Egg extract promotes cell migration and growth in primary culture of early embryos in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Gaku Akiduki*

Transgenic Animal Research Center, National Institute of Agrobiological Sciences; Tsukuba, Ibaraki 305–8634, Japan

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Abstract

Fetal bovine serum (FBS), commonly used in the culture of many insect cell lines, is a source of both nutrients and bioactive compounds that promote cell growth; however, because the growth-promoting compounds contained in FBS are not considered to be identical to those produced by insects, the response of insect cells to FBS is likely to differ from responses to endogenous growth-promoting compounds. The present study examined whether primary cultures of *Bombyx* embryonic tissue fragments respond differently to silkworm egg extract and FBS. It was observed that, in primary cultures, *Bombyx* egg extract promoted the migration and growth of cells from embryonic tissue fragments more potently than FBS. These results indicate that the extract of silkworm eggs in an early developmental stage contains molecules that strongly promote cell migration and growth. The use of egg extracts could also shorten the time required to establish a novel cell line.

Key words: *Bombyx mori*; egg extract; embryonic tissue; cell growth; primary culture

INTRODUCTION

The study of insect cell cultures originated with a report on the culture of testes from *Hyalophora cecropia* (Goldschmidt, 1915); subsequently, a continuously culturable cell line was established from *Antheraea eucalypti* ovary (Grace, 1962). This success was a crucial step in establishing culture methods for many insect cell types, and numerous continuous cell lines have been developed since. An important factor in establishing insect cell lines has been knowledge of the nutrients essential for insect survival and growth (Wyatt et al., 1956; Day and Grace, 1959; Jones, 1962; Brooks and Kurtti, 1971; Kunudson and Buckley, 1977; Marks, 1980). One of the most common additives to insect cell culture media is fetal bovine serum (FBS), which promotes the growth of both mammalian and insect cells in culture (Mitsuhashi and Maramorosch, 1964; Hink et al., 1974).

In the case of silkworm primary cells or cell lines, cell survival and growth are improved in media that contain FBS (Mitsuhashi and Maramorosch, 1964); therefore, FBS is a source of both nutrients and bioactive compounds that promote cell growth. However, it is not known whether the activity of these compounds in FBS resembles that of endogenous growth-promoting substances found in insects. Furthermore, the response of insect cells to FBS may differ from that elicited by endogenous bioactive molecules from insects. For example, the silkworm cell line NIAS-Bm-Ke1 is more sensitive to infection with *Bombyx mori* nucleopolyhedrovirus (BmNPV) when cultured in the presence of silkworm hemolymph than when cultured in FBS (Imanishi et al., 2006).

Therefore, studies are required to clarify differences in the growth-promoting effects of FBS and endogenous substances found in silkworm hemolymph or other tissues. The types of compounds and their levels in silkworm organs and tissues also need to be determined. Such studies should ultimately provide fundamental knowledge about the differential functions of these bioactive compounds in insects.

In the present study, differences were observed in the responses of primary cultures of *Bombyx* embryonic tissue fragments to silkworm egg ex-
tract and FBS, confirming that silkworm egg extract has different growth-promoting activity to FBS. The results show that early developmental egg extract is potently bioactive, promoting cell migration and growth, and that this extract could be used to shorten the time required to establish a novel cell line.

MATERIALS AND METHODS

Reagents. Fetal bovine serum was obtained from Tissue Culture Biologicals (Tulare, CA, USA), IPL-41 medium was from Invitrogen (Carlsbad, CA, USA), and COMPLETE-Protease Inhibitors Cocktail, EDTA-free, was from Roche (Indianapolis, IN, USA).

Preparation of Bombyx egg extract. Silkworms of the Daizo (Nagano) strain were obtained from Genebank (National Institute of Agrobiological Sciences, Tsukuba, Japan). Twenty-four hours after oviposition, silkworm eggs were dipped in 1.075 g/l HCl at 46°C for 5 min. After washing with tap water to completely remove the HCl, the eggs were kept at 80% humidity and 25°C for 1 d (used as Day 2 (D2) extract) or for 2 d (used as Day 3 (D3) extract). The eggs were then weighed and frozen in liquid nitrogen. All subsequent manipulations were performed on crushed ice. Frozen eggs were placed in a mortar and washed twice with cold phosphate-buffered saline (PBS; composition (in mM): NaCl 137; Na₂HPO₄ 8.1; KCl 2.68; KH₂PO₄ 1.47, pH 7.4). The eggs were then mashed completely with a pestle in an equivalent weight of cold DEI buffer (Castagnetti et al., 2000). Egg homogenate was transferred to 15-ml plastic tubes and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was collected and transferred to 1.5-ml tubes. These tubes were centrifuged at 13,500 x g for 10 min at 4°C. The supernatant was collected and filtered through a 0.20-μm acetylcellulose filter (Minisart; Sartorius, Goettingen, Germany). Aliquots (1 ml) of the purified supernatant were stored in 2-ml serum tubes at −30°C until use.

Silkworm embryos and culture conditions. Twenty-four hours after oviposition, silkworm eggs were dipped in 1.075 g/l HCl at 46°C for 5 min; D2 and D3 embryos, obtained as described above, were used for tissue culture. Eggs were surface-sterilized by dipping in 70% ethanol for 5 min and were then placed, one egg per well, in 96-well plates. The eggs were crushed using forceps and the embryos fragmented. Fragmented embryos were cultured at 25°C in IPL-41 medium (without additive) for 1 week and then washed in PBS. Thereafter, embryos were cultured at 25°C in IPL-41 medium supplemented with 1% or 5% (v/v) FBS, D2 extract, or D3 extract. In each case, the medium was replaced with fresh medium every month. Cell migration and growth were observed by phase-contrast microscopy and photomicrographs were taken using a digital camera (FinePix 2900Z; Fujifilm, Tokyo, Japan).

Judgment of cell survival. Cell survival in each well of 96-well plates was monitored by microscopic observation and cell count. Cells were judged as surviving when they showed morphological characteristics of healthy cells, such as glossy surfaces and full contents, and increased number and colony.

Measurement of ecdysteroids in egg extract. The concentration of ecdysteroids in each egg extract was determined using ELISA, according to the method of Shiotsuki et al. (2005). D2 and D3 egg extracts were prepared as described above. After storing at −30°C, some extracts were incubated for 1 week at 25°C. Then, 40-μl egg extract that had either been stored at −30°C after initial preparation or incubated at 25°C for 1 week was subjected to ELISA.

Detection of vitellin in egg extracts. Vitellin was determined in D2 and D3 egg extracts that had either been incubated for 1 week at 25°C or stored frozen at −30°C. Aliquots (10 μl) of the egg extracts were loaded onto a 12.5% acrylamide gel with a size marker and subjected to electrophoresis (EzRun C⁺; ATTO, Tokyo, Japan). The anti-vitellin heavy chain protein of B. mori antibody used in the present study was supplied by Dr Izumi (Yano et al., 1994). This anti-serum was from rabbits immunized by vitellin heavy chain. The secondary antibody was anti-rabbit IgG[H+L] (Goat), HRP Conjugate (Nacalai Tesque, Kyoto, Japan). Signals were detected using LAS-1000 (Fujifilm) in an ECL Advance Western Blotting Detection Kit (GE Healthcare UK, Buckinghamshire, UK).

RESULTS

Short-term results of embryo culture

Morphological changes of embryonic tissues
were evaluated to clarify the effect of FBS or silk-
worm egg extract on both cell migration and cell
growth. First, fragmented embryos were cultured at
25°C in IPL-41 medium without additives for 1
week to reduce the effects of any egg components
contained within the embryos. Then, D2 and D3
embryos were cultured at 25°C in IPL-41 medium
supplemented with 1% or 5% (v/v) FBS, D2 ex-
tract, or D3 extract. Ten embryos were cultured
under each condition. No morphological changes
were observed after 2 or 4 weeks of culture of D2
embryos (Fig. 1A, B, D, E, G, H). However, cell
migration and growth was observed after 12 weeks
culture of D2 embryos in media containing 5% D2
extract or 5% D3 extract (Fig. 1C, F); no changes
were observed for D2 embryos cultured in medium
containing 5% FBS (Fig. 1I). These results indicate
that it takes more than 8 weeks for cell migration
and growth to commence even when embryonic
tissues are cultured in IPL-41 medium containing
5% egg extract. Higher levels of cell migration and
growth were observed for embryos cultured in

![Fig. 1. Morphology of silkworm cells in a Day 2 (D2) embryonic primary culture after 2 (A, D, G), 4 (B, E, H) and 12 weeks
(C, F, I) culture. Fragmented embryonic tissues were cultured in 96-well plates and were cultured in the presence of (A–C) 5% D2
extract, (D–F) 5% D3 extract, or (G–I) 5% fetal bovine serum. Scale bar, 250 nm.](image-url)
media supplemented with 5% egg extract compared with culture media containing 1% egg extract or FBS (Table 1).

No morphological changes were observed after 2 or 4 weeks culture of D3 embryos (Fig. 2A, B, D, E, G, H); however, cell migration and growth were observed after 12 weeks for D3 embryos cultured in media containing 5% D2 extract, 5% D3 extract, or 5% FBS (Fig. 2C, F, I). Cell migration and growth were also visible in D3 embryonic tissues samples after 3 months culture in media containing either egg extract or FBS (Table 1). Supplementation of the culture medium with 5% egg extract induced marked cell migration and growth, whereas the effect of supplementation with 5% FBS was similar to that seen following supplementation of the culture medium with 1% egg extract (Table 1). These results suggest that the egg extracts have greater cell migration- and growth-promoting activity than FBS. In addition, D3 embryonic tissues are generally more responsive to the growth-promoting effects of egg extracts and FBS.

Fig. 2. Morphology of silkworm cells in a Day 3 (D3) embryonic primary culture after 2 (A, D, G), 4 (B, E, H) and 12 weeks (C, F, I) culture. Fragmented embryonic tissues were cultured in 96-well plates and were cultured in the presence of (A–C) 5% D2 extract, (D–F) 5% D3 extract, or (G–I) 5% fetal bovine serum. Scale bar, 250 nm.
than D2 embryonic tissues (Table 1).

**Long-term results of embryo culture**

After 5 months culture, the cell survival rate declined for D2 and D3 embryonic tissues grown with egg extract, whereas D3 embryonic tissues grown in FBS remained viable (Table 2). Thus, it appears that, unlike FBS, the bioactive molecules for cell growth and survival in egg extracts are less active at 5 months than at 3 months; however, different cell types are clearly seen in cells surviving at 5 months under each of the different culture conditions (Fig. 3). Thus, combining egg extracts with FBS may increase the cell survival rate and the diversity of established cell lines.

**Quantitative and qualitative changes of egg extracts**

Because the egg extracts were treated with protease inhibitors only, changes may occur in the composition of egg extracts over the culture duration. Thus, we determined qualitative and quantitative changes in important components of the egg extracts: yolk protein and ecdysteroids. The concentration of ecdysteroids and the composition of yolk protein in D2 and D3 egg extracts were compared between samples that had been stored at \(-30^\circ C\) (fresh extract) and those incubated for a further week at 25\(^\circ C\) (incubated extract).

Ecdysteroid concentrations increased in the incubated D2 extract compared with fresh extract, but concentrations decreased in the incubated D3 extract compared with fresh extract (Fig. 4A). Further, we analyzed changes in the whole protein profile by CBB staining (Fig. 4B) and in vitellin by immunoblotting analysis (Fig. 4C, left). The composition of yolk protein was almost the same in D2 and D3 extracts (Fig. 4B), except that two novel proteins were detected in the incubated extracts (Fig. 4B, arrows). Quantitative levels of vitellin were slightly different between D2 and D3 extracts (Fig. 4C, left). Moreover, another protein was recognized by the anti-vitellin heavy chain antibody in the incubated extracts (Fig. 4C, arrowhead). The position of this immunoreactive protein was consistent with that of the larger novel protein detected by CBB staining. Because the molecular weight of this protein was heavier than that of the vitellin heavy chain, this protein is not a partially degraded product. This protein was not identified in the present study, but its presence is interesting.

**DISCUSSION**

The present study has demonstrated that silkworm egg extracts promote cell migration and growth in primary cultures of silkworm early embryos. Importantly, the results show that the time period required to establish a silkworm cell line from a primary culture can be shortened from 6 months (Inoue and Mitsuhashi, 1984) to 3 months,

| Table 1. Cell migration and growth of embryonic tissue samples after 5 months culture in IPL-41 medium plus supplementation with fetal bovine serum or egg extracts |
|-----------------|-----------------|-----------------|
|                 | D2 embryos      | D3 embryos      |
| Supplement      | 1%              | 5%              | 1%              | 5%              |
| FBS             | 1/10            | 1/20            | 2/10            | 6/20            |
| D2 egg extract  | 1/10            | 8/20            | 4/10            | 13/20           |
| D3 egg extract  | 2/10            | 11/20           | 3/10            | 12/20           |
| Total           | 20/60           | 31/60           |

Data show the number of wells in which migration and growth were observed/total number of wells.

FBS, fetal bovine serum; D2, D3, Day 2 and 3 egg extract, respectively.

| Table 2. Survival of embryonic tissue samples after 5 months culture in IPL-41 medium plus supplementation with fetal bovine serum or egg extracts |
|-----------------|-----------------|-----------------|
|                 | D2 embryos      | D3 embryos      |
| Supplement      | 1%              | 5%              | 1%              | 5%              |
| FBS             | 0/1             | 0/1             | 2/2             | 6/6             |
| D2 egg extract  | 0/1             | 3/7             | 3/8             | 5/13            |
| D3 egg extract  | 0/2             | 5/9             | 5/11            | 2/12            |
| Total           | 8/20            | 13/31           |

Data show the number of wells in which migration and growth were observed after 5 months/that after 3 months.

FBS, fetal bovine serum; D2, D3, Day 2 and 3 egg extract, respectively.
because fully grown cells were observed in the culture at this time.

Differences in cell migration and growth were observed between D2 and D3 embryonic tissues after 3 months in culture. D2 embryonic tissues were only slightly stimulated by FBS or 1% egg extract, but showed a marked response to 5% egg extract. In contrast, D3 embryonic tissues were stimulated by FBS, D2 egg extract, and D3 egg extract, with higher levels of stimulation observed with higher concentrations of supplement. The difference in the response of D2 and D3 embryonic tissues to the supplements may be due to differences in the developmental stage of the embryos. D2 embryos are at the spatula formation stage, whereas D3 embryos are at the abdominal appendage formation stage (Takami and Kitazawa, 1960; Hong et al., 2006). At the spatula formation stage, organogenesis has only just started and mesoderm induction is weak; however, at the abdominal appendage formation stage, the neural groove has already formed and mesoderm induc-

Fig. 3. Effect of fetal bovine serum (FBS), Day 2 (D2) egg extract, or Day 3 (D3) egg extract on cell migration and growth of D2 and D3 embryonic tissues after 5 months in culture. The culture conditions were as follows: (A) D2 embryo, D2 extract 5%; (B) D2 embryo, D3 extract 5%; (C) D3 embryo, FBS 1%; (D) D3 embryo, FBS 5%; (E) D3 embryo, D2 extract 1%; (F) D3 embryo, D2 extract 5%; (G) D3 embryo, D3 extract 1%; (H) D3 embryo, D3 extract 5%. Scale bar, 100 nm.
tion is complete. In addition, at this stage, organogenesis is progressing and the abdominal appendages are formed. Therefore, the differences in cell growth observed between D2 and D3 embryos suggest that responsiveness to bioactive molecules and cell growth capability is dependent on the stage of embryonic development.

We found that the concentration of ecdysteroids in D2 egg extract increased after incubation of the extract at 25°C for 1 week. Moreover, an unidentified protein was recognized by the anti-vitellin heavy chain antibody in both D2 and D3 incubated extracts. Because the molecular weight of this protein signal was above that of the vitellin heavy chain, this signal may be due to some modifications of vitellin. These differences may be responsible for the different effects of the extracts on embryonic culture. The major components of silkworm eggs during early development are the yolk proteins, with the major silkworm yolk proteins being vitellin, egg-specific protein, and 30-kDa protein (Yamamoto and Takahashi, 1993). It is known that, in the silkworm, embryonic-specific proteinases degrade yolk proteins and that these
modified proteins can promote embryo development (Kunudson and Buckley, 1977; Yamahama et al., 2003). In addition to these proteins, specific modified ecdysteroids have been identified in early developmental stage embryos (Sonobe and Yamada, 2004). It has recently become clear that vitellin functions as a reservoir of maternal ovarian ecdysteroid phosphates in addition to being a nutritional source during embryonic development (Yamada et al., 2005). Ecdysteroid phosphates are dephosphorylated and function as active ecdysteroids during early embryogenesis (Yamada et al., 2005). Recent studies have also revealed that silkworm egg extract can induce the conversion of BmN4 cells into adipocytes (Akiduki and Imanishi, 2007). Taken together, these observations suggest that the egg extract exerts multiple effects, because cell growth and cell differentiation do not occur simultaneously and distinct signal cascades promote these phenomena. Therefore, these proteins and ecdysteroids are candidates for compounds that promote cell growth and cell differentiation in early embryonic tissues and established cell lines. It is important to clarify the functions of these molecules to further our understanding of early embryonic development.

In the present study, silkworm embryos were randomly fragmented owing to the crudeness of the dissection tool. Thus, many morphologically different cell types were observed and some samples showed no response (Fig. 1; Table 1). More precise dissection of embryos should enable observation of specific cell and tissue responses during culture. However, the molecular mechanism underlying the early development of the silkworm is currently not well understood, so a primary culture system for embryos, such as the one described herein, could be a useful tool for the analysis of molecular mechanisms and cellular responses.

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