JOURNAL OF THE EAST ASIAN SOCIETY OF DIETARY LIFE
Vol. 4, No. 3(1994)


# A Growth-Stimulating Protein in Cow's Milk

**Eun-Kyung Ki[1], Yong-Bok Park[1] and Myung-Sook Choi[2*]**

[1]Department of Genetic Engineering, College of Natural Sciences,
[2]Department of Food Science and Nutrition, College of Human Ecology,
Kyungpook National University, Taegu 701-702, Korea

**To whom correspondences should be addressed*

## Abstract

Growth-stimulating effects of cow's milk was examined using Vero cell culture. Medium containing whole cow's milk stimulated cell growth about the same degree as that containing fetal bovine serum. The growth-stimulating factor in cow's milk was purified using hydrophobic (phenyl-sepharose) and gel filtration (Sephadex G-100) column chromatographies. It appeared that the factor is a highly hydrophobic protein, since the major growth-stimulating activity was found in the fractions eluted with 50% ethylene glycol from the phenyl-sepharose column during the purification. The purified factor showed a single band on the polyacrylamide gel electrophoresis in the presence of 1 % (w/v) SDS. The factor has been found to have a relatively high molecular weight in the range of about Mr=100,000-150,000. In the presence of the purified factor (5%, w/v) in the culture medium, the incorporation of [3H]-thymidine into the cells was increased approximately 2,400-fold over that in the presence of 5% (w/v) fetal bovine serum. It seems that the growth-stimulating factor purified in this study is one of the major growth factors in the cow's milk.

Key word : growth stimulating factor, cow's milk.

## Introduction

Sera, obtained by allowing blood to clot, are widely used as a source of growth factor in the cell culture. Many factors in the serum capable of inducing cell growth in the culture have been isolated and studied.

Milk is another body fluid involved in growth and development. Milk, the secretion of the mammary gland, is composed of constituents of precu-

rsors from blood plasma ; accordingly, hormones that are transported in blood may be detectable in milk(1-3). This has generally been proven for protein and steroid hormones. Recent studies have shown that human breast milk also contains some biologically active growth factors (4-8). We previously observed that human breast milk contains a potent substance(s), which molecular weight ranges 10,000-100,000, capable of stimulating the fetal aortic proliferation(9). In this study, we characterized the growth-stimulating abilities of cow's milk by comparing with those in fetal bovine serum using vero cell(African green monkey cell line) culture. We also obtained a protein responsible for the stimulation of the cell growth in a highly purified state.

## Materials and methods

### Preparation of milk

Cow's milk was collected from a local farm on 15 days after delivery and cell-free milk was prepared using following procedure. Fresh milk was centrifuged at 1000× g for 15 min at 4 C to remove creamy layer and the supernatant was recentrifuged for 30 min under the same conditions to remove micelles and colloidal substances. The supernatant from the second centrifugation was filtered through the millipore membrane (0.2 μm pore) and kept at -20℃ until used.

### Cell Culture

Frozen vero cells were thawed, diluted to 2 × $10^4$ cell/ml and transferred into Falcon plates (Beckon Dickinson and Co, Maryland, USA). The cells were cultured in Medium 199 (Gibco, Grand Island Biological Co, NY, USA) supplemented with penicillin (20,000 U/ml), streptomycin (20,000 U/ml), amphotericin B (2.5 μℓ/ml), sodium deoxycholate (2.05 μg/ml), heat-inactivated (for 30 min at 65℃) fetal bovine serum (FBS, 5%, v/v, Gibco). For experiments, the Medium 199 was prepared in the same manner except replacing FBS for milk or various milk fractions. Dishes containing culture medium just enough to cover the dish surface were placed in a humidified incubator at 37℃ with regulated supply of 5% $CO_2$. At desired time, the culture medium was replaced with fresh medium. When cellular outgrowth began in medium 199 supplemented with 5% FBS, dishes were randomly devided into control and milk groups and media were substituted with 5% FBS and various milk fractions, respectively. When necessary, 1 μCi/ml of [$^3$H]-thymidine (73 Ci/mmol in 70% ethanol, ICN Radiochemical, Irvine, CA, USA) was added to medium for evaluation of cellular proliferation. The radioactivity incorporated in the cells was measured as an indication of cell proliferation. After an incubation period, the medium was removed and the cells were washed 3 times with nonradioactive PBS(phosphate buffered saline) and detached from the plates using 0.05% trypsin, 0.02% EDTA in PBS solution. Cells were diluted to a final volume of 1.2 ml with PBS. An aliquot of the sample (200 μl) was used for cell counting using the hemacytometer. Protein content was measured by the method of Bradford (10) after the sample was sonicated in the ultrasonicator using the defatted bovine serum albumin as a standard. Absorbance measurement at 280 nm (Hitachi Model 100-60) was used for the brief estimation of protein contents in the samples.

### Purification of Gowth Fctor in Milk

Growth-stimulating factor was purified from the cow's milk using phenyl-sepharose and gel filtration column chromatographies. Briefly, the centrifuged milk was loaded on a phenyl-sepha-

rose (Bio-Rad Laboratories, Richmond, Ca. USA) column (2.5× 15 cm) which was previously equilibrated with PBS, pH 7.4 and the column was washed with the same buffer until absorbance at 280 nm was negligible. Proteins bound to the column was eluted sequentially with deionized water, 50% ethylene glycol and 75% ethylene glycol in order. The fraction that expressed the highest growth-stimulating activity was collected and applied to Sephadex G-100 (Bio-Rad Laboratories) column (1.5× 90cm). The gel filtration column chromatography was conducted with a flow rate of 20 ml/hr using PBS buffer, pH 7.4. The purified milk fractions were used as a substituted component for FBS in the media to test cellular proliferation.

### Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (12%, w/v) was carried out in the presence of 1% SDS using low molecular weight standards as size markers (Pharmacia). The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue (Pharmacia) in methanol-glacial acetic acid-distilled water (3 : 1 : 6, v/v) and destained with methanol-glacial acetic acid (4 : 1, v/v) solution.

## Results and discussion

Vero cells ($2\times10^4$ cells) were grown in Medium 199 containing FBS (5%, v/v) for 18 hours and dishes were devided into 3 groups, FBS, milk and decasined milk media. Culture was continued for next 35 hours in the presence of the respective media (5%, v/v). Radiolabeled thymidine was added at 53 hours of culture and cultured for 19 hours thereafter. Cells reached at apparent confluence when cultures were terminated. After 3 days of culture, cells were harvested and the growth-stimulating effect of media was analyzed. Table 1 shows the effects of milk in cell growth. Total number of cells was significantly higher in milk group : thus, cell density was higher in milk group than FBS or decaseined milk groups. It was apparent that cell proliferation occurs more effectively, in its numbers, for milk group than FBS group. When DNA synthesis was expressed as cpm of [$^3$H]-thymidine incorporated into the cells per unit mass of cellular protein, it was slightly higher in milk group than FBS or decaseined milk groups. Figure 1 shows growth curves of cells grown in FBS and milk media. Cells of milk group reached to confluence with slower rate than FBS group, but final number of cells was higher than that in FBS groups. As shown in Fig. 2, the rate of protein synthesis by cells was greater in milk group than FBS group. It was found that stimulation of protein synthesis was more effective by milk media than by FBS media. The concentration of cellular proteins was continuously increased in milk group during entire period of culture and was significantly higher ($p<0.005$) than FBS group as shown in Fig. 2. Rates of protein synthesis were 1.1 g/hr and 0.625 g/hr in milk group and FBS group

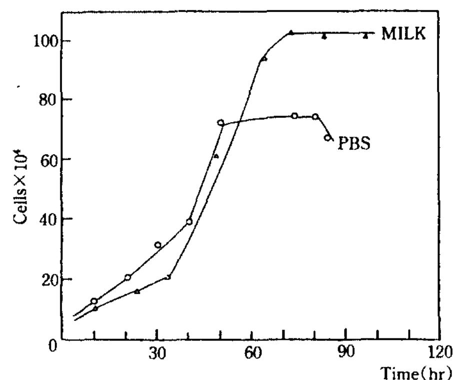


Fig 1. Stimulation of vero cell division by 5% milk and 5% FBS media. After every 12 hr, the cells were detached and the numbers of cell were counted. Each point represents the mean of cell number from 3 dishes.

Table 1. Effects of milk in vero cell culture

| media | CPM／1.2ml | Protein conc. in media | Total cell numbers | CPM／protein conc. in cells | DNA* synthesis |
|---|---|---|---|---|---|
| | $\times 10^2$ | mg | $\times 10^5$ | CPM/ μg | CPM |
| 5% FBS | 1808 ± 492[a] | 165.5[a] | 2.17 ± 0.02[a] | 1073.6 ± 78.9[a] | 1073.6 ± 78.9[a] |
| 5% milk | 1174 ± 353[b] | 71.0[b] | 3.01 ± 0.02[a] | 975.1 ± 60.1[ab] | 2242.7 ± 92.8[b] |
| 5% decasinated milk | 1053 ± 380[b] | 93.0[b] | 1.30 ± 0.01[b] | 884.9 ± 45.7[b] | 1592.8 ± 86.1[c] |

After the first 18 hr culture in control media (FBS media), dishes are devided into 3 groups and cultured for 35 hr. Radiolabeled thymidine was added at this time and cultured 19 hr.
* : CPM/protein conc. is normalized with protein conc. in each media. n ( number of dishes) = 5
Each values represents the mean ± SEM.
Numbers which do not share superscripts in same column are significantly different at $P<0.05$.

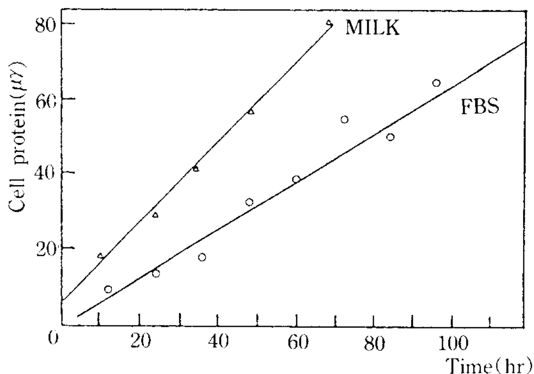


Fig 2. Stimulation of protein synthesis during cell division by 5% milk and 5% FBS media. After every 12 hr, the cells were detached and the cellular protein conc. are measured. Each point represents the mean of protein concentration from 3 dishes.

when calculated from Fig 2. From the elution profile from the phenyl-sepharose column chromatography in Fig. 3, fractions were pooled as indicated to test the growth-stimulating activity in the cell culture with a final concentration of 5% (v/v). As shown in table 2, protein concentrations in FBS media was significantly higher than milk media (166.5 mg/ml vs. 1.9 - 16.9 mg/ml in milk groups). Among the milk fractions, pool 3 and 4 were most effective in DNA synthesis when expressed as specific radioactivities. Electrophoretic pattern of these milk fractions are shown in Fig. 4. Pool 3 from phenyl-sepharose column chromatography (lane 5 on Fig. 4) was concentrated and applied to Sephadex G-100 column chromatography. Four fractions, pool 1′-4′ in Fig. 5, were pooled and concentrated to test the growth-stimulating activity in the cell culture. As the result (Table 3), pool 2′ was most effective in DNA synthesis. This fraction, pool 2′, was a compound with molecular weight of 100,000-150,000 (Fig. 7) when identified by gel electrophoresis.

Casein in cow′s milk appears to be present almost exclusively in micellar form. Therefore, casein does not appear to be one of the growth factors involved in this experiment. Several types of hormone-like growth-promoting factors have recently found in human milk (7, 11-13). Among them are epidermal growth factor (EGF), insulin, somatomedin-C (insulin-like growth factor), and transforming growth factor (TGF). These compounds are predominantly of low molecular weight compounds and are usually classified as peptides ; therefore they are different from the gro

Table 2. Effects of milk fractions (pooled from phenyl-sepharose column chromatography) in DNA synthesis of vero cell culture

| groups(media) | protein conc. in media | CPM/protein conc. in cell | Normalized DNA synthesis |
|---|---|---|---|
| | mg | CPM/ μg | CPM |
| I (FBS) | 165.5[a] | 1876 ± 120[a] | 1876 ± 120[a] |
| II (milk) | 19.0[a] | 139 ± 21[b] | 1208 ± 48[a] |
| III (pool 1) | 16.9[b] | 625 ± 53[c] | 6128 ± 55[b] |
| IV (pool 2) | 7.9[c] | 1073 ± 59[d] | 22432 ± 180[c] |
| V (pool 3) | 5.4[c] | 1238 ± 43[d] | 37936 ± 209[d] |
| VI (pool 4) | 1.9[d] | 361 ± 29[b] | 31495 ± 219[c] |

After the first 18 hr culture in control media (FBS media), dishes are devided into 3 groups and cultured for 35 hr. Radiolabeled thymidine was added at this time and cultured 19 hr. Refer fig 3 for designation of pool 1, 2, 3 and 4.

* : CPM/protein conc. is normalized with protein conc. in each media.

n ( number of dishes) = 5

Each values represents the mean ± SEM.

Numbers which do not share superscripts in same column are significantly different at $P<0.05$.

Table 3. Effects of milk fractions (pooled from gel filtration column chromatography) in DNA synthesis of vero cell culture

| groups(media) | protein conc. in media | CPM/protein in cell | Normalized DNA synthesis |
|---|---|---|---|
| | mg | CPM/ μg | CPM |
| A (FBS) | 165.5[a] | 2228 ± 134[a] | 2228 ± 134[a] |
| B (milk) | 47.3[b] | 699 ± 39[b] | 2447 ± 123[a] |
| C(pool 1') | 30.1[b] | 422 ± 31[c] | 2329 ± 161[a] |
| D(pool 2') | 0.05[c] | 1623 ± 115[a] | 5370475 ± 3009[b] |
| E(pool 3') | 24.3[d] | 314 ± 19[c] | 2139 ± 127[a] |
| F(pool 4') | 16.2[d] | 205 ± 17[d] | 2094 ± 135[a] |

After the first 18 hr culture in control media (FBS media), dishes are devided into 3 groups and cultured for 35 hr. Radiolabeled thymidine was added at this time and cultured 19 hr. Refer fig 5 for designation of pool 1', 2', 3' and 4'.

* : CPM/protein conc. is normalized with protein conc. in each media.

n ( number of dishes) = 5

Each values represents the mean ± SEM.

Numbers which do not share superscripts in same column are significantly different at $P<0.05$.

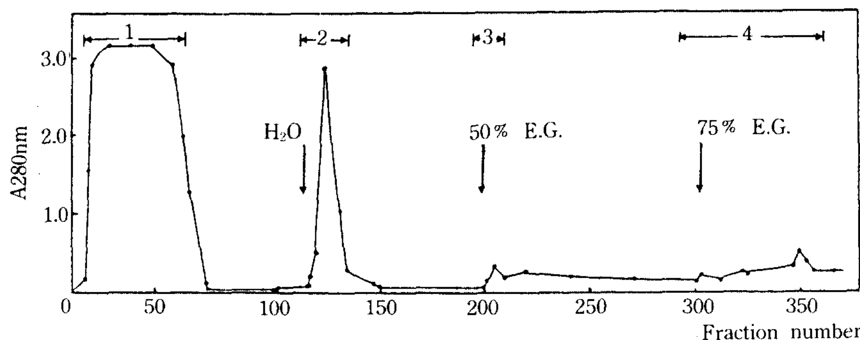


Fig 3. Elution profile of the phenyl-sepharose column chromatography. Centrifuged milk was loaded on the column, washed with PBS and eluted with distilled water, 50% ethylene glycol and 75% ethylene glycol sequentially at arrow position. Milk fractions were collected 2ml per tube.

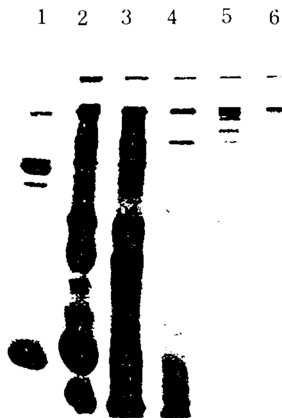


Fig 4. Electrophoretic pattern of milk fractions from phenyl-sepharose column chromatography. Lane 1 : stansard (BSA, lysozyme), Lane 2 : centrifuged milk, Lane 3 : pool 1, Lane 4 : pool 2, Lane 5 : pool 3, Lane 6 : pool 4.

wth factors we found from this experiments. We have isolated a new growth factor from the cow's milk which was very efficient in stimulating cell growth. This growth factor we have isolated has not been named yet, since it needs to be further studied for amino acid sequencing and other characterization.

The concentrations of the growth factors isolated so far are very low ; however, as hormones they certainly may have pronounced physiological activity even at low concentration. Growth factors can affect mucosal cell growth (and mass) and gastric pH and may consequently exert an effect on the absorption of nutrients(14). We have been working on investigation of physiological role of milk including function of growth factors and lipid-lowering factors present in milk. It can be expected that this rapidly emerging research area will give us significant information with on the importance of human milk or cow's milk for gut proliferation and maturation.

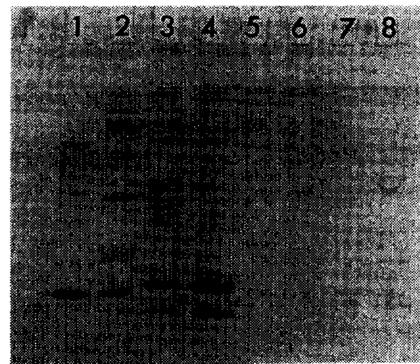


Fig 6. Electrophoretic patterns with the samples from sephadex G-100 column chromatography. Lane 1 : high Mwt stansard, Lane 2 : low Mwt standard, Lane 3 : centrifuged milk, Lane 4 : sample loading-washing, Lane 5 : eluate with distilled water, Lane 6 : pool 1', Lane 7 : pool 2', Lane 8 : pool 3'.

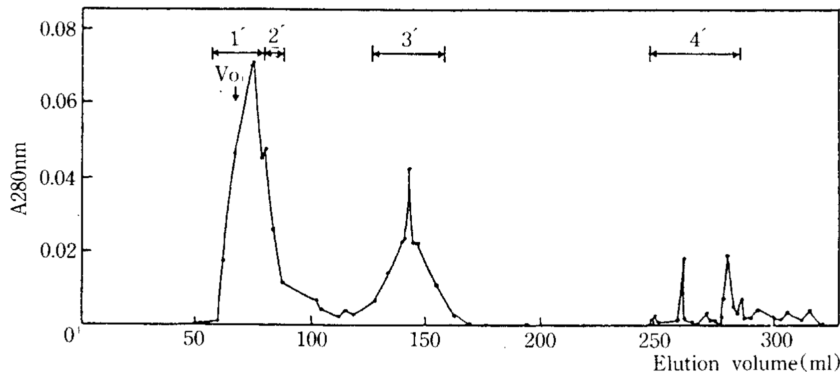


Fig 5. Elution profile of sephadex G-100 column chromatography with the pool 3 fraction from the phenyl-sepharose column chromatography. The elution buffer (PBS, pH 7.4) was used throught the chromatography. Two ml fractions were collected with the flow rate of 20 ml/hr.

## 요 약

이 실험에서는 소의 유즙에 있는 성장인자를 세포배양을 통해 확인하고 크로마토그래피를 사용하여 분리 정제 하였다. 5% 의 우유를 포함하는 배지를 FBS(fetal bovine serum)를 포함한 배지와 비교했을때 African green monkey kidney cell 의 성장 촉진 효과가 비슷하게 나타났으며, 우유의 성장인자를 분리 정제한 결과 FBS 배지에서보다 2,000 배 이상의 성장 촉진 효과가 나타났다. 이 성장인자는 hydrophobic column(phenyl-sepharose)과 gel-filtration column(sephadex G-100)을 사용하여 분리했으며 그 결과 milk-deriven growth factor 는 phenyl-sepharose 에서 50% ethylene glycol step 에서 용출되므로 hydrophobic protein임이 증명되었고 size exclusion column chromatography 로 부터 분자량이 100,000-15,0000 범위의 고분자 물질임이 밝혀졌다. 또한 우유의 성장인자는 매우 다양하며 그중 본 실험으로부터 정제된 물질이 주요 성장인자로 밝혀졌다.


## References


1. Schams, D., and H. Karg. Hormones in milk, Annals New york academy of sciences, 464, 75, 1984.
2. Mulloy, A. L., and P. V. Malven. Relationship between concentrations of porcine prolactin in blood serum and milk of lactating sows, J. Animal Science, 48, 876, 1979.
3. Malven, P. V. and I. R. Falconer. Transfer of prolactin from plasma into milk and associated physiological benefits to mammary cells, Endocrionol. Exp. 17, 283, 1983.
4. Bano, M., D. S. Salmon and W. R. Kidwell. Purification of amammary-derived growth factor from human milk and human mammary tumors. The Journal of Biological Chemistry, 260, 5745, 1985.
5. Subbiah, M.T.R., R. L. Yunker, M. Yamamoto, B. A. Kottke, and L. K. Bale. Human breast milk stimulates prostaglandin synthesis in cultured human skin fibroblasts, Biochemical and Biophysical Research Communications, 129, 972, 1985.
6. Bydlowski, S. P., R. L. Yunker, B. N. Campaigne, U. R. Kotogal and M.T.R. Subbiah. Human breast milk contains a factor which markedly stimulates aortic prostacyclin synthesis, Biochimica et Biophysica Acta, 878, 1, 1986.
7. Carpenter G. Epidermal growth factors is a major growth-promoting agent in human milk, Science, 210, 198, 1980.
8. Klagsbrun, M. Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts, Proc. Natl. Acad. Sci., 75, 5057, 1978.
9. Park, M.S.C. and Subbiah, M.T.R. Human breast-milk factors influencing lipid metabolism by fetal rabbit aorta in organ culture. American Journal of Clinical Nutrition, 48, 963, 1988.
10. Bradford, M. M. A rapid and senstive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Annals of Biochemistry, 72, 248, 1976.
11. Baxter, R.C., Z. Zaltsman, J. R. Turtle. Immunoactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk, J. Clin. Endocrinol. Metab. 58, 955, 1980.
12. Noda, K., M. Umeda and T. Ono. Transforming growth factor activity in human colostrum, Gann 75, 109, 1984
13. Read, L. C., F. M. Upton, G. L. Francis, J. C. Wallace, G. W. Dahlenberg and F. J. Ballard. Changes in the growth promoting activity of human milk during lactation, Pediatric Research, 18, 133, 1984.
14. Lonnerdal, B. O. Biochemistry and physiological function of human milk proteins, The American Journal of Clinical Nutrition, 42, 1299, 1985.