Influence of Chicken Embryo Extract on Protein Synthesis of Chicken Embryo Myoblasts Depends on Cell Density

K. Kita[1], K. Hiramatsu[2] and Jun-ichi Okumura
School of Agricultural Sciences, Nagoya University, Nagoya 464-01, Japan

ABSTRACT : The synergistic effect of fetal calf serum (FCS) and chicken embryo extract (CEE) on protein synthesis of chicken embryo myoblasts was examined. Myoblasts were derived from chicken embryo cultured for 14 days by trypsin digestion and cultured in 5% CO^2/95% air at 37℃. When myoblasts were cultured at the low level of cell density (20-50% of well), CEE enhanced the ability of FCS to stimulate protein synthesis of myoblasts. However, there was no significant effect of CEE to stimulate protein synthesis of myoblasts cultured at high level of cell density (100% of well).
(**Key Words:** Myoblasts, Chicken Embryo Extract Fetal Calf Serum, Protein Synthesis Cell Density)

INTRODUCTION

Skeletal muscle development involves two phases in which there are an initial period of myoblast proliferation and the following period of cell differentiation including multinucleated myotube formation. Myoblast primary culture has been widely used for investigating cell proliferation and terminal differentiation. As we reported previously, mammalian or avian serum is required for cell proliferation and for inducing protein synthesis in chicken embryonic cell cultured *in vitro* (Kita et al., 1996). Chicken embryo extract (CEE) has been also added into the culutre medium for myoblast primary culture prepared from chicken embryos (Konigsberg, 1963, 1971). However, the information about the interactive effect between CEE and mammalian serum on protein synthesis of avian myoblasts has been limited so far. Therefore, in the present study, we examined the synergistic effect of CEE and fetal calf serum (FCS) on protein synthesis and myotube formation of chicken embryo myoblasts.

MATERIALS AND METHODS

Primary culture of chicken embryo myoblasts

Ten fertilized eggs from single comb White Leghorn hens maintained in our laboratory were incubated for 14 days. At this time five embryos were taken from eggs, decapitated, rinsed in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS) including 0.25 mg/ml Fungizone (GIBCO LABORATORIES Life Technologies Inc., NY, U.S.A.) and 50 μg/ml gentamycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The breast muscle was removed and rinsed in DPBS including Fungizone and gentamycin. Thereafter, muscles were minced finely with scissors, mixed with 15 ml of DPBS containing 0.25% trypsin (TRYPSIN 1:250, DIFCO LABORATORIES, MI, U. S. A.), and incubated at 37℃ for 20 min. After the incubation, 15 ml of culture medium was added. The culture medium was prepared by mixing with Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Company, MO, U. S. A.) and Medium 199 (M199, GIBCO LABORATORIES Life Technologies Inc., NY, U. S. A.) (DMEM: M199 (1:1)) including 10% (v/v) FCS (GIBCO LABORATORIES Life Technologies Inc., NY, U. S. A.) and 2% (v/v) CEE. After centrifuge (500 × g, 1 min, room temperature), the supernatant was withdrawn. The muscle crumble was mixed with 15 ml of culture medium and suspended by pipetting. The suspended myoblasts were passed through an autoclaved cotton mesh. The cells were centrifuged down by centrifuge for 10 min at 2,000 × g, and then the supernatant was removed. After rinsing cell pellets with the culture medium twice, the cells were re-suspended in the culture medium and seeded into a non-collagen-coated culture dish to exclude fibroblasts which were easily attached on the dish. After 1 h of cell seeding, the medium including myoblasts was transferred to other culture dish. After 1 h, myoblasts floating into medium were collected again and seeded in a Type 1 collagen-

[1] Address reprint requests to K. Kita.
[2] Faculty of Agriculture, Shinshu University, Minami-minowa 8304, Kami-ina, Nagano 399-45, Japan.
Received December 30, 1997; Accepted May 11, 1998

coated 24-well plate and incubated at 37℃ in 5% CO_2/95% air.

Preparation of chicken embryo extract

Ten fertilized eggs from single comb White Leghorn hens maintained in our laboratory were incubated for 9 days. At this time all embryos were taken from eggs and decapitated. After removal of head, wings and legs, remained body was rinsed in DPBS including 0,25 mg/ml Fungizone and 50 μg/ml gentamycin, minced finely with scissors, and suspended with an equal wet weight of DPBS. The suspension was centrifuged at 6,000 × g for 20 min at 4℃. The supernatant was filtrated through 0.22 μm filter [Millex-GP (25 mm), Millipore, MA, U.S.A] and stored at −80℃ until use.

Experiment 1

To investigate the influence of CEE on protein synthesis, varying levels of CEE was added to the culture medium including 10% FCS. Myoblasts were seeded on a Type-1 collagen-coated 24-well plate at the low level of cell density (500 cells/cm^2) and incubated in DMEM: M199 (1 : 1) including 10% FCS and 2% CEE. When myoblasts were expended to approximate 10-20% of well surface, the medium was changed to DMEM : M199 (1 : 1) including 10% FCS and either 0, 1, 2, 3, 4 or 5% of CEE. Medium was changed every 2 days of incubation. After 4 days of incubation with the experimental culture medium, protein synthesis was measured by using the method of Ballard (1982) modified by Kita et al. (1996). The medium was taken out from the well and then 1 ml of fresh medium including L-[^{3}H] leucine (2.11 TBq/m mol, 37 MBq/ml, Amersham LIFE SCIENCE, Ltd., Tokyo, Japan) was added into the well, in which the radioactivity was 1 μCi/ml. Thereafter cells continued to be incubated for further 18 hours, and then the medium was removed and cells were rinsed with ice-cold DPBS twice. The intracellular free amino acids were removed by washing with ice-cold trichloroacetic acid twice. After rinsing with ice-cold water, 1 ml of 0.5M NaOH/0.1% Triton X-100 was added and incubated at room temperature for 30 min. After dissolving protein by pipetting, the radioactivity in NaOH/Triton X-100 solution was measured as an index of protein synthesis.

Experiment 2

The results derived from Experiment 1 showed that more than 2% of CEE attained the maximum protein synthesis when myoblasts was cultured at low density of cells. In Experiment 2, the synergistic effect of CEE and FCS on protein synthesis of myoblasts cultured at higher levels of cell density. Myoblasts were seeded in the well (3,000 cells/cm^2) and incubated in DMEM : M199 (1 : 1) including 10% FCS and 2% CEE. After 1 day of incubation (approximately 50% of well), the medium was changed to DMEM : M199 (1 : 1) including either 10% or 0% of FCS with either 0 or 2% of CEE. At Days 1 and 2 of incubation with experimental culture medium, protein synthesis was measured as described in Experiment 1.

Statistical analysis

Statistical analysis of data was performed by one-way (Experiment 1) and two-way ANOVA (Experiment 2) (Cochran and Cox. 1992) using the General Linear Model Procedures of SAS (SAS/STAT Version 6, SAS Institute, Cary NC, U.S.A.).

RESULTS

The influence of CEE to stimulate protein synthesis of chicken embryo myoblasts is shown in figure 1. The medium used in the present study contained 10% of FCS and either 0, 1, 2, 3, 4 or 5% of CEE. The lowest value for protein synthesis was observed in chicken embryo myoblasts cultured in DMEM : M199 without CEE.

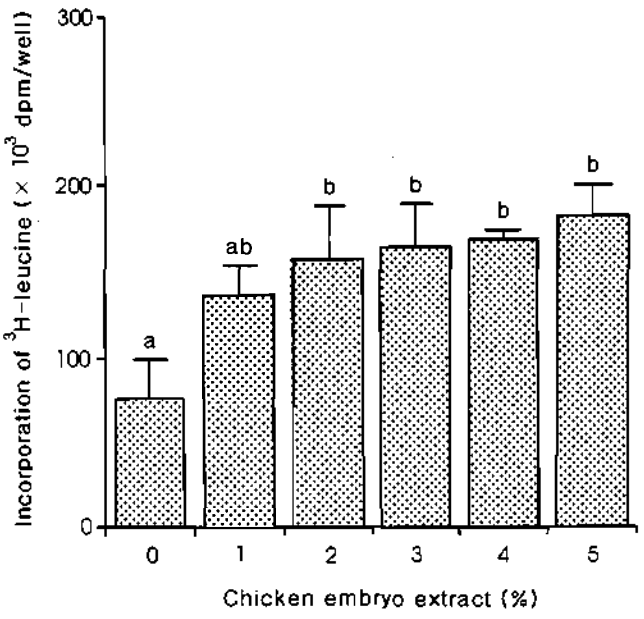

Figure 1. Protein synthesis of chicken embryo myoblasts cultured in the medium (DMEM : M199 (1 : 1)) containing 10% fetal calf serum and varying levels (0, 1, 2, 3, 4 and 5%) of chicken embryo extract. Incorporation of ^{3}H-leucine into protein was measured after 1 day of incubation as an index of protein synthesis. Means not sharing with the same letter are significantly differenct at $p < 0.05$ (a, b).

Protein synthesis was significantly increased by elevating CEE levels from 0 to 2%. Above 2% of CEE, no significant increase in protein synthesis was observed. Myotube formation was observed in all treatments (data not shown).

Figure 2 illustrates chicken embryo myoblasts cultured in the medium with and without FCS and/or CEE on day 1 of incubation. When myoblasts were seeded at the level of 50% of well in the medium containing 10% of FCS, cells proliferated rapidly and expanded in the well (100% of well) for 1 day of incubation. Myoblasts were fused to form multinucleated myotubes when cells were cultured in the medium without FCS, which was independent in the presence of 2% CEE. There was no apparent difference in myotube formation between day 1 and 2 of incubation (data not shown).

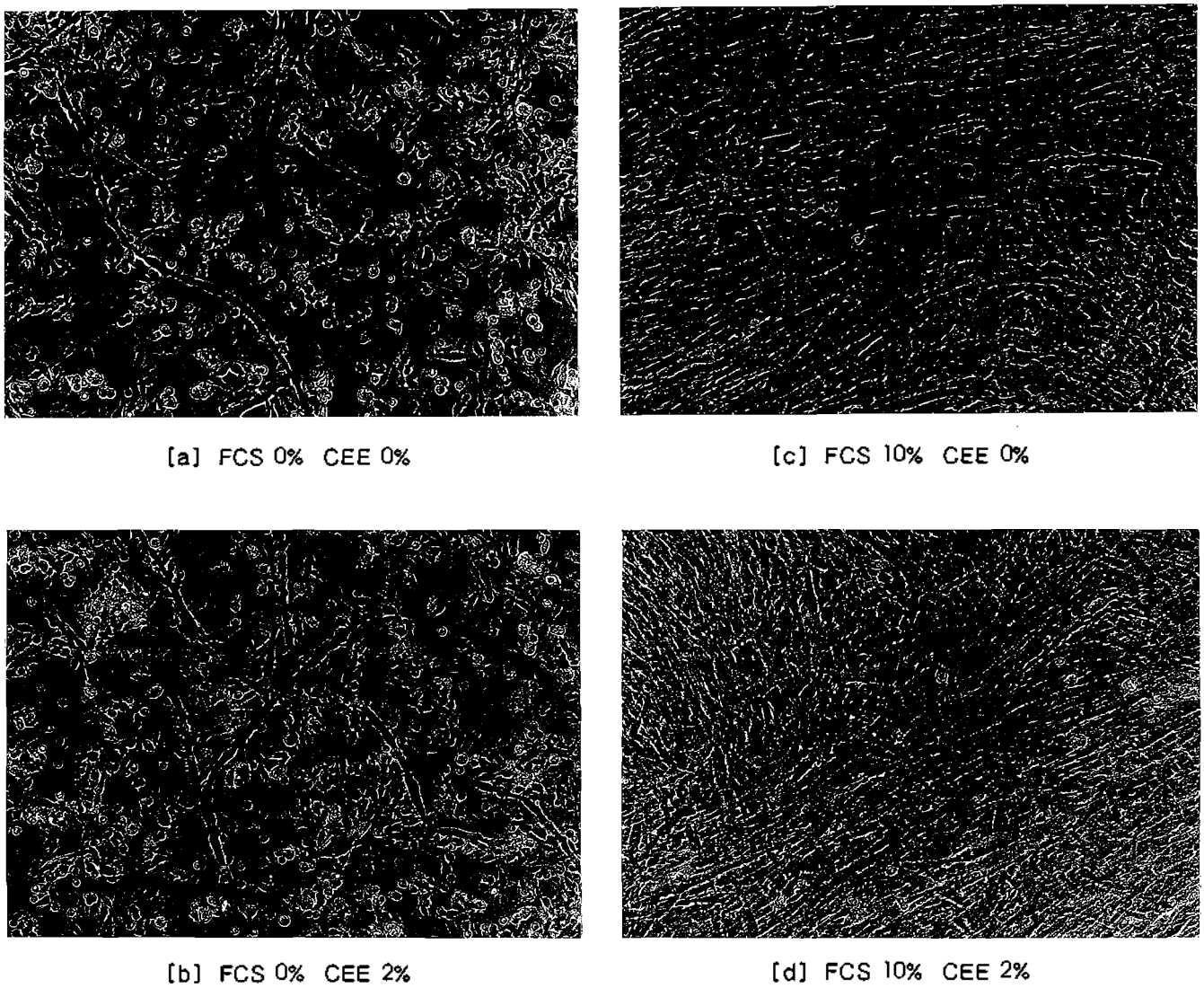

Figure 2. Chicken embryo myoblasts cultured for 1 day in the medium (DMEM : M199 (1 : 1)) containing either 10% or 0% fetal calf serum (FCS) with either 2% or 0% chicken embryo extract (CEE). [a] FCS 0%, CEE 0%, [b] FCS 0%, CEE 2%, [c] FCS 10%, CEE 0%, [d] FCS 10%, CEE 2%. Magnification was × 200.

Figure 3 shows the synergistic effect of FCS and CEE on protein synthesis of myoblasts. On day 1 of incubation, interactive effect of CEE and FCS was observed. Protein synthesis was not stimulated by 2% of CEE when myoblasts were cultured in the medium without FCS. However, when 2% of CEE was supplemented into the medium containing 10% of FCS, the higher value for protein synthesis was observed compared to the absence

in 2% of CEE. On day 2, protein synthesis on myoblasts cultured without FCS showed similar value for that observed on day 1. No interaction between FCS and CEE was found, and the value for protein synthesis of myoblasts with FCS was almost twice higher than that on day 1.

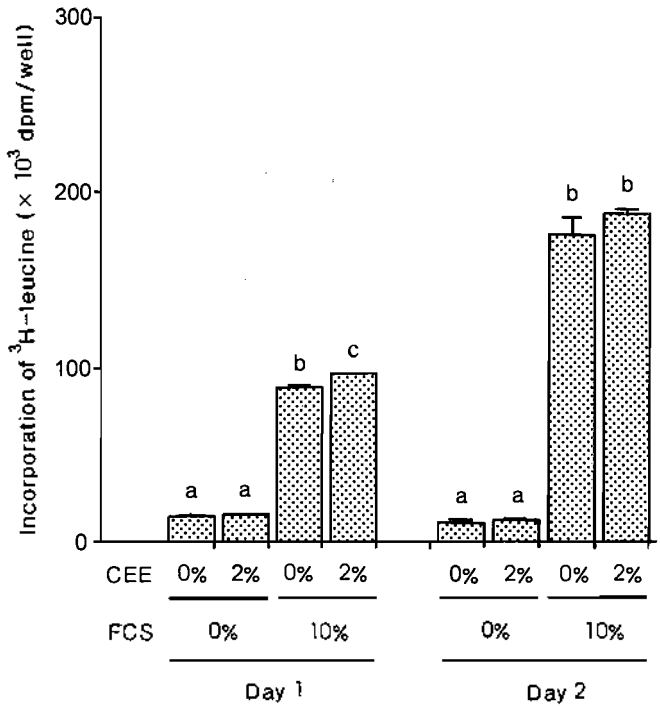

Figure 3. Protein synthesis of chicken embryo myoblasts cultured in the medium (DMEM: M199 (1 : 1)) containing either 10% or 0% fetal calf with either 2% or 0% chicken embryo extract. Incorporation of ^{3}H-leucine into protein was measured after 1 day of incubation as an index of protein synthesis. Means not sharing the same letter on each day are significantly different at $p < 0.05$.

DISCUSSION

As shown in figures 1 and 3, when myoblasts were seeded at low level of cell density (20-50% of well, on day 1), the highest protein synthesis was obtained in medium containing more than 2% of CEE. On the contrary, after myoblasts expanded fully (100% of well, on day 2), CEE did not influence on protein synthesis. These results indicate that when myoblasts were in the stage of cell proliferation, CEE is able to enhance the ability of FCS to stimulate protein synthesis of myoblasts.

Oh and Markelonis (1980) reported that a trophic protein (sciation), which has been originally purified from adult chicken sciatic nerves (Markelonis and Oh, 1979), is the component of CEE requires for myogenesis and is potent to stimulate the terminal differentiation when chicken embryo myoblasts were cultured in the medium containing 10% horse serum. In Experiment 1, however, myoblasts cultured in the medium containing 10% FCS without CEE fused to form multinucleated myotubes. This inconsistence may be resulted from the serum derived from different species because Yasin et al. (1981) reported that the striking growth effect of CEE on adult human muscle cell proliferation was seen using FCS but not using horse serum.

It has been well recognized that mammalian and avian cells cultured *in vitro* require whole serum obtained from animals. As shown in figure 2, when FCS was withdrawn from medium, chicken embryo myoblasts rapidly ceased to proliferate and commenced to form multinucleated myotubes. This alteration involved in the decrease in rates of protein synthesis as shown in figure 3, which is consistent with the previous finding reported by McElligott et al. (1988). Recent findings indicate that some components in whole-serum stimulate cell proliferation and inhibit myogenic differentiation. For instance, Spizz et al. (1986) and Clegg et al. (1987) demonstrated that fibroblast growth factor represses skeletal muscle differentiation through a mechanism dependent on protein synthesis and independent on cell proliferation. Yoshida et al. (1996) also indicated that lysophosphatidic acid, a bioactive phospholipid contained in serum, stimulates the growth and prevents the differentiation. Therefore, the change in protein synthesis dependent on FCS in the medium might be associated with the above substrates in serum, and this issue should be elucidated in the future.

REFERENCES

Ballard, F. J. 1982. Regulation of protein accumulation in cultured cells. Biochem. J. 208:275-287.

Clegg, C. H., T. A. Linkhart, B. B. Olwin and S. D. Hauschka, 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. 105:949-956.

Kita, K., M. Miyazaki and J. Okumura. 1996. Influence of food-deprived chicken serum on protein synthesis of chicken embryo fibroblasts. Jpn. Poult. Sci. 33:339-346.

Konigsberg, I. R. 1963. Clonal analysis of myogenesis. Its relevance to the general problem of the stability of celltype in cultured animal cells is discussed. Science 140:1273-1284.

Konigsberg, I. R. 1971. Diffusion-mediated control of myoblast fusion. Dev. Biol. 26:133-152.

Markelonis, G. and T. H. Oh. 1979. A sciatic nerve protein has a trophic effect on development and maintenance of skeletal

muscle cells in culture. Proc. Natl. Acad. Sci. U. S. A. 76:2470-2474.

McElligott, M. A., L. −Y. Chaung, V. Baracos and E. A. Gulve, E. 1988. Prostaglandin production in myotube cultures. Influence on protein turnover. Biochem. J. 253:745-749.

Oh, T. H. and G. J. Markelonis. 1980. Dependence of *in vitro* myogenesis on a trophic protein present in chicken embryo extract. Proc. Natl. Acad. Sci. U. S. A. 77:6922-6925.

Spizz, G., D. Roman, A. Strauss and E. N. Olson. 1986. Serum and fibroblast growth factor inhibit myogenic differentiation through a mechanism dependent on protein synthesis and independent of cell proliferation. J. Biol. Chem. 261:9483-9488.

Yasin, R., D. Kundu and E. J. Thompson. 1981. Growth of adult human cells in culture at clonal densities. Cell Different. 10:131-137.

Yoshida, S., A. Fujisawa-Sehara, T. Taki, K. Arai and Y. Nabeshima. 1996. Lysophosphatidic acid and bFGF control different modes in proliferating myoblasts. J. Cell Biol. 132:181-193.