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Effect of Human Milk and Epidermal Growth Factor on Growth of Human Intestinal Caco-2 Cells

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ABSTRACT

Background: Epidermal growth factor (EGF) in human milk has been thought to be mitogenic for cell growth. This study investigated the effects of human milk and EGF on the growth of human intestinal Caco-2 cells to determine whether the action occurred through regulation of the cell cycle or through c-*jun* expression.

Methods: Cells were incubated with 5% human milk, 0.375 nmol/L EGF (relevant to EGF concentration in 5% human milk, 0.05 × EGF), 7.5 nmol/L EGF (1 × EGF), or 75 nmol/L EGF (10 × EGF). Cell numbers; cellular RNA, DNA, and protein concentrations; DNA content in the cell cycle, and expressions of c-Jun protein and mRNA were analyzed.

Results: Cell numbers increased in the 1 × and 10 × EGF groups at 48 hours. Cellular RNA increased in the 5% human milk and 10 × EGF groups. DNA and protein contents increased in the 1 × and 10 × EGF groups. The 1 × and 10 × EGF groups increased DNA content in the G1 phase compared with the 5% human milk group at 24 hours. The greatest c-jun protein expression was 2.6, 1.4, 1.8, and 1.9 times the control, and the c-jun mRNA increased by 202%, 14%, 150%, and 181%, respectively, in the 5% human milk, 0.05 ×, 1 ×, and 10 × EGF groups.

Conclusions: In a dose-dependent manner, EGF stimulated intestinal growth in vitro, by increasing DNA content in the G1 phase and c-jun mRNA expression. However, low concentrations of human milk (5%) and its equivalent EGF did not affect cell growth. ***JPGN 34:394–401, 2002.*** **Key Words:** Human milk—Epidermal growth factor—Caco-2 cells—Cell growth—c-Jun.

Human milk is rich in nutrients, hormones, growth factors, and immunoreactive molecules, which influence the growth, development, and immune status of newborn infants (1). In vivo and in vitro studies showed that human milk stimulates intestinal development (2), DNA synthesis, and cell proliferation (3). Epidermal growth factor (EGF), an acid polypeptide of 53 amino acids with 3 disulfide bonds, has been thought to be the main mitogen in human milk that stimulates cell division (4). The concentration of EGF is highest in colostrum and gradually decreases in mature milk to a concentration of approximately 5.0 to 6.7 nmol/L (5). Epidermal growth factor is acid stable and trypsin resistant, allowing it to survive passage through the gastrointestinal tract and to act directly on the intestinal cells (6). Immunohistochemical investigation found EGF receptors on the intestinal mucosa (7). Epidermal growth factor may regulate intestinal growth through the EGF receptor on the basolateral membrane (8).

Epidermal growth factor can induce tyrosine phosphorylation on the EGF receptor and can stimulate growth-related signal transduction to regulate the cell cycle and enhance cell proliferation. Sato et al. (9) demonstrated that EGF stimulated 3T3 fibroblasts from the G0 into the G1 phase of the cell cycle. Epidermal growth factor has been found to stimulate the expression of protooncogenes, such as c-*fos*, c-*myc*, and c-*jun* (10). When EGF interacts with the EGF receptor, c-*jun* NH_2-terminal kinase is activated, and Ser63 and Ser73 residues on Jun are phosphorylated (10,11). Phosphorylated Jun protein then stimulates transcription. Additionally, the synthesized Fos (c-Fos, Fos-B, Fra-1, and Fra-2) and Jun proteins (c-Jun, Jun-B, and Jun-D) form activator protein-1 (AP-1), a complex of heterodimer or homodimer (12). The transcription factor AP-1 activates the 12-O-tetradecanoyl-1,2-phorbol 13-acetate (TPA)–response element on the genes and initiates gene transcription.

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The physiologic functions of EGF in the gastrointestinal tract have been reported and include inhibition of gastric acid secretion; stimulation of intestinal DNA synthesis and cell division; regulation of sucrase and lactase activities; increased water, glucose, and sodium absorption; increased cellular calcium concentration; activation of ion transport; and modulation of prostaglandin synthesis and secretion (7,13,14). The evidence suggests that EGF in human milk plays an important role in fetal or postnatal intestinal growth and development. Epidermal growth factor in human milk has been shown to protect neonates from necrotizing enterocolitis (15).

A previous study showed that [^{3}H]-thymidine incorporation was dose dependent and reached maximal effect at a concentration of 5% human milk in the human fetal small intestinal FHs 74 Int cells (16). Additionally, EGF concentration in human milk significantly correlated with the growth-promoting activity measured by [^{3}H]-thymidine incorporation (16). However, the mechanism of the growth-promoting activity for human milk or for EGF in human intestinal cells has not been clearly stated. To study the effects of human milk and EGF on intestinal growth, a human colon adenocarcinoma (Caco-2) cell line was used. Caco-2 cells possess EGF receptors, and their morphology is similar to normal human intestinal cells. Therefore, the intestinal epithelial Caco-2 cell is a good model for studying the physiologic roles of exogenous growth factors in intestinal growth and development. The purpose of this study was to investigate the effects of human milk and various dosages of EGF on the growth of human intestinal cells, determined by cell numbers and by cellular RNA, DNA, and protein concentrations. To further study the mechanism of the growth-promoting activity, the regulation of human milk and EGF on cell cycle and c-*jun* gene expression in Caco-2 cells also was determined.

MATERIALS AND METHODS

Cell Line and Chemicals

Caco-2 cells (human colon adenocarcinoma, CCRC No. 60018) were purchased from Culture Collection and Research Center (CCRC) at Food Industry Research and Development Institute (Hsinchu, Taiwan, Republic of China). Unless otherwise stated, chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.) and Life Technologies GIBCO BRL (Taipei, Taiwan, Republic of China). Human EGF immunoassay (Quantikine, DEG00) was purchased from Research and Diagnostics Systems, Inc. (Minneapolis, MN, U.S.A.). Cycle Test Plus DNA reagent kits were obtained from Becton Dickinson and Company (Franklin Lakes, NJ, U.S.A.). Bicinchoninic acid assay was purchased from Pierce (Rockford, IL, U.S.A.). Mouse antihuman Jun/AP-1 antibody and goat antimouse immunoglobulin G–horseradish peroxidase conjugate were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech Asia Pacific Ltd. (Taipei, Taiwan, Republic of China).

Human Milk Collection

Seven healthy mothers who delivered full-term infants at Taipei Medical University Hospital in Taipei volunteered to provide 5 mL of milk after 8, 12, 16, and 20 days parturition. All volunteers gave informed, written consent. Human milk samples were collected in sterile tubes, placed on ice, and immediately sent to the laboratory. Samples were centrifuged at 3,750*g* for 2 minutes to remove fat and cell debris. Supernatant aliquots of 1.5 mL were stored at −80°C for EGF analysis.

Measurement of EGF in Human Milk

Twenty milk samples were diluted to 1:400, and EGF in human milk was measured using an ELISA kit (17). Diluted milk samples (200 μL) were incubated with murine monoclonal EGF antibody coated on the 96-well plate at room temperature for 2 hours. After several washes, polyclonal EGF antibody conjugated to horseradish peroxidase (200 μL) was added to the plate and reacted for 1 hour. Samples were then incubated with 200 μL substrates (tetramethylbenzidine:H_2O_2 = 1:1) for 20 minutes after several washes. The reaction was terminated with 50 μL sulfuric acid (1 mol/L). Epidermal growth factor concentrations were determined at 450 nm and corrected at 540 nm using an ELISA reader (Multiskan RC; Labsystems, Helsinki, Finland).

Treatments and Cell Numbers

Caco-2 cells were grown in minimum essential medium, containing nonessential amino acids, 20% fetal bovine serum, 50,000 U/L penicillin, and 50 mg/L streptomycin, at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2). Cells (passage number, 32 to 34) were seeded in a 55-cm^2 Petri dish at a density of 1.0×10^6 and grown to approximately 50% confluency. Before treatments, cells were synchronized by incubating them in basal medium (serum-free complete medium without antibiotics) for 12 hours to eliminate interfering factors present in the serum. Cells were then incubated with 5% human milk, 0.375 nmol/L EGF (0.05 × EGF, equivalent to the concentration in 5% human milk), 7.5 nmol/L EGF (1 × EGF, equivalent to the physiologic concentration in human milk), or 75 nmol/L EGF (10 × EGF) for 12 to 48 hours. The medium was not changed during the incubation period. Cell numbers were measured using the trypan blue exclusion method. Cells were then frozen and stored at −80°C for further analyses.

Quantitation of Cellular RNA, DNA, and Protein

Total cellular RNA, DNA, and protein in Caco-2 cells were extracted using TRIZOL reagent (Life Technologies GIBCO BRL, Taipei, Taiwan, Republic of China) (18). Cells were lysed in TRIZOL reagent (1 mL/10^7 cells), and mixed with 200 μL chloroform. After centrifugation at 12,000*g* for 15 minutes at 4°C, RNA was separated in the upper aqueous phase. The interphase and lower organic phase were retained for DNA and protein analyses. The aqueous phase RNA was mixed with 500 μL cold 100% isopropanol for 10 minutes. The RNA pellet was then washed with 1 mL cold 75% ethanol, air dried, and rehydrated in 10 μL to 20 μL diethylpyrocarbonate water. The amount of RNA was measured spectrophotometrically at 260

nm (U2000 double-beam spectrophotometer; Hitachi, Tokyo, Japan), and expressed as $\mu g/10^5$ cells. DNA in the interphase and organic phase was precipitated with 300 μL of 100% ethanol. After centrifugation at 2,000*g* for 10 minutes at 4°C, the supernatant was saved for protein isolation. The DNA pellet was mixed with 1 mL of 0.1 mol/L sodium citrate and 10% ethanol, washed with 2 mL of 75% ethanol, air dried, and dissolved in 200 μL of 8 mmol/L NaOH. The amount of DNA was measured spectrophotometrically at 260 nm and expressed as $\mu g/10^5$ cells. The supernatant containing protein was added to 100 μL of 2 mol/L NaOH, placed on ice for 30 minutes, and centrifuged at 12,000*g* for 10 minutes at 4°C. Protein content ($mg/10^5$ cells) in the supernatant was then quantitated by bicinchoninic acid assay (19).

DNA Content in Cell Cycle

Cells were grown on a 25-cm^2 flask to approximately 60% to 70% confluency, and switched to basal medium for 12 hours. The medium was added to 5% human milk, 0.05 × EGF, or 1 × EGF for 12 and 24 hours. Cells were stained using Cycle Test Plus DNA reagent kit (20). The cell suspension (10^6 cells) was mixed with 1 mL citrate buffer (250 mmol/L sucrose, 20 mmol/L trisodium citrate, 50 g/L dimethyl sulfoxide; pH 7.6), and centrifuged at 300*g* for 5 minutes. After three washes, the cell pellet was incubated with 250 μL solution A (0.5 mmol/L Tris, 10 mmol/L spermine tetrahydrochloride, 1 g/L NP40, 0.03 g/L trypsin; pH 7.6) at room temperature for 10 minutes. The cell suspension was then mixed with 200 μL solution B (0.5 mmol/L Tris, 10 mmol/L spermine tetrahydrochloride, 1 g/L NP40, 0.05 g/L trypsin inhibitor, 0.001 g/L ribonuclease A; pH 7.6) for 10 minutes. Then 200 μL solution C (0.5 mol/L Tris, 50 mmol/L propidium iodide; pH 7.6) was added in the dark for another 10 minutes. Finally, DNA content in the cell cycle was detected by flow cytometer (FAC Scan; Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) and analyzed using Cell Fit (Becton Dickinson and Company).

Measurement of c-Jun Protein

The cell suspension (30 μg protein) was mixed with an equal volume of 2 × SDS-PAGE sample buffer (0.125 mol/L Tris-HCl, pH 6.8; 40 g/L SDS; 20% (vol/vol) glycerol; 10% (vol/vol) 2-mercaptoethanol) (21), denatured at 100°C for 3 minutes, and applied to SDS-PAGE (Hoefer SE 250, 80 × 100 × 1 mm, Pharmarcia Biotech Asia Pacific Ltd., Taipei, Taiwan, Republic of China). Proteins were separated using 12.5% resolving gel with 4% stacking gel in the running buffer (25 mmol/L Tris, pH 8.3; 192 mmol/L glycine; 1 g/L SDS) at 40 V for 30 minutes followed by 120 V for 1.5 hours. After separation on the gel, proteins were then transferred onto the nitrocellulose membrane (0.45 μm) using a semidry transfer unit (Hoefer TE 70, Pharmarcia Biotech Asia Pacific Ltd.) in Towbin buffer (25 mmol/L Tris, 192 mmol/L glycine, 1.3 mmol/L SDS, 10% (vol/vol) methanol) (22) at 90 mA for 2 hours. The membrane was washed briefly with phosphate buffered saline (PBS) and incubated with blocking buffer (50 g/L skim milk, 0.1% (vol/vol) Tween-20 in PBS) for 1 hour. After blocking, the membrane was incubated with 250 μg/L mouse antihuman Jun/AP-1 antibody at room temperature for 2 hours. The membrane was washed three times with wash buffer (0.1% (vol/vol) Tween-20 in PBS) and incubated with 10 μg/L goat antimouse immunoglobulin G–horseradish peroxidase conjugate for 1 hour. The blot was washed again three times with wash buffer, incubated with 1 mL enhanced chemiluminescence (ECL) solution (ECL1:ECL2 = 1:1) for 1 minute, and exposed to an x-ray film for 20 minutes to overnight. The bands were quantitated using an image analysis system (Gel analysis system; EverGene Biotechnology, Taipei, Taiwan, Republic of China) and Phoretix 1D Lite software (Phoretix International Ltd., Newcastle upon Tyne, United Kingdom).

Reverse Transcription–Polymerase Chain Reaction

To detect the early response gene, the level of mRNA for c-*jun* was determined using reverse transcription–polymerase chain reaction after 0.5-hour incubation. Total cellular RNA (10 μg) as a template was heated at 65°C for 10 minutes, followed by incubation with 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, and 2.5 mmol/L deoxy-NTP transcriptase (M-MLV RT; Life Technologies GIBCO BRL, Taipei, Taiwan, Republic of China) at 37°C for 1 hour, and then heated at 65°C for 10 minutes to terminate the reaction. Polymerase chain reaction was performed with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute and 30 seconds for 25 cycles. Synthetic primers for c-*jun* (20-mer sense strand, 5′-ACGACCTTCTATGACGATGC-3′; 20-mer antisense strand, 5′- CCGTTGCGTGACTGGATTAT-3′) and for β-actin (32-mer sense strand, 5′-ATCTGGCA CCACACCTTCTACAATGAGCTGCG-3′; 32-mer antisense strand, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′) were used to produce 238- and 1761-bp products for c-*jun* and β-actin mRNA, respectively. The sequences of polymerase chain reaction products were analyzed with ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA, U.S.A.) according to Sanger dideoxy chain termination method. Polymerase chain reaction products were then separated using 2% agarose gel electrophoresis. The expression of c-*jun* and β-actin mRNA was quantitated using Phoretix 1D Lite.

Statistical Analysis

Data were analyzed using SAS (version 6.12, SAS Institute, Cary, NC, U.S.A.). A one-way analysis of variance was used to determine the main effect of human milk or EGF on Caco-2 cells. Fisher's least significant difference test was used to make post hoc comparisons if the treatment effect was demonstrated. Dunnett's test was used to compare cell numbers between the treatment and the control groups. Statistical significance is assigned at the 0.05 level.

RESULTS

Cell Proliferation

The average concentration of EGF in human milk collected on postpartum days 8, 12, 16, and 20 from seven healthy mothers was 7.5 nmol/L ± 0.3 nmol/L (45.0 ± 1.8

TABLE 1. *Effect of human milk and epidermal growth factor (EGF) on cell proliferation in Caco-2 cells**

Time	Control	Cell number (×10^5/dish) 5% HM†	0.05 × EGF	1 × EGF	10 × EGF
0 h	2.2 ± 0.4	—‡	—	—	—
12 h	2.5 ± 0.2^a	2.9 ± 0.5^{ab}	2.8 ± 0.3^{ab}	3.2 ± 0.5^{ab}	3.3 ± 0.5^b
24 h	2.9 ± 0.4^a	3.3 ± 0.5^a	3.1 ± 0.6^a	3.6 ± 0.7^a	3.6 ± 0.7^a
36 h	3.1 ± 0.8^a	3.7 ± 0.8^a	3.5 ± 0.8^a	4.0 ± 0.8^a	4.2 ± 0.9^a
48 h	3.8 ± 0.4^b	2.8 ± 0.5^a	4.3 ± 0.6^{bc}	5.2 ± 0.8^{cd}	6.2 ± 0.8^d

* Data are mean ± SD (n = 3). Values in a row not sharing a superscript letter differ significantly ($P < 0.05$) by Fisher's least significant difference test.
† Human milk.
‡ Not measured.

μg/L). In this study, 5% human milk, 0.05 × EGF (0.375 nmol/L), 1 × EGF (7.5 nmol/L), or 10 × EGF (75 nmol/L) EGF was added to Caco-2 cells. Table 1 shows cell proliferation after 12-, 24-, 36-, or 48-hour incubation. After 12-hour incubation, the 10 × EGF group increased cell numbers by 32% ($P < 0.05$) compared with the control group. However, cell numbers did not significantly differ among the four treatment groups at 12, 24, and 36 hours. After 48-hour incubation, the 5% human milk group had lower ($P < 0.05$) cell numbers compared with the others. However, the 1 × EGF and 10 × EGF groups had significantly increased cell numbers by 28% and 52% ($P < 0.05$), respectively, compared with the control group at 48 hours. Cell numbers did not significantly differ between the control and the 0.05 × EGF groups or between the 0.05 × EGF and 1 × EGF groups, or between the 1 × EGF and 10 × EGF groups at 48 hours.

Cellular RNA, DNA, and Protein Content

The peak for cell proliferation occurred between 36 and 48 hours, followed by the period between 12 and 24 hours. The 5% human milk group had dramatically decreased cell numbers at 48 hours, probably because of the formation of toxic substances from decomposed milk after 48-hour incubation at 37°C. Therefore, cellular RNA, DNA, and protein contents were measured after incubation with 5% human milk, 0.05 × EGF, 1 × EGF, or 10 × EGF for 12 hours. Cellular RNA, DNA, and protein contents were 3.01 ± 1.72 μg/10^5 cells, 2.97 ± 0.35 μg/10^5 cells, and 100.0 ± 52.1 μg/10^5 cells in the control group, respectively. The results showed that cellular RNA content significantly increased by 62% and 288% ($P < 0.05$) in the 5% human milk and 10 × EGF groups, respectively, compared with the control group (Fig. 1). However, RNA content did not significantly differ among the control, 0.05 × EGF, and 1 × EGF groups, or among the 5% human milk, 0.05 × EGF, and 1 × EGF groups. Cellular DNA content significantly increased by 32% and 79% ($P < 0.05$) in the 1 × EGF and 10 × EGF groups, respectively, compared with the control group. DNA content did not change in the 5% human milk and 0.05 × EGF groups. Cellular protein content significantly increased by 67% and 79% ($P < 0.05$) in the 1 × EGF and 10 × EGF groups, respectively. However, protein content did not differ significantly among the treatment groups, or among the control, 0.05 × EGF, and 1 × EGF groups.

DNA Content in the Cell Cycle

To compare the regulation of human milk and EGF on DNA content in the cell cycle of human intestinal cells, the percentage of DNA in the G1, S, G2+M phases was analyzed using flow cytometer after adding 5% human milk, 0.05 × EGF, 1 × EGF, or 10 × EGF for 12 and 24 hours. The results showed that the percentage of DNA in any phase at 12 hours did not differ significantly among the groups (Table 2). The percentage of DNA increased significantly ($P < 0.05$) during the G1 phase in the 1 × EGF and 10 × EGF groups at 24 hours compared with the 5% human milk group. Although 5% human milk increased significantly the percentage of DNA in the S phase at 24 hours, the percentage of DNA in the G2+M phase at 24 hours did not differ between the treatment groups and the control group. However, adding 0.05 × EGF did not change the percentage of DNA very much in the G1, S, and G2+M phases compared with the control group.

Expression of c-Jun Protein

After adding 5% human milk, 0.05 × EGF, 1 × EGF, or 10 × EGF for 12, 24, and 36 hours, c-Jun protein expression was analyzed using Western blotting and is shown in Figure 2. Figure 3 shows the results of c-Jun

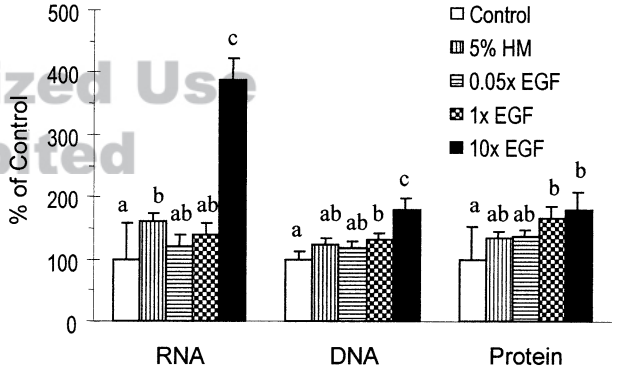

FIG. 1. Cellular RNA, DNA, and protein content (% of control) in human colon adenocarcinoma (Caco-2) cells after adding 5% human milk, 0.05 × EGF (0.375 nmol/L, equivalent to the concentration in 5% human milk), 1 × EGF (7.5 nmol/L, equivalent to physiologic concentration in human milk [HM]), or 10 × EGF (75 nmol/L) for 12 hours. Data are means ± SD (n = 3). Means not sharing a letter differ significantly ($P < 0.05$) by Fisher's least significant difference test. Cellular RNA, DNA, and protein contents were 3.01 ± 1.72 μg/10^5 cells, 2.97 ± 0.35 μg/10^5 cells, and 100.0 ± 52.1 μg/10^5 cells in the control group, respectively.

TABLE 2. *Effect of human milk and epidermal growth factor (EGF) on cell cycle in CaCo-2 cells**

Time	Phase	Control	Percentage of DNA (%) 5% HM†	0.05 × EGF	1 × EGF	10 × EGF
0 h	G1	46.1 ± 2.8	—‡	—	—	—
	S	23.9 ± 1.9	—	—	—	—
	G2 + M	30.0 ± 3.4	—	—	—	—
12 h	G1	43.2 ± 2.1[a]	42.4 ± 3.7[a]	41.0 ± 0.7[a]	40.2 ± 1.9[a]	39.0 ± 4.0[a]
	S	25.1 ± 4.8[a]	29.6 ± 2.0[a]	28.3 ± 5.7[a]	27.0 ± 7.4[a]	30.7 ± 8.8[a]
	G2 + M	31.8 ± 2.7[a]	28.0 ± 4.0[a]	30.8 ± 6.2[a]	32.8 ± 9.2[a]	30.3 ± 5.4[a]
24 h	G1	41.6 ± 2.5[ab]	39.1 ± 2.1[a]	41.5 ± 4.3[ab]	45.7 ± 2.3[b]	45.0 ± 2.1[b]
	S	23.2 ± 3.2[a]	29.8 ± 2.0[b]	22.3 ± 1.9[a]	22.5 ± 2.0[a]	19.7 ± 3.8[a]
	G2 + M	35.2 ± 1.6[ab]	31.1 ± 0.6[a]	36.2 ± 4.2[b]	31.8 ± 0.5[a]	35.3 ± 2.6[ab]

* Data are mean ± SD (n = 3). Values in a row not sharing a superscript letter differ significantly ($P < 0.05$) by Fisher's least significant difference test.
† Human milk.
‡ Not measured.

expression quantitated by the image analysis system. The expression of c-Jun protein decreased with incubation time in the control group (Fig. 3A). In the 5% human milk group at 12 hours, the expression of c-Jun protein was 264% of that at 0 hour (Fig. 3B). The expression of c-Jun protein in the 0.05 × EGF group decreased at 12 and 24 hours (Fig. 3C). After 36-hour incubation, the content of c-Jun increased to 141% of that at 0 hour in the 0.05 × EGF group. In the 1 × EGF and 10 × EGF groups, cell proliferation was significantly stimulated at 12 hours compared with that at 0 hour. The content of c-Jun also increased at 12 hours (Fig. 3 D and E). In the 1 × EGF group, the expression of c-Jun protein increase at 12 hours to 167% of that at 0 hour, and at 24 hours to 176% (Fig. 3D). After 36-hour incubation, c-Jun protein increased by 87% in the 10 × EGF group (Fig. 3E).

c-*jun* mRNA

The level of c-*jun* mRNA was determined indirectly using reverse transcription–polymerase chain reaction. Figure 4 shows the results. After incubation for 0.5 hours, c-*jun* gene transcription increased with increased EGF treatment. The level of c-*jun* mRNA was 114%, 250%, and 281% of the control in the 0.05 × EGF, 1 × EGF, and 10 × EGF groups, respectively. The expression of c-*jun* mRNA increased to 302% of the control in the 5% human milk group.

DISCUSSION

The concentration of EGF in human milk collected on postpartum days 8, 12, 16, and 20 was 7.5 nmol/L ± 0.3 nmol/L. Similar to the findings of previous studies, EGF concentrations were 11.5 (22) and approximately 5.0 to 6.6 nmol/L (5) in mature human milk from postpartum days 7 and 8 determined by radioimmunoassay and by radioreceptor assay, respectively. Moran et al. (24) found that duration of gestation, time of day, and duration of lactation did not affect EGF concentrations. However, other studies reported that EGF concentration was higher in colostrum than in mature milk (16,25–26). Milk EGF content was equivalent or higher in milk from mothers who delivered prematurely compared with EFG content of term milk when assayed by radioimmunoassay and radioreceptor assay, respectively (27). Because all mature milk was collected from mothers who delivered at term, only a small variation (coefficient of variation, 4%) was seen in this study.

Our data showed that cell proliferation was comparable among the control, 0.5% human milk, 0.05 × EGF, 1 × EGF, and 10 × EGF groups after 48-hour incubation. After 48-hour incubation, EGF tended to stimulate cell proliferation in a dose-dependent response. Cell numbers increased significantly in the 1 × EGF and 10× EGF

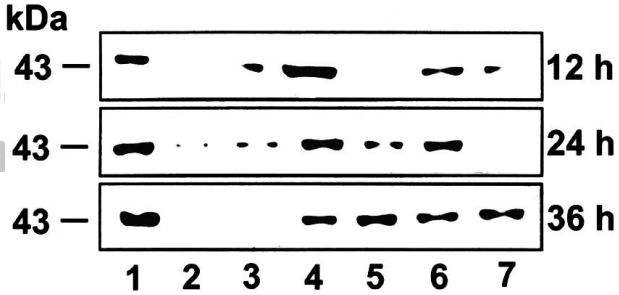

FIG. 2. The expression of c-Jun protein with the molecular weight of 43 kDa after adding 5% human milk, 0.05 × EGF (0.375 nmol/L, equivalent to the concentration in 5% human milk), 1 × EGF (7.5 nmol/L, equivalent to physiologic concentration in human milk), or 10 × EGF (75 nmol/L) for 12, 24, or 36 hours in human colon adenocarcinoma (Caco-2) cells. Cellular proteins (30 μg) were separated using 12.5% SDS-PAGE, and c-Jun protein was analyzed with Western blotting using mouse antihuman Jun/activator protein 1 antibody. Samples were obtained from three independent experiments (n = 3). Lane 1, RSV-3T3 cell lysate as a positive control; lane 2, control at 0 hour; lane 3, control; lane 4, 5% human milk; lane 5, 0.05 × EGF; lane 6, 1 × EGF; lane 7, 10 × EGF.

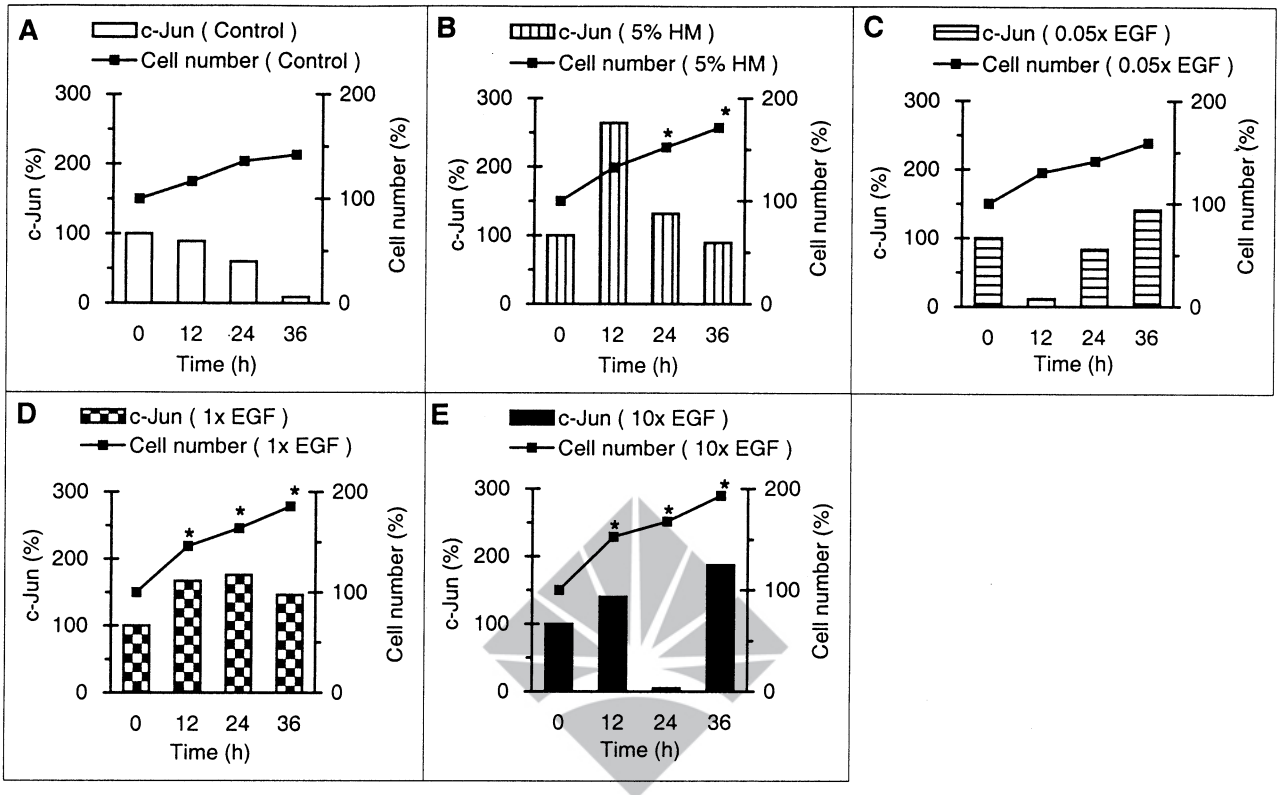

FIG. 3. Cell numbers (right *y* axis) and the level of c-Jun protein expression (left *y* axis) in human colon adenocarcinoma (Caco-2) cells after incubation with 5% human milk, 0.05 × EGF (0.375 nmol/L, equivalent to the concentration in 5% human milk), 1 × EGF (7.5 nmol/L, equivalent to physiologic concentration in human milk [HM]), or 10 × EGF (75 nmol/L) for 12, 24, or 36 hours. Data are expressed by % of the control at 0 hour. Because of an insufficient number of cells in one dish, samples from three independent experiments (n = 3) were pooled for one measurement of c-Jun expression. **A:** Control. **B:** 5% Human milk. **C:** 0.05 × EGF. **D:** 1 × EGF. **E:** 10 × EGF. *Significantly different in cell numbers from the control group at 0 hour ($P < 0.05$) by Dunnett's test.

groups compared with the control group. The 10 × EGF group had significantly increased cell proliferation compared with the 0.05 × EGF group. The doubling time for Caco-2 cells without EGF stimulation was 53.4 ± 5.0 hours (n = 3) in this study. Cell numbers of Caco-2 cells increased obviously at 48 hours after adding EGF. However, 5% human milk had decreased cell numbers after 48-hour incubation, possibly because of the formation of toxic substances in deteriorated human milk after long-term incubation at 37°C. Ichiba et al. (16) reported that DNA synthesis determined by [^{3}H]-thymidine incorporation in normal fetal human small intestinal (FHs 74 Int) cells increased 3 times in 5% human milk compared with 1% human milk. Because it requires more time to observe an increase in cell numbers, which may lead to deteriorated human milk and toxicity to the cells in vitro, the possibility that human milk could increase cell proliferation of human intestinal cells in vivo cannot be ruled out.

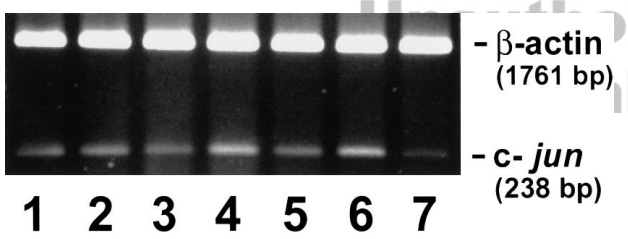

FIG. 4. The expression of c-*jun* mRNA (238 bp) in human colon adenocarcinoma (Caco-2) cells after adding 5% human milk, 0.05 × EGF (0.375 nmol/L, equivalent to the concentration in 5% human milk), 1 × EGF (7.5 nmol/L, equivalent to physiologic concentration in human milk), or 10 × EGF (75 nmol/L) for 0.5 hours. The level of c-*jun* mRNA was detected by reverse transcription–polymerase chain reaction (RT-PCR). To ensure the equal addition of reverse-transcribed RNA, the parallel amplification of β-actin mRNA (1,761 bp) was performed as an internal control. The products of RT-PCR were subjected to 2% agarose gel. Lane 1, control at 0 hour; lane 2, control at 0.5 hour; lane 3, 0.05 × EGF; lane 4, 5% human milk; lane 5, 1 × EGF; lane 6, 10 × EGF; lane 7, RSV-3T3 cell lysate as a positive control.

Cell numbers and DNA content of Caco-2 cells significantly increased in the 1 × EGF and 10 × EGF groups. The results also showed that human milk and its equivalent EGF (0.05 × EGF) had similar action on cellular RNA, DNA, and protein contents. Ichiba et al. (16) found that the incorporation of [^{3}H]-thymidine in FHs 74 Int cells doubled after adding 5 nmol/L EGF for 24 hours compared with adding 0.005 nmol/L EGF. They also found the DNA synthesis stimulating response of EGF

was dose dependent. Compared with normal fetal human intestinal (FHs 74 Int) cells, DNA content in Caco-2 cells increased dose-dependently by 7.5 nmol/L and 75 nmol/L EGF after 12-hour incubation. A previous study showed that the growth-promoting activity and the concentration of EGF in human milk correlated positively ($r = 0.42$, $P < 0.05$) (16). However, the concentration of insulin or insulin-like growth factor-1 (IGF-1) in human milk did not correlate with the growth-promoting activity of FHs 74 Int cells. Epidermal growth factor, rather than insulin or IGF-1, may be the main growth-promoting factor in human milk for proliferation of human fetal intestinal cells (16).

Other growth factors such as IGF-1 and transforming growth factor-α (TGF-α) in human milk, rather than EGF, may be involved in enhancing gene transcription or cell proliferation. Insulin-like growth factor-1 or EFG increased cell proliferation of rat intestinal (IEC-6) cells, and combined IGF-1 and EGF increased proliferation more (28). The synergic action on cell proliferation may be possible because EGF can stimulate the cell cycle from G0 to G1 phase, allowing IGF-1 to progress the cell cycle from G1 to S phase, and to enhance the growth-promoting activity of EGF (28). The stability of exogenous EGF in the intestinal fluid varied with individual maturity and source. After 30-minute injection of I^{125}-EGF in vivo, EGF degraded more quickly in weaned pigs than in sucking pigs, and recombinant EGF degraded more quickly than milk-borne EGF (29). Therefore, 5% human milk may have more effective growth-promoting activity than its equivalent EGF (0.05 × EGF). However, in our study, cell numbers, cellular RNA, DNA, and protein contents did not differ significantly between the 5% human milk and 0.05 × EGF groups at 12 hours.

Epidermal growth factor affects the cell cycle of human colon LIM 1215 cells (30). Incubation with EGF in the S phase resulted in only slight stimulus to proliferation when the cells returned to the G1 phase. The addition of EGF before cell division may stimulate cell growth in a short time. When the cells have entered the G1 phase, it may take 10 hours to stimulate the cells to enter another cell cycle (30). A previous study suggested that EGF may regulate cell growth through stimulating the cell cycle from the G0 to the G1 phase (28), and our study showed that 1 × EGF and 10 × EGF increased the percentage of DNA in the G1 phase compared with 5% human milk at 24 hours. However, 5% human milk elevated DNA content in the S phase at 24 hours.

In our study, the stimulation of c-Jun protein occurred earlier with human milk or with a higher concentration of EGF. Our data showed that the expression of c-Jun in Caco-2 cells during 36-hour incubation was highest after 12- or 36-hour addition of human milk or 0.05 × EGF, respectively. Thereafter cell proliferation reached a peak at 36 hours. The level of c-Jun increased with 1 × EGF and 10 × EGF at 12 hours; cell numbers also increased at 12 hours. Therefore, c-Jun expression may relate to cell proliferation, which was more clearly seen in c-*jun* mRNA expression. The expression of c-Jun changed variously within 36 hours, perhaps because of the image analysis system's high sensitivity and the short turnover rate of c-Jun per se. Although dramatic decreases of c-Jun expression occurred in the 0.05 × EGF and 10 × EGF groups at 12 and 24 hours, the unexplained degradation of c-Jun may occur during analysis.

The reverse transcription–polymerase chain reaction analysis showed that the level of c-*jun* mRNA increased dose-dependently with the addition of EGF at 0.5 hours, indicating that increased c-*jun* mRNA expression may be involved in stimulating cell proliferation by EGF. The level of c-*jun* mRNA was stimulated most by 5% human milk, suggesting that other factors in milk may also regulate c-*jun* gene transcription. However, Simmons et al. (37) found that EGF stimulated the expression of c-*jun* mRNA in IEC-6 cells, but IGF-1 did not. Therefore, adding EGF alone or combined EGF and IGF-1 had a similar effect on the level of c-*jun* mRNA in IEC-6 cells (30). A previous study also found that EGF enhanced transcription of c-Jun through phosphorylation of its activation domain at Ser63 and Ser73 in primary rat hepatocytes (32). Because the expression of c-Jun protein and c-*jun* mRNA did not consistently correlate, EGF may stimulate cell proliferation of Caco-2 cells through regulating c-*jun* at the transcriptional level rather than at the translational level.

Our study found that cell numbers and the percentage of DNA in the G1 phase of Caco-2 cells increased dose-dependently in higher concentrations of EGF (1 × EGF and 10 × EGF). Cellular DNA level also increased dose-dependently with the addition of EGF. The concentrations of c-*jun* mRNA also increased by EGF dose at 0.5 hours. The expression of c-Jun protein increased only slightly, from 141% in the 0.05 × EGF group to 187% of the control in the 10 × EGF group after 36-hour incubation. However, both 5% human milk and 0.05 × EGF had less effect on cell numbers and on cellular DNA and protein contents, possibly because the concentration was too low to affect cell growth. In conclusion, EGF could dose-dependently stimulate intestinal cell growth by increasing DNA content in the G1 phase and c-*jun* mRNA expression. However, 5% human milk and its equivalent 0.05 × EGF did not affect cell growth, probably because of insufficient concentrations. These results suggest that physiologic (7.5 nmol/L) or high (75 nmol/L) concentrations of EGF can stimulate intestinal growth in vitro, which may be helpful in promoting healing of the damaged intestinal cells or promoting growth of newborn intestinal epithelia, especially for premature or very-low-birth-weight infants. More in vivo studies are required to identify the role of EGF in human milk.

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