Identification and Partial Purification of a Basic Fibroblast Growth Factor-Like Growth Factor Derived from Bovine Colostrum

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ABSTRACT

Bovine colostrum that had been collected up to 6 h postpartum was fractionated by ammonium sulfate precipitation, and various fractions were examined for basic fibroblast growth factor activity. Activity that stimulated cell growth was detected in the cream fraction, which was purified by isoelectric focusing and heparin affinity chromatography. Three peaks were eluted from the heparin affinity column at approximately 0.5, 1, and 1.75 M NaCl. Although activity that stimulated cell growth was detected in the second and third peaks, a reaction with antibasic fibroblast growth factor antibody was observed only in the third peak. Fractions in the second and third peaks were examined by SDS-PAGE and Western blot analysis. Activity that stimulated cell growth was detected in the second and third peaks; however, after Western blot analysis using antibasic fibroblast growth factor, only the third peak yielded positive bands at 15 and 28 kDa. These fractions were further subjected to a neutralization test using antibasic fibroblast growth factor antibody. The activity that stimulated cell growth in the second peak was virtually unchanged; however, the activity in the third peak was diminished, showing a relative activity of less than 10% at 1.25 μg/ml. Therefore, neutralization of the activity that stimulates cell growth by antibasic fibroblast growth factor antibody suggests that the third peak, which was eluted at approximately 1.5 to 2 M NaCl in heparin affinity chromatography, might be a basic fibroblast growth factor-like growth factor. (Key words: bovine, colostrum, fibroblast growth factor, heparin-sepharose affinity chromatography)

Abbreviation key: C = cream fractions (used with number indicating separation by 50 and 100% saturated ammonium sulfate), FGF = fibroblast growth factor, H = heparin affinity (used with number indicating fractions 1 and 2), MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, W = whey fractions (used with number indicating separation by 50, 75, and 100% saturated ammonium sulfate).

INTRODUCTION

Milk contains an abundance of physiologically active proteins that defend against infections (3, 4, 9, 15) that are associated with nutrition and metabolic control (1, 2, 5, 23, 26, 34). Human milk or bovine colostrum in place of serum in cell cultures supports normal growth of various cell types, such as epithelial cells, fibroblasts, and smooth muscle cells (19, 22, 39). Physiologically active substances have been found in trace amounts at specific stages during lactation: epidermal growth factor (30), insulin-like growth factor (6), growth factor-like growth factor derived from platelets (36, 37), transforming growth factor-α (28), and transforming growth factor-β (8, 16). Apart from these growth factors, basic fibroblast growth factor (FGF), which is present universally in the body (10, 11), has been reported in human milk (14). However, Schams (33) conducted immunological studies on bovine milk using antibodies against basic FGF and detected substances that were positive for antibasic FGF ranging from 0.5 to 1 ng/ml of milk during the lactation. Kirihara and Ohishi (21) detected a 35-kDa protein that reacted with antibasic FGF antibody in the cream fraction of bovine colostrum. Those researchers (21) proposed that the protein was a basic FGF-like bovine colostrum growth factor and reported that this factor was almost absent in the skim milk or whey fractions and could not be detected in the milk after 3 d postpartum. Those reports suggested that a basic FGF-like growth factor is present in bovine milk, as it is in human milk. Because the basic FGF-like growth factor has been
detected only by immunological methods using antibasic FGF antibody and because this factor has not been isolated, its character remains largely unknown. In this study, we identified partially purified basic FGF-like growth factor and characterized the bovine colostral fraction that contained the basic FGF-like growth factor.

**MATERIALS AND METHODS**

**Purification of Growth Factor**

**Milk samples.** Colostrum samples were obtained within 6 h after parturition from healthy Holstein cows on Sato Farm (Hannou, Saitama, Japan) and were stored at −80°C until assayed.

**Whey and cream fractions of bovine colostrum.** Defrosted colostrum was diluted with 3 vol of deionized water and was centrifuged (3000 × g for 30 min at 4°C) to separate the skim milk and cream fractions. The skim milk was adjusted to pH 4.5 using 1 M HCl and was incubated at 37°C for 30 min. The resulting casein aggregates were removed by centrifugation. The resulting whey (W) was fractionated further using ammonium sulfate precipitation, yielding 50, 75, and 100% saturated ammonium sulfate fractions (W50, W75, and W100, respectively). The cream fraction was diluted with 3 vol of 3 M NaCl and was mixed by a homogenizer. The butter mass was removed after centrifugation to produce a buttermilk fraction. Caseinate was removed as in skim milk to obtain a cream extract fraction (C). Ammonium sulfate was added to prepare 50 and 100% saturated ammonium sulfate fractions (C50 and C100, respectively) (Figure 1). The various fractions were dissolved in deionized water, dialyzed against deionized water, and lyophilized.

**Isoelectric focusing.** Isoelectric focusing was performed using Rotofor® (Bio-Rad, Richmond, CA) in the presence of 2% (vol/vol) carrier ampholyte (pH 3 to 10), which was electrophoresed at a constant current of 12 W at 4°C for 5 h. The fractions were collected (2.5 ml each) after electrophoresis, and the pH and absorbance at 280 nm of each fraction were measured. After the buffer was replaced with 25 mM PBS, each fraction was assayed for activity that stimulated cell growth.

**Heparin affinity chromatography.** The fractions that stimulated cell growth activity (fraction numbers 18 to 20) as detected by isoelectric focusing were pooled. After the buffer was replaced with 10 mM Tris-HCl-buffered saline (pH 7.2), the preparation was loaded onto a heparin-Sepharose CL-6B (Pharmacia Biotechnology, Uppsala, Sweden) column (1.5 × 10 cm) equilibrated with the same buffer. The column was washed with the same buffer and eluted with a linear gradient of 0.15 to 3 M NaCl; the eluted fraction was collected in volumes of 1 ml per tube.

Each fraction was examined for reactivity with antibasic FGF antibody and for activity that stimulated cell growth. The fractions showing any activity that stimulated cell growth were pooled as the heparin affinity (H) fraction 1 (H1; fraction numbers 51 to 64) and 2 (H2; fraction numbers 70 to 100). These fractions were dialyzed against deionized water and lyophilized.

**SDS-PAGE and Electroblotting**

The SDS-PAGE was performed according to the methods of Laemmli (25). A precast gradient gel (1 × 80 × 90 mm) containing 10 to 20% (wt/vol) acrylamide was used. Each sample was electrophoresed for 90 min at constant current of 20 mA per gel. After electrophoresis, the protein in the gel was stained using Coomassie Brilliant Blue R (Pharmacia Biotechnology).

Electroblotting was performed using a semidry method (24, 31); polyvinylidene difluoride membrane was the transfer membrane, and 25 mM Tris-HCl containing 192 mM glycine and 20% (vol/vol) methanol was the transfer buffer. The transfer was...
performed using a constant current of 2 mA/cm² for 120 min. The transfer membrane was stained by Coomassie Brilliant Blue R or was subjected to Western blot analysis.

Western Blot Analysis

The membrane that had undergone electroblotting was blocked with 1% (wt/vol) human serum albumin (Sigma Chemical Co., St. Louis, MO). Anti-bovine brain basic FGF antibody (R&D Systems, Inc., Minneapolis, MN) was used as the first antibody, and anti-rabbit IgG antibody conjugated with horseradish peroxidase (R&D Systems, Inc.) was used as the second antibody. The Western blot analysis was developed using the substrate diaminobenzidine.

Detection of Basic FGF by Surface Plasmon Resonance

Surface plasmon resonance (12, 13, 18) (BIAcore™ Pharmacia Biosensor AB; Pharmacia Biotechnology) was used to detect basic FGF. Immobilization of antibody on the sensor chip was performed according to the Pharmacia protocol using 100 μl of 10 mM acetate-buffered saline (pH 4.5) containing 100 μg/ml of anti-bovine brain basic FGF antibody. A sample that had been fractionated according to the previously mentioned methods was desalted using an ultrafiltration membrane cartridge (5-kDa cutoff) and replaced with 10 mM HEPES-buffered saline containing 3.4 mM EDTA (pH 7.4). An aliquot of 25 μl of each sample was reacted with the sensor chip for basic FGF measurement.

Cell Culture and Assay for Activity that Stimulated Cell Growth

The NIH 3T3 cells, obtained from Nippon Medical School (Kawasaki, Kanagawa, Japan), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% (vol/vol) fetal bovine serum, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin; the cells were then incubated at 37°C under 5% CO₂.

Activity that stimulated cell growth was assayed by the addition of various concentrations of a sample to 1 × 10³ cells per well in a 96-well microplate and incubating the plates for 3 d at 37°C in 5% CO₂. The cell number in each well was estimated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (27) (Chemicon International Inc., Temecula, CA), and the relative activity was calculated. In brief, after 10 μl of the MTT reagent were added to each well and incubated at 37°C for 4 h, 100 μl of the SDS and HCl solution [10% (wt/vol) SDS and 10 mM HCl] were added and incubated at 37°C for 16 h. The difference in absorbance of each well at 560 and 690 nm was measured using a microplate reader. Relative activity (RA) of cell growth was calculated by the following expression:

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RA\ percentage = \left(\frac{OD+}{OD-} - 1\right) \times 100
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where \(OD = \) optical density with (+) or without (−) growth factor.

Affect on Activity that Stimulated Cell Growth by Antibasic FGF Antibody and Dithiothreitol

A sample was diluted to various concentrations in a medium containing 1 μg/ml of antibasic FGF antibody or 5 mM dithiothreitol and incubated at 37°C for 30 min. The sample dilutions were then added to the cells in a microplate that was prepared similarly to that for the assay for activity that stimulated cell growth. After 3 d of incubation, cell growth was measured by the MTT assay.

Assay of Protein Concentration

The protein concentration was measured using Bio-Rad protein assay (Bio-Rad). Bovine serum albumin was used as the standard.

RESULTS

Basic FGF-like growth factor was purified from bovine colostrum that had been collected up to 6 h postpartum. Whey and cream fractions were prepared by the ammonium sulfate precipitation method, and activity that stimulated cell growth was assayed in each fraction. An extremely low activity of 5 to 10% cell growth at 10 μg/ml was detected in the W50, W75, W100, and C100 fractions. In the C50 fraction, however, a high activity (approximately 30% relative activity) at 5 μg/ml was observed, which was 1/25 of the activity of the commercially available bovine basic FGF standard derived from the brain (R&D Systems Inc.) (Figure 2). These fractions were subjected to SDS-PAGE (Figure 3A) and Western blot analysis (Figure 3B). Bands at approximately 15 and 28 kDa were detected in the C50 fraction, but no reaction was detected in W50, W75, W100, or C100 by Western blot analysis (Figure 3B).

Fractions of C50 that were purified by isoelectric focusing were tested for any activity that would
stimulate cell growth. Activity was observed in fractions 13 and 14 (pH 5.64 to 6.27) and in fractions 18 to 20 (pH 8.72 to 10.49) (Figure 4). Because the isoelectric point of basic FGF is at pI 9 to 10 (10), fractions 18 to 20, which showed activity that stimulated cell growth, were purified by heparin affinity chromatography. The heparin affinity products were eluted with a linear gradient ranging from 0.15 to 3 M NaCl, and three peaks were eluted from the column at approximately 0.5, 1, and 1.75 M NaCl (Figure 5A).

Each fraction was tested for reactivity with anti-basic FGF antibody using surface plasmon resonance and activity that stimulated cell growth. Of the two peaks that were eluted near 1 M NaCl (H1; fractions 51 to 64) and 1.75 M NaCl (H2; fractions 70 to 100), only H2 showed both reactivity with anti-basic FGF antibody (Figure 5A) and activity that stimulated cell growth (Figure 5B). Although activity that stimulated cell growth was detected in the H1 fraction, the reactivity with antibody (resonance unit value) was very weak (Figure 5A). In H2, the resonance unit value and cell proliferation were in agreement and were distributed as a broad peak from 1.5 to 2 M NaCl.

Although several bands were detected by SDS-PAGE for both fractions (Figure 6A), H1 yielded a slight reaction with a molecular mass of 28 kDa in Western blot analysis. Fraction H2 showed bands with molecular masses of 15 and 28 kDa in the Western blot analysis, which were similar to the molecular masses of these bands found in the C50 fraction (Figure 6B).

Both H1 and H2 stimulated cell growth similarly. Both demonstrated activity beginning at a concentration of 0.06 µg/ml, which increased in a dose-dependent manner, producing a relative activity of 46% (H1) and 42% (H2) at 2 µg/ml (Figure 7). These two fractions were further subjected to a neutralization test using anti-basic FGF antibody or dithiothreitol. The anti-basic FGF antibody neutralized the activity of the basic FGF standard, resulting in a relative activity of less than 10%, except at 1 µg/ml, but dithiothreitol did not neutralize activity (Figure 8A). Activity in H1 was virtually unchanged by the addition of the antibody but was inactivated by the addition of dithiothreitol (Figure 8B). The activity in H2 was reduced by the addition of antibody...
Figure 4. Isoelectric focusing of cream fraction separated by 50% saturated ammonium sulfate and activity that stimulated cell growth of the fractions that were revealed with isoelectric focusing. The cream fraction separated by 50% saturated ammonium sulfate was prepared by isoelectric focusing. Each fraction was measured for pH (◊) and for absorbance at 280 nm (○) and was assayed for activity that stimulated cell growth (●) after the buffer was replaced.

Figure 5. Heparin affinity chromatography. A. Immunoreactivity of heparin affinity fractions. B. The cell growth that promoted activity of the heparin affinity fraction. The fractions that were revealed by isoelectric focusing (fractions 18 to 20; Figure 4), which had activity that promoted cell growth, were applied to a heparin-sepharose CL-6B (Pharmacia Biotechnology, Uppsala, Sweden) column and eluted with a linear gradient of 0.15 to 3 M NaCl (± ± ± ±). Each fraction was measured for absorbance at 232 nm (◇), for reactivity with antibasic fibroblast growth factor antibody (○), and for the activity that stimulated cell growth (●).

as in the basic FGF standard; less than 10% cell growth occurred up to 1.25 μg/ml, and approximately 13% occurred at 2.5 μg/ml. However, cell growth was not inactivated by the addition of dithiothreitol (Figure 8C). The activity in H1 was inactivated only with the addition of dithiothreitol, and, in H2, activity was neutralized with the addition of antibody but was not inactivated with addition of dithiothreitol as in the basic FGF. The neutralization of activity that stimulated cell growth in H2 using antibasic FGF antibody suggests that a basic FGF-like growth factor is present in the H2 fraction.

DISCUSSION

A positive immunological reaction and marked stimulation of cell growth were detected only in the C50 cream fraction. The whey fractions and the C100 fraction did not show immunological reaction or activity that stimulated cell growth. These findings support the report of Kirihara and Ohishi (21) that basic FGF-like growth factor in bovine colostrum was found mainly in the cream fraction. The C50 fraction was partially purified by isoelectric focusing and heparin affinity chromatography. The presence of a positive reaction with antibasic FGF antibody and with stimulation of cell growth were observed in the fractions that were purified by isoelectric focusing at pH 9 and higher. Two peaks were eluted at approximately 1 M NaCl (H1) and 1.75 M NaCl (H2) using heparin affinity chromatography (Figure 7). These peaks contained several components with molecular masses from 15 to 40 kDa in SDS-PAGE. Although the activity that stimulated cell growth in H1 and H2 was similar, the activity in H1 was lost by dithiothreitol treatment (Figure 8). Shing and Klagsbrun (36, 37) examined growth factors in bovine colostrum and identified a platelet-derived growth factor with a molecular mass about 30 kDa and a pI of 10, which was inactivated by dithiothreitol treatment. Fraction H1 had the same range of molecular mass and was inactivated by dithiothreitol treatment but was not neutralized by antibasic FGF, suggesting that H1 might contain growth factor derived from platelets.

The 15-kDa band was detected in the H2 fraction and was similar to the molecular mass of basic FGF (Figure 6A and B). The 28-kDa band showed positive reactions with antibasic FGF antibody in both heparin affinity fractions (H1 and H2) and in the C50 fraction. Therefore, in the C50 fraction, a positive reaction was also detected around the 100-kDa band. Spivak-Kroizman et al. (38) reported that acidic FGF preparations by binding with heparin. The oligomer revealed a molecular mass of 32 kDa and a higher mass under reducing conditions. Spivak-Kroizman et al. (38) also determined that basic FGF...
Basic FGF has strong affinity to heparin and heparan sulfate proteoglycan (20, 40) and may be bound to the heparan sulfate proteoglycan on the cell surface or in the extracellular matrix (7, 32). Kirihara and Ohishi (21) demonstrated that human basic FGF-like growth factor was present on the surface of mammary gland cell. Similarly, milk fat globule membrane containing heparan sulfate proteoglycan is present in milk (35), and milk fat globule membrane is derived from the plasma membrane of the mammary gland secretory cell (17, 29). Fukushima et al. (14) and Kirihara and Ohishi (21) demonstrated the synthesis of human milk basic FGF by mammary gland cells. Those reports and the results of this study suggest that the basic FGF in bovine colostrum might also be synthesized in mammary gland cells and might be bound to the heparan sulfate proteoglycan in the milk fat globule membrane. However, Schams (33) reported that an almost constant concentration of basic FGF-like growth factor was detected in bovine milk throughout lactation and suggested that this factor might have transmigrated from blood. The basic FGF could either be synthesized by the mammary gland or alternatively transmigrated from blood; this controversy requires further study. The physiological significance of the presence of basic FGF in colostrum is not clear. Perhaps basic FGF is involved in the stimulation of cell growth in the mammary gland or is used by the newborn for some undetermined proliferative function.
Figure 8. Neutralization of activity that stimulated cell growth by antibasic fibroblast growth factor antibody to heparin affinity fractions. Various concentrations of the samples were diluted in a medium containing 1 μg/ml of anti-bovine basic fibroblast growth factor antibody and added to the cells in a microplate to assay activity that stimulated cell growth. After 3 d of incubation, cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. A. Activity that stimulated cell growth of basic fibroblast growth factor with antibody (▲), with (partially shaded symbol) or without dithiothreitol (●). B. Activity that stimulated cell growth of heparin affinity fraction 1 with antibody (●), with (partially shaded symbol) or without dithiothreitol (○). C. Activity that stimulated cell growth of heparin affinity fraction 2 with antibody (●), with (partially shaded symbol) or without dithiothreitol (○).

REFERENCES