Effects of Chicken Egg White on the Proliferation and Neurite Outgrowth of Mammalian Cells

Chao Zou, Kunihiko Kobayashi, and Akio Kato*

Department of Biochemistry, Yamaguchi University, Yamaguchi 753, Japan

The growth-promoting and neurite-growth-promoting effects of chicken egg white on mammalian cell were studied by using CV-1, BHK-21, IEC-18, and PC12 cell lines. Chicken egg white promoted the proliferation of CV-1, BHK-21, and IEC-18 cell lines. The growth-promoting activity of egg white was dose dependent and seeding cell density dependent. Although chicken egg white did not exhibit cell-growth-promoting activity as strong as that of serum, it promoted the cell growth in cooperation with serum. In addition to growth-promoting activity, egg white exhibited a strong activity to induce the process growth of CV-1 cells. During the 12-h cell culture, about half of the CV-1 cells having long processes grew in the egg white supplemented medium. Further evidence of the existence of a process outgrowth-promoting component in egg white was obtained by using the PC12 cell line, which extends neurites in response to nerve growth factor.

It is well-known that there are many similar functions between egg white proteins and blood proteins, because egg white proteins evolved from blood proteins. Therefore, egg white may contain proteins similar to serum proteins which have biological functions for mammalian cells.

Evans et al. (1957) reported that whole egg extract from either fertile or nonfertile eggs exhibited growth-promoting activity for three mammalian cell strains. Fujii and Gospodarowicz (1983) have indicated that chicken egg yolk has proliferation activity for several mammalian cells, whereas there is a cell growth inhibitory substance in chicken egg white. Yamada et al. (1989) have reported that egg yolk lipoprotein has stimulating activity for immunoglobulin production in human–human hybridoma HB4C5 cells. In addition, phosphatidylcholine in egg yolk has been reported to be involved in the proliferation of mammalian cells (Fujii et al., 1983). Thus, it has been proved that there are some components which have activities for mammalian cell culture in egg yolk. Recently, Azuma et al. (1989) reported peptide substances having cell-growth-promoting activity in acidic extracts of egg white using Balb/c 3T3 cells as an indicator. However, it is still unclear whether the cell-growth-promoting activity of egg white was also exhibited for cell lines other than the Balb/c 3T3 cell line and which proteins or polypeptides in egg white have the cell-growth-promoting activity. Except for the cell-growth-promoting activity of egg white, there has been little information about other biological activity such as that inducing cell differentiation.

This paper describes the effects of egg white on cell proliferation, spreading, morphological alteration, and neurite outgrowth using various mammalian cell lines, CV-1, BHK-21, IEC-18, and PC12, as indicators.

MATERIALS AND METHODS

Materials. CV-1 cell line, kidney fibroblast cell from *Cercopithecus aethiops*, was provided by Dr. K. Takimoto, Faculty of Agriculture, Yamaguchi University. BHK-21 (kidney fibroblast cell from *Mesocricetus auratus*), IEC-18 (rat epithelial cell), and PC12 (rat adrenal pheochromocytoma) were all purchased from American Type Culture Collection. Eagle's medium (MEM) was purchased from Nissui Pharmaceutical Co. Ltd., RPMI 1640 medium (RPMI) from Flow Laboratories, and fetal bovine serum (FBS) from Whittaker M. A. Bioproducts Inc.

Preparation of Aseptic Egg White. Chicken eggs were collected on the chicken farm within 24 h after laying. Egg white was separated from egg yolk and homogenized. The egg white was allowed to stand overnight at 4 °C after it was diluted with a 3-fold volume of water, adjusted to pH 6.0 with 0.1 N HCl, and then readjusted to pH 7.4 with 0.1 N NaOH and centrifuged at 10000*g* for 20 min to remove the precipitate. The supernatant was filter-sterilized through a membrane filter (0.22 μm, Millipore) for the experiments. The filtrated supernatant is abbreviated EW.

Cell Proliferation Assay. Confluent cells were twice washed with phosphate-buffered saline (PBS) and trypsinized with 0.5% trypsin solution containing 0.5% EDTA for 1 min. After removal of the trypsin solution, cells were suspended in serum-free MEM and seeded into plastic tissue culture dishes at a seeding density of 10^5 cells/35-mm dish and then incubated in 5% CO_2/95% air for 2 h at 37 °C. Medium in each dish was replaced by test media (containing EW, FBS, or MEM alone) and renewed every day. The number of cells in each dish was counted after incubation for various periods of time.

Measurement of Effective Seeding Density of Cell. CV-1 cells were suspended in MEM and seeded at various seeding densities. To determine exact seeding density, the survival rate of cells was measured after cells were incubated in 5% CO_2/95% air at 37 °C for 2 h. The effective seeding density is represented as the survival cell numbers attached to 1 mm^2 of surface area of plastic tissue culture dish.

Cell Spreading Assay. Confluent CV-1 cells were suspended by serum-free MEM, seeded into plastic tissue culture dishes at a seeding density of 5×10^4 cells/35-mm dish, and incubated for 1 h to get cell adhesion, and then 10% EW or FBS was added. After incubation in 5% CO_2/95% air at 37 °C for 2, 5, 10, or 24 h, the percentages of the spreading cells in each medium were calculated by randomly counting over 100 cells in each dish. The cell spreading rate is represented as the percentage of spreading cells to total attached cells.

Microscopic Observation of Cell. Cell morphologic alteration was photographed by a phase contrast microscope (Nikon, Diaphot-TMD).

Cell Process Outgrowth Assay. CV-1 cells were seeded into plastic tissue culture dishes at a seeding density of 5×10^4 cells/35-mm dish and incubated for several hours. The medium was replaced by EW-supplemented medium, and the cells were reincubated for 12 h. The percentages of the cells with process were calculated by randomly observing and counting over 100 cells in each dish.

Neurite Outgrowth Assay in PC12. PC12 cells were exposed to the RPMI medium containing 15% FBS at a seeding density of 2×10^4 cells/35-mm dish for 1 day, and then the medium was replaced by different test media. The media were exchanged daily for 1 week. The neurite growth of cells in each medium was photographed with a phase contrast microscope.

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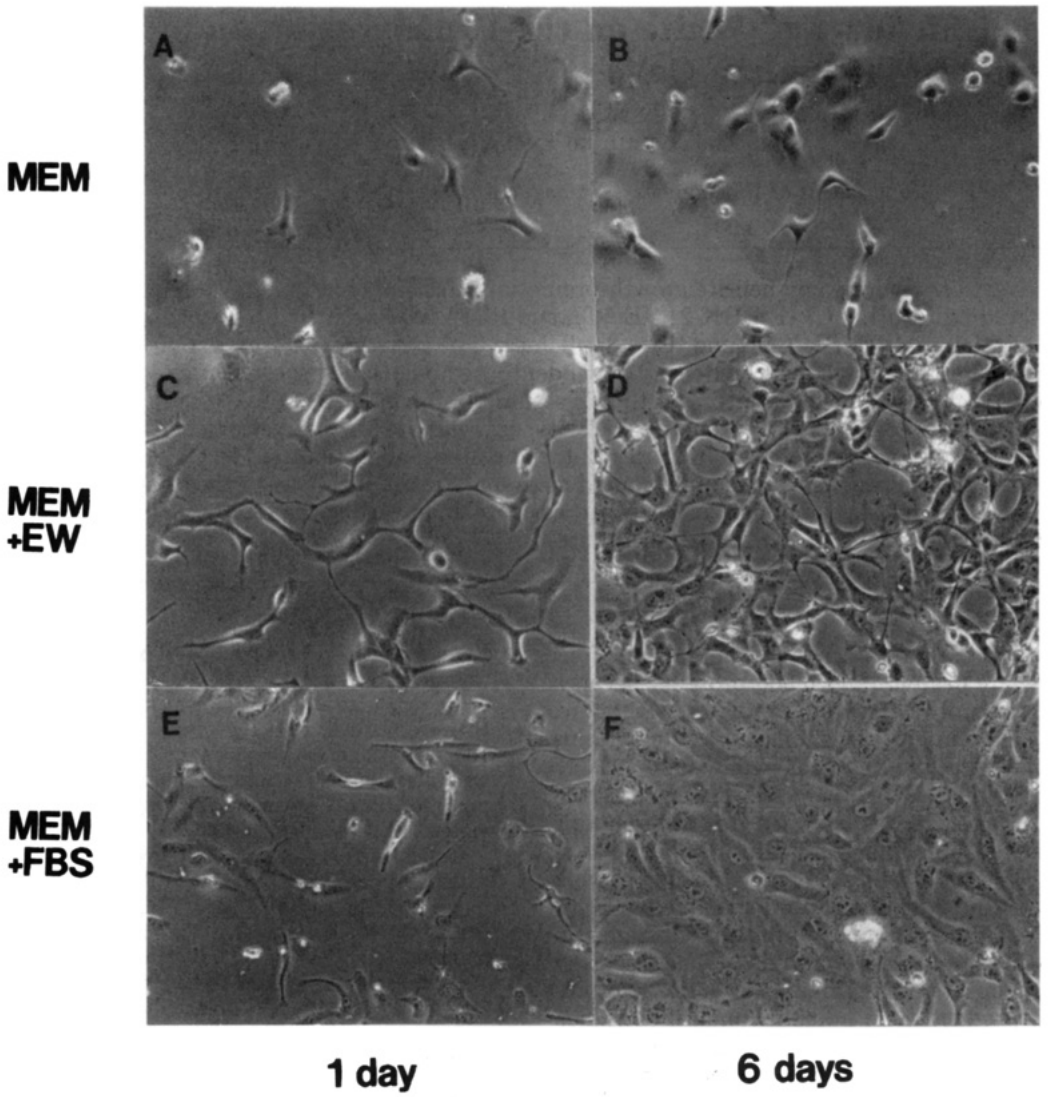

Figure 1. Phase contrast micrographs (×200) of CV-1 cells cultured in MEM for 1 day (A) and 6 days (B), in MEM plus 10% EW for 1 day (C) and 6 days (D), and in MEM plus 10% FBS for 1 day (E) and 6 days (F). (Published figure is 75% of original.)

RESULTS

The effect of egg white on the growth of mammalian cells was investigated by using CV-1, BHK-21, and IEC-18 cell lines. The cell-growth-promoting activity of egg white was estimated by comparing the cell numbers cultured in EW-supplemented medium with those in FBS-supplemented medium or MEM. Figure 1 shows the phase contrast microscope of CV-1 cells in different media. When CV-1 cells were cultured in the presence of EW or FBS, they apparently proliferated with different behavior. In the EW-supplemented culture, cells grew with marked long processes. These morphologic changes were hardly detected in the FBS-supplemented culture or the MEM culture.

The time course of CV-1 cell proliferation was followed by counting cell numbers in each medium during various periods of culture time after the test medium was added. The cell number in EW-supplemented culture was about 2 times higher than that in unsupplemented medium (MEM) after 4 days of incubation (Figure 2).

The optimum growth-promoting effect of egg white on CV-1 cells was examined. Figure 3 shows changes in the cell growth in serum-free medium supplemented with various concentrations of EW for 4 days. The growth-promoting activity of egg white was increased in proportion to the concentration of EW. The optimal concentration of EW to obtain the best growth-promoting effect on CV-1 cells is about 10% (v/v) in medium. In addition, the cell-growth-promoting activity of egg white was dependent on the cell seeding density, the cell numbers attached to 1 mm^2 of surface area of plastic tissue culture dish, as shown in Figure 4. The cell-growth-promoting activity of egg white was estimated by measuring the time when the cell number was doubled in each seeding density. The growth of CV-1 cells is a function of the cell seeding density, and the dependence of the cell seeding density on the cell growth in EW-supplemented medium was greater than that in FBS-supplemented medium. The value of the best cell seeding density for CV-1 cells to proliferate in EW-supplemented culture was about 80 cells/mm^2, at which CV-1 cells doubled 67 h after seeding, while that in FBS-supplemented culture was about 50 cells/mm^2, at which cells doubled after only 57 h.

The cooperative effect of egg white with serum on the proliferation of CV-1 cell was examined in the medium supplemented with the various concentrations of FBS in the presence of 10% (v/v) EW. As shown in Figure 5, the growth of cells in the presence of EW was significantly higher than that in the absence of EW in all concentrations of FBS-supplemented medium.

Figure 6 shows the effect of heat treatment of egg white on the CV-1 cell-growth-promoting activity. The growth-

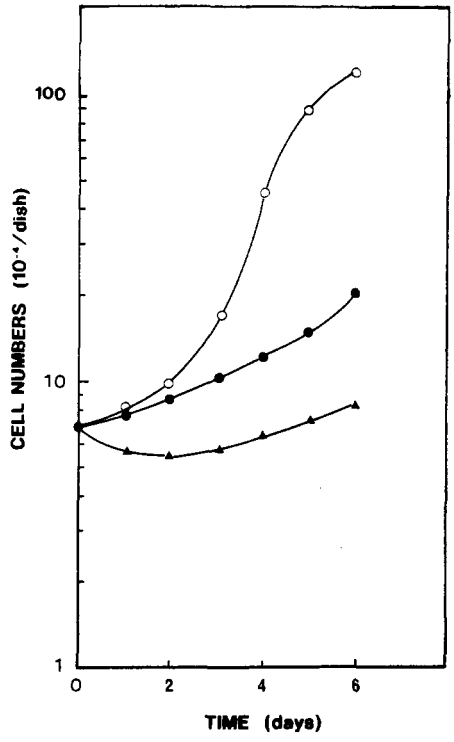

Figure 2. Proliferation of CV-1 cells in 10% FBS-supplemented MEM (○), in 10% EW-supplemented MEM (●), and in MEM (▲).

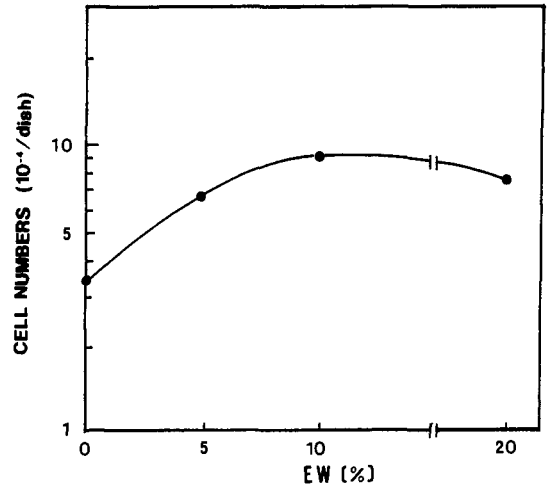

Figure 3. Effect of concentrations of EW in serum-free culture on CV-1 cell growth. The numbers of CV-1 cells were counted after incubation for 4 days.

promoting activity of egg white was sharply reduced by heating egg white at the transition temperature of 58 °C. This suggests that the growth-promoting substance might be proteinlike.

The effect of egg white on the spreading of CV-1 cells was also examined. CV-1 cells were exposed to EW-supplemented or FBS-supplemented MEM, and MEM alone. After incubation for various periods of time, both attaching and spreading cells in each dish were counted. Figure 7 shows the rate of spreading cells to attaching cells in different media. The cells exposed to EW-supplemented or FBS-supplemented medium exhibited higher spreading speeds than those exposed to unsupplemented MEM at each incubation time. After 5 h, the spreading cells in FBS- or EW-supplemented medium reached about 90%, while those in MEM reached 75%.

Thus, the growth-promoting effects of egg white on CV-1 cells were confirmed as described above. A further attempt

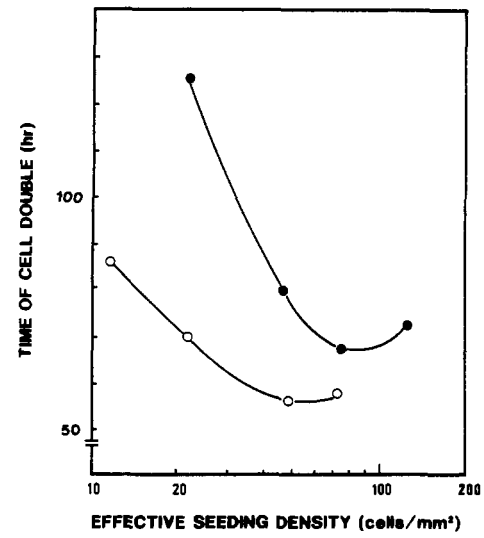

Figure 4. Effect of effective seeding density on CV-1 cell-growth-promoting activity in 10% EW-supplemented MEM (●) and in 10% FBS-supplemented MEM (○). Each dish with different seeding density was counted every 24 h, and cell growth is represented as the cell doubling time.

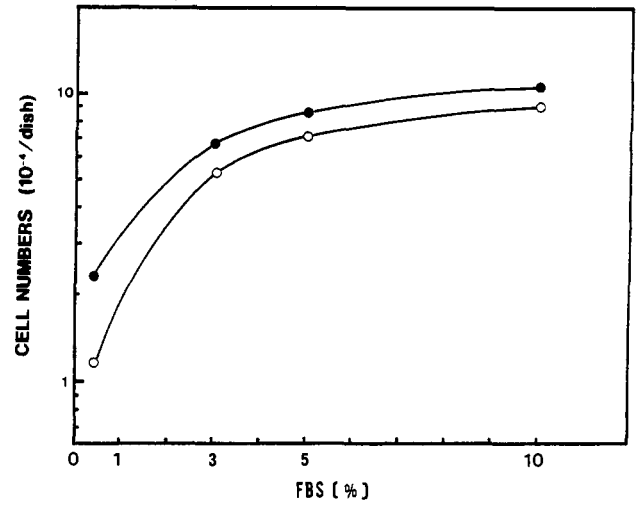

Figure 5. Cooperative effect of EW with FBS on CV-1 cell proliferation. CV-1 cells were cultured in various concentrations of FBS-supplemented MEM in the presence of 10% EW (●) and in the absence of EW (○). The cell numbers were counted after incubation for 4 days.

to confirm the effects was done by using BHK-21 and IEC-18 cell lines. As shown in Figure 8, egg white exhibited growth-promoting activity for these mammalian cells in the presence of 2% FBS, although it showed no growth-promoting effect on either cell line in serum-free medium.

In addition to the cell-growth-promoting effect of egg white, remarkable morphological changes were also observed. When CV-1 cells were exposed to EW-supplemented medium, their morphology and movement dramatically changed, as shown in Figure 9. CV-1 cells in EW-supplemented medium began to change their morphology when they were exposed to EW-supplemented medium for 3.5 h (data not shown). When cells were exposed to EW-supplemented medium for 12 h, their morphology dramatically changed with extended long processes, while cells did not exhibit these phenomena in the FBS-supplemented medium and MEM. The rate of the process outgrowth of CV-1 cells cultured in different media is shown in Figure 9. During a 12-h culture, about 50% of cells grew with long processes in EW-supplemented

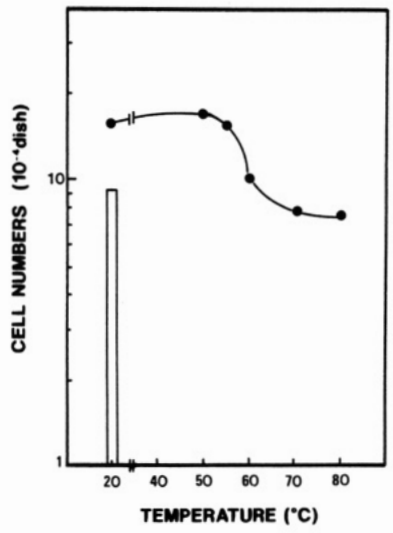

Figure 6. Effect of heating of EW on CV-1 cell-growth-promoting activity. EW was dissolved in MEM at a final concentration of 10% (v/v), heated at various temperatures for 10 min, and then filter-sterilized. CV-1 cells were cultured in the heat-treated medium for 4 days, and then cell numbers in each dish were counted. The bar indicates the cell number cultured in MEM.

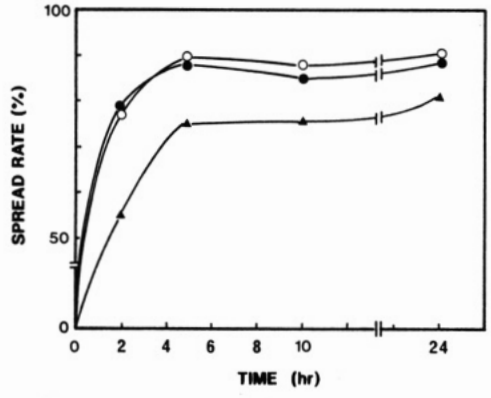

Figure 7. Effect of egg white on CV-1 cell-spreading activity in 10% FBS-supplemented MEM (○), in 10% EW-supplemented MEM (●), and in MEM (▲). The cell-spreading rate is represented as the percentage of spreading cells to attached cells.

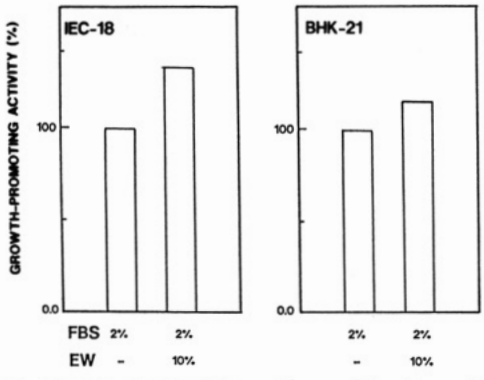

Figure 8. Effects of egg white on the proliferation of BHK-21 and IEC-18 cells. Cells were exposed to MEM containing 2% FBS or 2% FBS plus 10% EW, and cell numbers were counted after 4 days of culture.

medium, while less than 10% of cells had short processes in FBS-supplemented and unsupplemented media.

This observation suggests the ability of egg white to induce neurite outgrowth of mammalian cells. Therefore, an attempt was made using the PC12 cell, a neurite-inducing cell in response to nerve growth factor (NGF), as an indicator. As shown in Figure 10, PC12 cells with

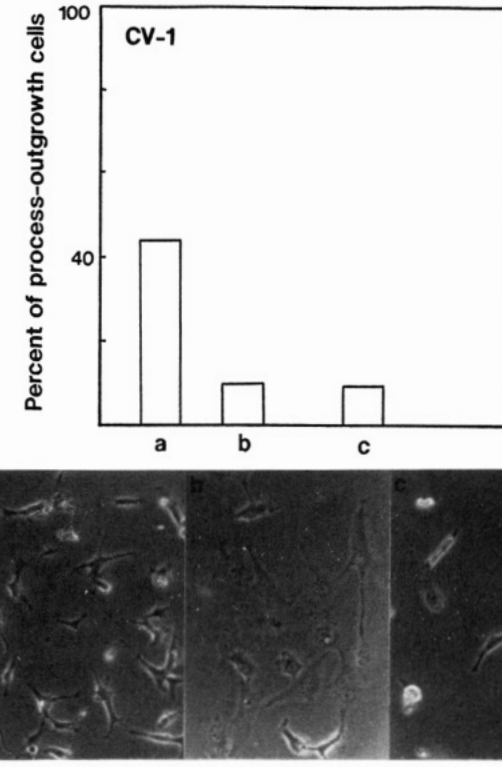

Figure 9. Process outgrowth of CV-1 cell in EW-supplemented MEM (a), in FBS-supplemented MEM (b), and in MEM (c). Phase contrast micrographs were taken under 200× magnification after 12 h of incubation. (Published figure is 55% of original.)

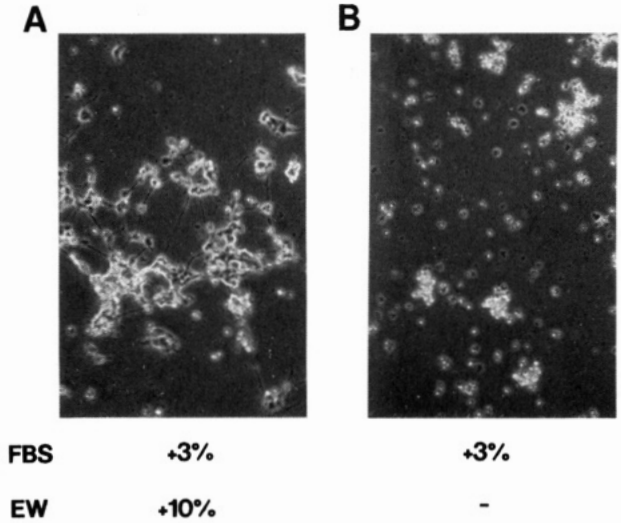

Figure 10. Neurite outgrowth of PC12 cells in 10% EW-supplemented MEM containing 3% FBS (A) and in 3% FBS without EW (B). Phase contrast micrographs were taken under 200× magnification after 1 week of incubation. (Published figure is 50% of original.)

long neurites were observed in the EW-supplemented medium, while neurites were hardly observed in the control cells in the medium without EW.

DISCUSSION

Although the component was unidentified, chicken egg white seems to contain a cell-growth-promoting factor and a neurite growth factor. The growth-promoting effects of egg white may be common for many mammalian cell lines because it was observed for the BALB/c 3T3 cell (Azuma et al., 1989), the CV-1 cell, the BHK-21 cell, and the IEC-18 cell, which was confirmed here. In this paper, the

growth-promoting effects for the CV-1 cell were studied in detail, because the cell was more sensitive to egg white than other cells.

It is unclear whether the efficiency of egg white on growth promotion of the CV-1 cell results from the proliferation-promoting activity or the spreading-promoting activity. Folkman et al. (1978) found that the spreading and adhesion of cells are tightly coupled to DNA synthesis and growth in nontransformed cells. Therefore, the spreading-promoting activity of EW may lead to the faster growth of CV-1 cells in EW-supplemented culture.

In addition to cell proliferation, CV-1 cells exhibit morphological alteration, which may be differentiation, in EW-supplemented culture. The morphological alteration with process outgrowth is a main behavior of CV-1 cells in EW-supplemented medium because the proliferation of CV-1 cells in EW-supplemented medium occurred only in the high cell density, where the growth of processes was inhibited by cell contact, while cells were easy to grow their process and difficult to proliferate in low density. On the other hand, in the medium containing both 10% EW and 10% FBS, the main behavior of CV-1 cells is proliferation and the process outgrowth was not observed as CV-1 cells exhibited in the EW-supplemented medium.

The most striking finding is that the neurite-growth-inducing activity of egg white was observed by using the PC12 cell which extends neurites in response to NGF (Greene et al., 1976), suggesting that NGF-like protein exists in chicken egg white. Since the ability of the PC12 cell to attach to the dish surface is very weak and egg white has a cell adhesion inhibition activity, the neurite-outgrowth-inducing activity of egg white was examined in the presence of 3% serum to promote the adhesion of PC12 cells.

The components having these activities in chicken egg white ramain unknown. Further study is needed for the identification of the components responding to the multiple effects on mammalian cell. It has been reported that laminin and fibronectin have cell adhesion-, spreading- and neurite-outgrowth-promoting activities (Sasaki et al., 1987; Tashiro et al., 1989). Therefore, the active component may be laminin or fibronectin-like material in egg white.

In conclusion, chicken egg white not only promotes cell spreading and proliferation but also induces cell morphological changes such as process outgrowth in the CV-1 cell line and neurite outgrowth in the PC12 cell line. These results suggest that it is possible to use widely chicken egg white in mammalian cell culture and develop new functional proteins for mammalian cell.

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