar results.

The effectiveness of DTIBF as a medium was enhanced by a 1-day preincubation of the cells in serum- and EE-supplemented medium. Previous studies have also noted that prior exposure to serum (and EE) is necessary for optimal serum-free culture of chick and mammalian myogenic cells (Florini and Roberts, 1979; Link and Nishi 1997). However, this preculture introduces the possibility that some unidentified substance(s) critical for growth or differentiation might be carried over into the serum-free culture system. In this regard, we cultured cells in serum-free media in gelatin-coated dishes pretreated with BCM/EE or MEM for 24 hours and found no difference in myogenic growth promotion (data not shown). This preliminary result suggests that there was no significant carryover of an unknown bioactive substance(s) into DTIBF following preincubation in BCM/EE.

The composition of DTIBF is very simple compared with the serum-free media hitherto reported for chick primary myogenic cells. The four protein components added are relatively easy to obtain and have often been employed in serum-free media for primary or cell line myogenic cells from other species. As a result, the composition of DTIBF medium is very similar to that of the medium reported by Link and Nishi (1997). Their medium is composed of L15, insulin, transferrin, BSA and selenium, and is not very potent unless FGF is also added.

Importantly, it must also be noted that DTIBF medium was remarkably effective in suppressing the proliferation of contaminating fibroblasts compared to serum-containing media (Fig. 1), as reported in previous papers by Florini and Roberts (1979), Allen *et al*. (1985), and Vallette *et al*. (1986).

In summary, our serum-free medium is completely chemically defined, of simple composition, relatively potent compared to serum-supplemtnted media, inhibitory of fibroblast proliferation, and suitable for the sensitive assay of test substances affecting myoblast proliferation and differentiation. DTIBF supports the proliferation and subsequent differentia-

tion of chick primary myogenic cells without any medium change. Although it remains to be evaluated whether DTIBF medium is adequate for the maintenance and growth of myotubes in good condition for a longer period of time, we believe this medium could greatly facilitate future research of myogenesis using chick primary cells.

ACKNOWLEDGMENTS

We thank Dr. Y. Etoh (Cent. Res. Lab. Ajinomoto Co. Inc.) for kind gift of activin A. and Dr. M. Lamphier for reading manuscript. This work was supported in part by Research Grants from Waseda University (95A-297, 96A-290, 97A-360 and 98A-650) and by research-in-aids from the Ministry of Education, Science and Culture of Japan (08558078).

REFERENCES

Allen RE, Dodson MV, Luiten LS, Boxhorn LK (1985) A serum-free medium that support the growth of cultured skeletal muscle satellite cells. In Vitro Cell Dev Biol 21: 636–640

Bader D, Masaki T, Fischman DA (1982) Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J Cell Biol 95: 763–770

Barnes D, Sato G (1980) Methods for growth of cultured cells in serum-free medium. Anal Biochem 102: 255–270

Dollenmeier P, Turner DC, Eppenberger HM (1981) Proliferation and differentiation of chick skeletal muscle cells cultured in a chemically defined medium. Exp Cell Res 135: 47–61

Florini JR, Roberts SB (1979) A serum-free medium for the growth of muscle cells in culture. In Vitro 15: 983–992

Florini JR, Margi KA (1989) Effects of growth factors on myogenic differentiation. Am J Physiol 256: C701–711

Ii I, Kimura I, Ozawa E (1982) A myotrophic protein from chick embryo extract: Its purification, identity to transferrin, and indispensability for avian myogenesis. Dev Biol 94: 366–377

Kimura I, Hagiwara T, Ozawa E (1982) Indispensability of iron-bound chick transferrin for chick myogenesis in vitro. Dev Growth Differ 24: 369–380

Kimura I, Gotoh Y, Ozawa E (1989) Further purification of a fibroblast growth factor-like factor from chick embryo extract by heparin-affinity chromatography. In Vitro Cell Dev Biol 25: 236–242

Konigsberg IR (1963) Clonal analysis of myogenesis. Science 140: 1273–1284

Konigsberg IR (1979) Skeletal myoblasts in culture. Methods in Enzymol 58: 511–527

Link BA, Nishi R (1997) Opposing effects of activin A and follistatin on developing skeletal muscle cells. Exp Cell Res 233: 350–362

Lyles JM, Amin W, Weill CL (1992) Matrigel enhances myotube development in a serum-free defined medium. Int J Dev Neurosci 10: 59–73

Lyles JM, Amin W, Bock E, Weill CL (1993) Regulation of NCAM by growth factors in serum-free myotube cultures. J Neurosci Res 34: 273–86

Nie Z, Ham RG (1991) Protamine stimulates serum-free growth of human muscle cells. In VItro Cell Dev Biol 27A: 677–680

Ozawa E (1989) Transferrin as a muscle trophic factor. Rev Physiol Biochem Pharmacol 113: 90–141

Pietrzkowski Z, Reiss K, Korohoda W (1988) Dextran T-500 improves survival and spreading of chick embryo cells in serum-free medium. Folia Histochem Cytobiol 26: 123–132

Saito K, Hagiwara Y, Hasegawa T, Ozawa E (1982) Indispensability of iron for the growth of cultured chick cells. Dev Growth Differ 24: 571–580

Shiozuka M, Momoi T, Kimura I (1997) Activin A inhibits differentiation of chick myogenic cells in vitro. Zool Sci, 14: 327–330

Shiozuka M, Kimura I (1999) Developmental stage-related sensitivity for chick primary myogenic cells to differentiation-inhibiting effect of activin A. J Human Sci 11: 56–63 (in Japanese)

Vallette FM, Vigny M, Massoulie J (1986) Muscular differentiation of chicken myotubes in a simple defined synthetic culture medium and in serum supplemented media: Expression of the molecular forms of acetylcholinesterase. Neurochem Int 8: 121–133

(Received September 1, 1999 / Accepted September 25, 1999)