Human milk induces fetal small intestinal cell proliferation – involvement of a different tyrosine kinase signaling pathway from epidermal growth factor receptor

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Abstract

Breast milk has non-nutritional protective effects on recipient infants. It has been speculated that bioactive substances present in human milk have important roles in protecting infants. However, the mechanisms by which such substances protect newborns are unclear. Therefore, we analyzed the growth-promoting activity of human milk and the intracellular signaling mechanism thereof using human fetal small intestinal (FHS 74 Int) cells. Epidermal growth factor (EGF) stimulated the proliferation of these cells. However, this stimulation was less effective than that of aqueous milk (5% vol/vol). The bioactivity of human milk was heat stable but protease sensitive. EGF receptor tyrosine kinase inhibitor did not repress the milk-induced growth-promoting effect on fetal small intestinal cells. Regarding the intracellular signaling pathway, the milk-induced cell proliferation pathway was tyrosine kinase

dependent but was neither mitogen-activated protein (MAP) kinase nor phosphatidylinositol-3 (PI-3) kinase dependent. On the other hand, EGF-induced cell proliferation was tyrosine kinase, MAP kinase, and PI-3 kinase dependent. Rapid tyrosine phosphorylation of several intracellular proteins was detected after milk stimulation. Furthermore, the time course of phosphorylation induced by milk was different from that induced by EGF. The sizes of the proteins phosphorylated in response to milk were different from those of the Shc proteins phosphorylated in response to EGF. These results suggest that human milk induces fetal intestinal cell proliferation through a unique tyrosine kinase pathway different from the EGF receptor signaling pathway.

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Introduction

Breast milk has been shown to have many benefits for infants, including not only a reduced risk of neonatal necrotizing enterocolitis, gastroenteritis, respiratory infection and immunologically based disease but also improved later cognitive development (Wagner *et al.* 1996, Lucas *et al.* 1998, Schanler & Atkinson 1999). These clinical findings indicate that milk may exert protective effects on infants during neonatal maturation. It is well known that milk contains a variety of proteins, peptides, and steroids that possess biological activity (Grosvenor *et al.* 1993). It is speculated that these bioactive substances play important roles in the non-nutritional effects of human milk.

The newborn gastrointestinal tract undergoes maturational changes in the weeks after birth. Human milk is known to stimulate gastrointestinal mucosal proliferation and maturation in animal models and is thought to protect the neonatal infant from harmful environmental factors by affecting the mucosal barrier (Wagner *et al.*

1996). Some of the growth factors in human milk, such as epidermal growth factor (EGF), transforming growth factor alpha (TGF α) and insulin-like growth factors (IGFs), have been shown to stimulate the proliferation of intestinal cells and the formation of the mucosal barrier (Corps & Brown 1987, Ichiba *et al.* 1992, Wagner *et al.* 1998, Wagner & Forsythe 2000). Among them, EGF is thought to be the main stimulator in human milk (Grosvenor *et al.* 1993).

Numerous studies have been performed to investigate the intracellular signaling pathways of many growth factors and cytokines in a variety of cells in the past decade. Both EGF and TGF α bind to the same EGF receptor (EGFR). Ligands binding to EGFR activate the receptor tyrosine kinase activity, resulting in both autophosphorylation and phosphorylation of various intracellular effector proteins (Moghal & Sternberg 1999). Shc is an adapter molecule that links receptor tyrosine kinase to downstream signaling pathways. The activated EGFR recruits and phosphorylates Shc and subsequently activates

the Ras-mitogen-activated protein (MAP) kinase pathway. The activated EGFR also tyrosine phosphorylates p85, a phosphatidylinositol-3 kinase (PI-3 kinase) subunit, and increases PI-3 kinase activity (Moghal & Sternberg 1999). IGFs (IGF-I and IGF-II) bind to the IGF-I receptor (IGF-IR). The activated IGF-IR activates both the Ras-MAP kinase pathway and the PI-3 kinase pathway (Blakesley *et al.* 1996).

However, the proliferative effects of such growth factors on human small intestinal cells are thought to account only partially for the growth promotion by human milk (Ichiba *et al.* 1992, Wagner *et al.* 1998), and the signal transduction pathway by which human milk stimulates intestinal cell proliferation remains unknown. Therefore, in this study we analyzed the mechanism by which milk induces human fetal intestinal cell proliferation.

Materials and Methods

Growth factors, cytokine, antibodies and kinase inhibitor

Human recombinant EGF and insulin were purchased from Sigma-Aldrich (St Louis, MO, USA). The antibodies used were anti-phosphotyrosine monoclonal antibody (PY20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-phosphotyrosine-EGFR polyclonal antibody (Tyr1173, Santa Cruz Biotechnology) and anti-Shc polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) for Western blotting. The specific inhibitor of the tyrosine kinase activity of EGF receptor (AG1478, Sigma-Aldrich), tyrosine kinase inhibitor (genistein, Sigma-Aldrich), JAK-2 tyrosine kinase inhibitor (AG490, Sigma-Aldrich), PI-3 kinase inhibitor (wortmannin, Sigma-Aldrich) and MEK inhibitor (PD-98059, Sigma-Aldrich) were dissolved in DMSO, and 1 µl of these stock solutions (1% vol/vol) was added to the culture medium at the concentrations indicated in the Results section. Control cells were treated with 1 µl DMSO (1% vol/vol) to control for the cytotoxic effect of DMSO itself.

Cells and cell culture

A human fetal small intestinal cell line, FHs 74 Int (ATCC CCL241), was purchased from Health Science Research Resources Bank (Osaka, Japan) and was cultured in DMEM (Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA) containing 10% fetal calf serum (FCS; CSL, Parkville, Victoria, Australia), EGF (30 ng/ml) and insulin (10 µg/ml) as described previously (Wagner *et al.* 1998).

Milk samples

Six samples of human mammary secretions from six mothers of full-term infants were obtained 2 to 4 days after

parturition. Subjects donated milk samples after giving written informed consent. The samples were stored at 4 °C and processed within 12 h of expression and then pooled. Milk was centrifuged for 20 min at 1000 *g* to remove fat and cellular debris as described previously (Smith-Kirwin *et al.* 1998). The aqueous layer was stored at -20 °C until it was assayed. To deplete the steroid hormones, aqueous human milk was treated with dextran-coated charcoal (Sigma-Aldrich) as described previously (Provost *et al.* 2000). To inactivate heat-sensitive proteins and peptides in milk, aqueous milk was boiled for 5 min and then centrifuged for 20 min at 1000 *g*. The aqueous layer obtained thereby was used for experiments. Heat inactivation of EGF was carried out in the same way as heat inactivation of milk. For protease digestion, human milk was incubated at 50 °C for 24 h with 1.5 mg/ml predigested pronase (Roche Molecular Biochemicals, Mannheim, Germany) as described previously (Pimental *et al.* 1996). To concentrate and desalt aqueous milk, the sample was centrifuged using a Microcon Centrifugal Filter Device (YM-100, which could retain the molecules above 100 kDa) (Nihon Millipore, Tokyo, Japan) and reconstituted in PBS.

Cell proliferation assay

Cell proliferation was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Cells were placed in complete medium (10% FCS with EGF and insulin) at a concentration of 5×10^3 cells/well in 96-well plates for 24 h. Subsequently, the cells were incubated in 100 µl EGF and insulin-free DMEM with FCS and bovine serum albumin (1 mg/ml, Sigma-Aldrich) for another 24 h. Milk samples or growth factors were added at the concentrations indicated in the Results section and the cells were further cultured for 24 h. Then the MTS assay was performed as recommended by the manufacturer. All experiments were performed in quadruplicate and repeated at least three times with similar results. Results are expressed as the change in cell proliferation. The average values of data from a representative experiment are presented with standard deviations indicated by bars. Statistical analysis was performed by the unpaired *t*-test. Statistical significance was set at $P < 0.05$.

Immunoprecipitation, SDS-PAGE and Western blotting

Cells were harvested and lysed for 60 min in 1 ml lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulfonyl fluoride, 15% glycerol, and 10 µg/ml each of aprotinin, pepstatin, and leupeptin) as described (Lutticken *et al.* 1994). Proteins in

the lysates from 2×10^5 cells were immunoprecipitated with the anti-phosphotyrosine monoclonal antibody. Immune complexes were separated by SDS-PAGE (7.5%), and transferred to an Immobilon-P nylon membrane (Nihon Millipore), which was then immunoblotted with the relevant primary antibody as described (Takeda *et al.* 1994). Proteins were detected by enhanced chemiluminescence (ECL; Amersham Biosciences Corp. Piscataway, NJ, USA) and rehybridization was performed as described (Takeda *et al.* 1994).

Results

Human milk and EGF stimulate the proliferation of fetal intestinal cells

We tested the growth-promoting activity of milk and EGF on human fetal intestinal cells by using the MTS assay (Fig. 1A and B). Both of these factors could induce cell proliferation dose-dependently, but EGF was not as effective as milk. As the concentration of EGF in breast milk has been reported to be 30–50 ng/ml (Ichiba *et al.* 1992), the final concentration of this growth factor in 5% (vol/vol) milk medium was calculated as 1.5–2.5 ng/ml. In previous reports, it was demonstrated that a plateau in the stimulation of cell proliferation by EGF was reached at the 3–300 ng/ml concentration range (Ichiba *et al.* 1992, Wagner *et al.* 1998). The concentrations of EGF used in these experiments (0.5–500 ng/ml) were thus sufficient to induce observable effects and were much higher than those in 5% breast milk. These facts indicate that the growth-promoting activity of milk is not due solely to the effect of EGF. Although it has been reported that in the case of fibroblast proliferation EGF is a major growth-promoting agent in human milk (Carpenter 1980), our data suggest that there is some other bioactive substance in the case of intestinal cell proliferation. In the following experiments we characterized the growth-promoting agent in milk.

Growth-promoting activity of milk is sensitive to pronase digestion, but not to charcoal treatment and heat inactivation

It has been reported that steroid hormones, including estradiol, estriol, progesterone and other steroid hormones, are present in human milk (Grosvenor *et al.* 1993). We examined whether removing steroids from milk by charcoal treatment affects the growth-promoting activity of milk for intestinal cells (Fig. 2A). Stripping the steroids from milk did not affect the growth-promoting activity, suggesting that steroid hormones are not the major growth-promoting factors in human milk.

Next we analyzed the effect of heat inactivation of milk and EGF on their growth-promoting activity (Fig. 2B). Boiled milk could activate cell proliferation as effectively as

Figure 1 Growth-promoting activity of human milk and EGF. FHs 74 Int cells were treated with (A) human milk (1–10% vol/vol), or (B) EGF (0.5–500 ng/ml) for 24 h. Cell proliferation was measured using the MTS assay, in which the relative cell proliferation was estimated by measuring the change in optical density, measured by spectrophotometry at 490 nm, and expressed relative to that in the control cells taken as 1. The values are averages of quadruplicates of a representative experiment; more than three independent experiments were carried out with similar results. ** $P < 0.01$ versus control.

non-boiled milk. In contrast, boiled EGF could not activate cell proliferation at all. This result shows that the growth-promoting activity of milk is heat stable, suggesting the possibility that this bioactive factor is not a protein or a peptide. To investigate this possibility, we digested milk with pronase at 50 °C (Fig. 2C). Pronase digestion abolished the growth-promoting activity of milk. However, incubation of milk at 50 °C for 24 h without pronase did not affect the growth-promoting activity. These results show that the growth-promoting factors in milk are heat-stable proteins or peptides. To study whether the growth-promoting effects of milk were due to components other than proteins, we concentrated the protein fraction of milk and reconstituted it in PBS. The exchange of buffer and the depletion of small molecule substances did not change

Figure 2 The growth-promoting factor in milk is a heat-stable protein or peptide. (A) Steroid-stripping of milk did not impair the growth-promoting activity. FHs 74 Int cells were treated with human milk (5% vol/vol) or charcoal-treated milk (5% vol/vol) for 24 h. Steroid-stripping of milk by charcoal treatment was carried out as described in Materials and Methods. $^{**}P < 0.01$ versus control. No significant difference was observed between Milk and Milk (charcoal treated) groups. (B) The growth-promoting activity in milk is heat stable. FHs 74 Int cells were treated with human milk (5% vol/vol), heat-inactivated milk (Milk (boiled), 5% vol/vol), EGF (50 ng/ml) and heat-inactivated EGF (EGF (boiled), 50 ng/ml) for 24 h. Heat inactivation of milk and EGF by boiling were carried out as described in Materials and Methods. No significant difference was observed between Milk and Milk (boiled) groups. $^{**}P < 0.01$, EGF versus EGF (boiled). (C) The growth-promoting activity in milk is pronase sensitive. FHs 74 Int cells were treated with pronase-containing buffer (control, 5% vol/vol), human milk in buffer (Milk, 5% vol/vol), human milk diluted in buffer and incubated at 50 °C for 24 h (Milk (heat treated), 5% vol/vol) or human milk digested with pronase (Milk (pronase digested), 5% vol/vol) for 24 h. Pronase digestion of milk was carried out as described in Materials and Methods. No significant difference was observed between Milk and Milk (heat treated). $^{**}P < 0.01$, Milk (pronase digested) versus human milk (Milk). (D) The growth-promoting factor in milk is located in the protein fraction. FHs 74 Int cells were treated with human milk (5% vol/vol) or the protein fraction of milk (5% vol/vol) for 24 h. The protein fraction of milk was concentrated with an ultrafiltration device as described in Materials and Methods. $^{**}P < 0.01$ versus control. No significant difference was observed between Milk and Milk (protein fraction) groups. Cell proliferation was measured using the MTS assay and expressed relative to that of the control cells taken as 1. Values were calculated as described in the legend to Fig. 1. The values are averages of quadruplicates of a representative experiment; more than three independent experiments were carried out with similar results.

the growth-promoting activity (Fig. 2D). This result indicates that bioactive substances other than proteins in milk do not affect the proliferation of intestinal cells.

EGFR-mediated signaling is not the major pathway of milk-induced intestinal cell proliferation

We analyzed the effect of a specific inhibitor of the tyrosine kinase activity of EGFR (AG1478) on the

milk-induced growth-promoting activity (Fig. 3). AG1478 inhibited the EGF-induced cell proliferation effectively, but did not impair the growth-promoting activity of milk. This result indicates that EGFR-mediated signaling is not the major pathway of milk-induced intestinal cell proliferation. As shown in Fig. 3, milk-induced cell proliferation rates seemed to be higher than those seen in Figs 1 and 2. In this experiment we treated control cells with medium containing 1% DMSO. The

Figure 3 EGFR-mediated signaling is not the major pathway in milk-induced intestinal cell proliferation. An inhibitor of the tyrosine kinase activity of EGFR (AG1478) did not repress the growth-promoting activity of milk. FHs 74 Int cells were treated with EGF (50 ng/ml) or with human milk (5% vol/vol) with or without AG1478 (2.0 μ M) for 24 h. Control cells were treated with DMSO (1% vol/vol). Cell proliferation was measured using the MTS assay and expressed relative to that of the non-stimulated control cells (–) taken as 1. Values were calculated as described in the legend to Fig. 1. The values are averages of quadruplicates of a representative experiment; more than three independent experiments were carried out with similar results. ** $P < 0.01$ versus control cells (–).

cell proliferation of the 1% DMSO-treated cells was about 80–90% that of untreated cells and this cytotoxic effect of DMSO was completely abrogated by treatment with 5% milk (data not shown). Accordingly, the milk-induced cell proliferation rates with medium containing 1% DMSO are higher than those without DMSO.

Milk-induced intestinal cell proliferation depends on a tyrosine kinase pathway, but not on the MAP kinase or PI-3 kinase pathway

Many growth factors act via intracellular signaling pathways that involve protein tyrosine kinases (PTKs). There are two major signaling pathways that involve PTKs, the MAP kinase and PI-3 kinase pathways (Moghal & Sternberg 1999). We studied the effects of a PTK inhibitor (genistein), a MEK inhibitor (PD-98059) and a PI-3 kinase inhibitor (wortmannin) on milk- or EGF-induced intestinal cell proliferation (Fig. 4A and B). Genistein repressed the growth-promoting activity of milk, but PD-98059 and wortmannin did not. On the other hand genistein and PD-98059 repressed the growth-promoting activity of EGF completely and wortmannin repressed it partially. These results indicate that milk-induced intestinal cell proliferation depends on a PTK pathway, but not the MAP kinase or the PI-3 kinase pathway, and that

Figure 4 Milk-induced intestinal cell proliferation depends on a PTK pathway, but not on the MAP kinase or PI-3 kinase pathway. FHs 74 Int cells were treated with (A) human milk (5% vol/vol) or (B) EGF (50 ng/ml) with or without PTK inhibitor (genistein, 25 μ g/ml), MAP kinase inhibitor (PD-98059, 50 μ M), or PI-3 kinase inhibitor (wortmannin, 1000 nM) for 24 h. Control cells were treated with DMSO (1% vol/vol). Cell proliferation was measured using the MTS assay and growth-promoting activity of milk was expressed as fold induction. Fold induction was calculated as the ratio of proliferation of cells treated with milk (A) or EGF (B) to that of cells incubated with DMSO (1% vol/vol). The values are averages of quadruplicates of a representative experiment; more than three independent experiments were carried out with similar results. ** $P < 0.01$ versus Milk only in (A) and EGF only in (B).

the EGF-induced signal transduction pathway is different from the milk-induced pathway.

JAK kinase pathway has a repressive effect on the growth-promoting activity of milk

Studies on the signal transduction of interferons have shown that a novel tyrosine kinase, JAK kinase, plays a major role in signal transduction for a variety of cytokines and hormones (Hirano 1998). EGF and IGFs are also

Figure 5 Repressive effect of the JAK kinase pathway on the growth-promoting activity of milk. FHs 74 Int cells were treated with human milk (5% vol/vol) with or without the JAK kinase inhibitor (AG490, 2.5 µM or 5 µM) for 24 h. Control cells were treated with DMSO (1% vol/vol). Cell proliferation was measured using the MTS assay and the growth-promoting activity of milk was expressed as fold induction. Fold induction was calculated as the ratio of proliferation of cells treated with milk to that of cells incubated without milk. The values are averages of quadruplicates of a representative experiment; more than three independent experiments were carried out with similar results. ** $P < 0.01$ versus Milk only.

known to activate the JAK kinase pathway in addition to the MAP kinase pathway and the PI-3 kinase pathway (Zhong *et al.* 1994, Zong *et al.* 2000). We examined whether JAK kinase is involved in milk-induced intestinal cell proliferation (Fig. 5). JAK-2 tyrosine kinase inhibitor (AG490) increased the growth-promoting activity of milk. This indicates that the JAK kinase pathway has a repressive effect on the growth-promoting activity of milk.

Milk induces rapid tyrosine phosphorylation by activating a pathway different from that induced by EGF

We analyzed whether milk could induce tyrosine phosphorylation of cellular proteins (Fig. 6A upper panel). At least two proteins ranging in size from 46 kDa to 66 kDa were rapidly tyrosine phosphorylated in cells treated with milk. The peak of phosphorylation occurred at 15 min and the peak level of phosphorylation was sixfold higher than that in control cells. Next, we analyzed the EGF-induced tyrosine phosphorylation (Fig. 6B upper panel). The molecular sizes of the rapidly phosphorylated proteins seemed to be the same as those of the milk-induced proteins, but the peak of phosphorylation occurred at 5 min. The peak level of EGF-induced phosphorylation was only 1.2-fold higher than that in control cells (Fig. 6B upper panel). As the sizes of these phosphorylated proteins resembled those of Shc isoforms (46, 52 and 66 kDa), we reprobed the same membrane with anti-Shc antibody after stripping (Fig. 6A and B lower panels). The sizes of these proteins were different from those of the proteins whose

tyrosine phosphorylation was induced by milk. The peak protein levels occurred at 5 min in EGF-stimulated cells, but the protein levels were not changed in milk-stimulated cells.

Next, we studied the effects of a PTK inhibitor (genistein), an EGFR tyrosine kinase inhibitor (AG1478), a MEK inhibitor (PD-98059), a PI-3 kinase inhibitor (wortmannin) and a JAK kinase inhibitor (AG490) on milk or EGF-induced tyrosine phosphorylation (Fig. 6C). Genistein repressed the milk and EGF-induced phosphorylation, but AG1478 repressed only EGF-induced phosphorylation. These results indicate that the milk-induced tyrosine phosphorylation pathway is different from the EGFR-mediated signaling pathway. Other kinase inhibitors, PD-98059, wortmannin and AG490 did not repress the milk- or EGF-induced phosphorylation. These results indicate that the milk- and EGF-induced tyrosine phosphorylation pathways are not downstream of MEK kinase, PI-3 kinase or JAK kinase. As the EGF-induced cell proliferation was very weak (Fig. 1B), it is possible that these cells have no EGFR and EGF activates another growth factor receptor. We further confirmed that EGFR was tyrosine phosphorylated in EGF-treated cells, but not in milk-treated cells by reprobing the same membrane with anti-phosphotyrosine-EGFR antibody after stripping (Fig. 6D). EGF tyrosine phosphorylated EGFR effectively, and genistein and AG1478 repressed this phosphorylation completely (Fig. 6D lower panel). On the other hand, we could not detect the tyrosine phosphorylated EGFR in milk-treated cells (Fig. 6D upper panel).

Discussion

This is the first report about the intracellular events involved in milk-induced intestinal cell proliferation. In this work we have shown that (1) EGFR-mediated signaling is not the major pathway in milk-induced intestinal cell proliferation, (2) the growth-promoting factor in milk is a heat-stable protein or peptide, (3) milk-induced intestinal cell proliferation depends on a PTK pathway, not the MAP kinase or PI-3 kinase pathway, (4) the JAK kinase pathway has a repressive effect on the growth-promoting activity of milk, and (5) milk induces rapid tyrosine phosphorylation by activating a pathway different from that induced by EGF.

In previous reports, the growth-promoting activities of EGF, TGF α , IGF-I and insulin were examined using the same intestinal cells (FHs 74 Int cells) (Ichiba *et al.* 1992, Wagner *et al.* 1998, Wagner & Forsythe 2000). As a much higher concentration of IGF-I than that present in milk was necessary to observe the growth-promoting activity of IGF-I (Wagner & Forsythe 2000), IGF-I-induced cell proliferation seems not to be a physiological phenomenon. Although human colostrum contains high concentrations

Figure 6 Milk induces rapid tyrosine phosphorylation by activating a pathway different from that induced by EGF. FHs 74 Int cells were treated for the indicated times with (A) boiled milk (5% vol/vol) or (B) EGF (50 ng/ml). Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. Immune complexes were separated by SDS-PAGE and blotted with anti-phosphotyrosine antibody (A, B upper panels). The blots were then stripped and reprobed with anti-Shc antibody (A, B lower panels). The two arrows in A and B (upper panels) indicate the tyrosine-phosphorylated proteins induced by milk or EGF. The two arrows in A and B (lower panels) indicate Shc proteins (p46 and p52). (C) Milk-induced tyrosine phosphorylation depends on the PTK pathway, not on the EGFR tyrosine kinase, MAP kinase, PI-3 kinase or JAK kinase pathways. FHs 74 Int cells were pretreated with PTK inhibitor (genistein, 25 µg/ml), EGFR tyrosine kinase inhibitor (AG1478, 2.0 µM), MAP kinase inhibitor (PD-98059, 50 µM), PI-3 kinase inhibitor (wortmannin, 1000 nM) or JAK kinase inhibitor (AG490, 2.5 µM) for 1 h. Control cells were treated with DMSO (1% vol/vol). Then the cells were treated with boiled milk (5% vol/vol) (C, upper panel) or with EGF (50 ng/ml) (C, lower panel) for 15 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. Immune complexes were separated by SDS-PAGE and blotted with anti-phosphotyrosine antibody. The blots were then stripped and reprobed with anti-phosphotyrosine-EGFR antibody (anti-p-EGFR) (D).

of insulin (3.75 ± 0.88 nM) (Read *et al.* 1984), FHs 74 Int cells are not responsive to insulin in the range of concentrations found in milk (Ichiba *et al.* 1992). On the other hand, EGF can activate cell proliferation at a concentration expected in human milk (3 ng/ml), but its growth-promoting activity was weaker than that of human milk (Wagner & Forsythe 2000). These previously reported results and our data are in accord (Fig. 1). It is possible that

FHs 74 Int cells have no EGFR or EGFR is not activated efficiently by EGF stimulation. Our data showed that EGF could tyrosine phosphorylate EGFR effectively (Fig. 6D lower panel).

Our data also showed that a PTK-dependent pathway stimulated by milk plays an important role in intestinal cell proliferation (Figs 4 and 6). PTK-dependent pathways also play roles in the signal transduction of other regulators of

cell proliferation. Many growth factors (such as EGF, TGF α and IGF-I) use the Ras-MAP kinase pathway and the PI-3 kinase pathway, which are known signaling molecules downstream of PTK, but these pathways seem not to be important in milk-induced cell proliferation (Fig. 4). JAK family tyrosine kinases have been shown to function as central non-receptor tyrosine kinases in a pathway that has been characterized as a not-Ras-MAP kinase and a not-PI-3 kinase pathway (Hirano 1998). Therefore, we expected that this pathway might be involved in milk-induced cell proliferation. Contrary to our expectations, our data showed that the JAK kinase pathway played a repressive role in milk-induced cell proliferation. It has been reported that interleukin-6 (IL-6) inhibits normal intestinal cell proliferation (Booth & Potten 1995). The JAK kinase pathway plays an important role in the IL-6 signal transduction pathway. In cases of chorioamnionitis, a high concentration of IL-6 is present in the amniotic fluid and fetal plasma. A systemic fetal inflammatory response, as reflected by an elevated fetal plasma concentration of IL-6, is an independent risk factor for the occurrence of intestinal diseases with severe neonatal morbidity, such as necrotizing enterocolitis (NEC) (Gomez *et al.* 1998). The repressive effect of IL-6 on intestinal cell proliferation via the activated JAK kinase pathway may explain this correlation between chorioamnionitis and NEC. Although the details of this milk-induced PTK-dependent pathway remain unknown, such signaling pathways (PTK-dependent but MAP kinase- and PI-3 kinase-independent) are also present in the lung adenocarcinoma cells that are responsible for preventing apoptosis in tumor cells (Wei *et al.* 2001). It would be interesting to study whether the milk-induced PTK-dependent pathway plays some role in the signal transduction pathway of apoptosis.

Recently, it has been reported that an α -lactalbumin complex from acid-precipitated human milk casein (MAL) induces apoptosis in tumor cells and immature cells, but not in mature differentiated cells (Hakansson *et al.* 1995). That report showed that the human colonic cancer cell lines, Caco-2 and HT-29, were sensitive to the cytotoxic effects of MAL. FHs 74 Int cells, which we used in our study, were derived from human fetal intestine and show mature epithelial-like characteristics (Wagner *et al.* 1998). Milk has growth-promoting activity for FHs 74 Int cells as shown in our report and other studies (Ichiba *et al.* 1992, Wagner *et al.* 1998, Wagner & Forsythe 2000). FHs 74 Int cells seem to be resistant to the cytotoxic effect of MAL. We further confirmed that acid-precipitated human milk casein has no cytotoxic effect on this cell line (our unpublished data). Milk may help the neonatal intestinal epithelium towards maturity and away from neoplasia due to the selective sensitivity of tumor cells to MAL.

In this report we showed that the growth-promoting activity of breast milk was contained in the aqueous

phase. One of the largest differences between breast milk and artificial formulas is the presence of cellular components in breast milk. Epithelial cells of the mammary gland, macrophages, lymphocytes and neutrophils are present in breast milk and secrete growth modulators into the milk. These cells could survive in the gastrointestinal tract and may also stimulate intestinal cell proliferation by cell-to-cell interactions (Wagner *et al.* 1996). Fresh whole milk containing living cells may have much higher growth-promoting activity than the aqueous phase of milk used in this study, but fresh whole milk samples did not show any difference from the aqueous phase with respect to growth-promoting activity in our system (our unpublished data). It is thus possible that bioactive substances present in the aqueous phase of milk play a major role in milk-induced intestinal cell proliferation.

While we were preparing this manuscript, Hirai *et al.* (2002) reported that genistein completely inhibited the growth-promoting activity of milk using the same FHs 74 Int cells. Their results and our data are in accord. Furthermore, in this study, we show for the first time the presence of an unknown signaling pathway, which is different from the previously characterized one induced by EGF. Although our data demonstrated the presence of the unknown signaling pathway, it remains unknown whether this pathway is relevant to the proliferation on the intestinal cells. Further characterization of this signaling pathway will need to show the direct interaction of this pathway to cell proliferation. Our final goal is to identify the bioactive substance in human milk and to characterize the unknown signaling pathway. We are now trying to identify this substance by performing protein purification. As shown in Fig. 2D, this substance is present in the retained fraction of a Microcon Centrifugal Filter Device (YM-100). In the process of protein purification, we confirmed using HPLC that this fraction did not contain any of the low molecular weight substances below 2 kDa in size and that most of the substances were above 20 kDa (our unpublished data). Further examination of the mechanism of the effects of this substance will provide new insights into the regulation of milk-induced intestinal cell proliferation and may help to explain the protective effects of milk on the gastrointestinal tract. This may aid in the development of novel preventive and therapeutic strategies for intestinal diseases of newborn infants such as NEC.

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