The Effect of Human Milk on DNA Synthesis of Neonatal Rat Hepatocytes in Primary Culture

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ABSTRACT. We studied the effect of human milk on DNA synthesis of neonatal hepatocytes to elucidate the physiologic role of human milk in growth of the liver. Neonatal hepatocytes were isolated from 5-d-old rats and cultured in serum-free medium. Human milk stimulated DNA synthesis of these hepatocytes in a concentration-dependent manner. The stimulatory activity of 7.5% (vol/vol) human milk plus 0.1 $\mu\text{mol/L}$ insulin was five times that of control and was almost the same as that of 20 $\mu\text{g/L}$ human epidermal growth factor (hEGF) plus insulin. The effect of human milk was additive with treatment with hEGF and insulin. The milk associated with prolonged jaundice of infants was significantly more active than the milk that was not associated with jaundice, although the concentration of hEGF was not different between the two types of milk. The mitogenic activity of milk was heat-labile, inactivated by DTT and stable after treatment with trypsin. Three peaks of the activity were detected in milk by gel filtration and the fraction containing proteins of molecular weight between 36 000 and 76 000 showed the highest activity. Anti-hEGF antibody did not inhibit this activity completely. These results suggested the presence of mitogens other than hEGF or a more active form of hEGF in human milk. The milk associated with breast-milk jaundice exerts a different influence on cell growth and may affect maturation of the liver function related to bilirubin metabolism. The mitogenic activity of milk might be important for growth and development of the liver in infants. (*Pediatr Res* 29: 251–255, 1991)

Abbreviations

hEGF, human epidermal growth factor
mEGF, mouse epidermal growth factor
Mr, molecular weight

Human milk is a rich mixture of biologic factors and hEGF is present in a high concentration in milk (1, 2). Klagsbrun (3) first reported that human milk contains a mitogenic factor that stimulates DNA synthesis and cell division of fibroblast *in vitro*. Subsequent studies have shown that this response to human milk can be demonstrated in several types of cells (1, 3–5) and hEGF is now known to be one of the major growth factors in milk (5). Parenterally administered mEGF can exert a trophic effect on the liver of adult rats (6). It is also reported that ingested mEGF is absorbed by the intestine and distributed to the liver (7) and

that orally administered mEGF enhances hepatic incorporation of [^3H]thymidine into DNA and increases the liver weight of newborn rats (8, 9). Therefore, we hypothesized that human milk has the ability to enhance growth in the human neonatal liver and attempted to determine whether this ability is due to the presence of hEGF. We performed our study using neonatal rat hepatocytes in primary culture to isolate the effect of a single factor on DNA synthesis.

It is well known that human milk is associated with prolonged jaundice of breast-fed infants (10). The etiology of the prolonged jaundice is still obscure. Several studies have found that milk has inhibitory effects on uptake, conjugation, and excretion of bilirubin in hepatocytes (11–13). Thus it is thought that some factors in milk might be affecting the immature liver function of bilirubin metabolism. It has also been observed that certain growth factors have an effect on specific liver functions (9, 14–17). Orally administered mEGF induces precocious maturation of liver enzymes (9). We therefore examined the second hypothesis, that human milk from mothers whose infants had prolonged jaundice exert a different influence on the growth of the neonatal liver, to elucidate the physiologic role of milk in the immature liver.

MATERIALS AND METHODS

Human milk samples. Human milk was collected from three groups of mothers at The Tottori University Hospital as shown in Table 1. In two groups, milk was collected at a healthy baby clinic 1 mo after birth (milk-1 and milk-2) and in one group (milk-3) milk was taken from mothers soon after delivery at the bedside to obtain milk at an earlier stage of lactation. All donors had healthy full-term infants on a breast-feeding regimen and had given informed consent to the study. The first group (milk-1) was a control group. None of the infants in this group had jaundice and the serum total bilirubin level in all infants was under 103 $\mu\text{mol/L}$. The second group (milk-2) consisted of mothers whose infants had prolonged jaundice. The prolonged jaundice with hyperbilirubinemia (serum total bilirubin > 205 $\mu\text{mol/L}$) from d 21 to 37 after birth was diagnosed as breast-milk jaundice with no evidence for disease other than breast-feeding. Standard laboratory investigation of prolonged neonatal jaundice (18) was negative. The mean serum indirect-reacting bilirubin level was 239 $\mu\text{mol/L}$ (range 207–267 $\mu\text{mol/L}$). The infants in the third group had no significant jaundice and they required no treatment.

Milk samples were withdrawn manually and 5 mL midmilk from each mother were obtained for subsequent experiments. All samples from each group were pooled as a large sample and were frozen at -20°C for 1 to 3 mo before analysis. Subsequently, they were thawed and centrifuged at $15\,000 \times g$ for 60 min at 4°C to remove fat and cellular debris. The supernatant was filtered through a 0.22- μm filter (MILLEX-GV, Millipore Co., Bedford, MA) and added to culture in various volumes. The effect of milk on DNA synthesis was tested using milk-1, milk-2, and milk-3. The concentration of total protein in the super-

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natant was measured by the method of Lowry *et al.* (19) using BSA as a standard and hEGF was quantified by RIA using anti-hEGF MAb (20).

Treatment of human milk. The supernatant of milk-1 was subjected to the following conditions to characterize the growth-promoting substance in milk. Milk-1 was: 1) heated at 70°C for 20 min; 2) boiled at 90°C for 30 s; 3) treated with 1 mol/L acetic acid and made up to pH 4.5; 4) treated with 6 mol/L HCl and made up to pH 1.3; 5) treated with 50 mg/L trypsin for 2 h at 37°C; and 6) treated with DTT for 1 h at 25°C.

Gel filtration of milk. A Sephadex G-75 (Pharmacia, Uppsala, Sweden) column (16 mm × 0.70 m) was equilibrated with distilled water. The column was calibrated by chromatography of proteins of known Mr and the absorbance was monitored at 280 nm. The supernatant of 10 mL of milk-1 was applied to the column and eluted at a flow rate of 17.2 mL/h to estimate the

Table 1. Characteristics of human milk samples and of suckling infants*

	Number of mothers	Days after delivery	Presence of jaundice	Total protein (g/L)	hEGF (μg/L)
Milk-1	13	21-37	-†	11.8	25.2
Milk-2	10	21-42	+‡	11.5	24.7
Milk-3	14	2-6	-§	13.5	62.3

* Human milk was collected from mothers by manual suction on the days after delivery as indicated, pooled, and frozen at -20°C. After centrifugation, the concentrations of total protein (Lowry method) and hEGF (RIA) in the supernatant were measured.

† Without prolonged jaundice (total bilirubin < 103 μmol/L).

‡ Jaundice (total bilirubin > 205 μmol/L) of infants at 1 mo without any cause other than breast-feeding was diagnosed as the breast-milk jaundice.

§ Without treatment-requiring jaundice.

Table 2. Stimulatory effect of human milk on DNA synthesis of neonatal rat hepatocytes*

Additions to primary culture	[³ H]thymidine incorporation (dpm/h/mg protein × 10 ⁻⁴)
None	1.33 ± 0.13
I (0.1 μmol/L)	2.11 ± 0.22
I + hEGF (20 μg/L)	6.25 ± 0.37
Milk-1 (7.5%, vol/vol)	4.95 ± 0.24
Milk-1 + I	6.62 ± 0.60
Milk-1 + I + hEGF	10.21 ± 0.62†

* Values are means ± SD of six dishes of two separate experiments. I, insulin.

† $p < 0.01$ compared with I + hEGF.

Fig. 1. The stimulatory effect of hEGF (A) and human milk (B) on DNA synthesis of neonatal rat hepatocytes. hEGF and human milk were added to the culture medium with 0.1 μmol/L insulin at various concentrations. DNA synthesis was assayed by measuring incorporation of [³H]thymidine in 20 h. **Analysis of variance indicated significant differences between milk types ($p < 0.01$) that were concentration dependent.

Fig. 2. The stimulatory effect of human milk on DNA synthesis of neonatal (d 2 and 5) and adult (8 wk, male) rat hepatocytes cultured at low (initial density of 0.65×10^3 cells/mm²) and high (1.3×10^3 cells/mm²) cell densities. Milk-1 was added with 0.1 μmol/L insulin at various concentrations. **Analysis of variance indicated significant differences between low and high density only in the adult hepatocytes ($p < 0.01$) that were concentration dependent.

Table 3. Effects of various treatments on DNA synthesis-stimulating activity in human milk*

Treatment	[³ H]thymidine incorporation (dpm/h/mg protein × 10 ⁻⁴)	Residual activity (%)†
No addition	1.35 ± 0.18	
I (0.1 μmol/L)	1.98 ± 0.27	
Milk-1 (7.5%, vol/vol) + I (0.1 μmol/L)		
No treatment	6.50 ± 0.33	100
70°C, 20 min	1.89 ± 0.15	0
90°C, 30 s	1.85 ± 0.39	0
pH 4.5 (1 mol/L acetic acid)	6.54 ± 0.62	100
pH 1.3 (6 mol/L HCl)	2.60 ± 0.40	14
50 mmol/L DTT (25°C, 1 h)	1.77 ± 0.22	0
50 mg/L trypsin (37°C, 2 h)	5.06 ± 0.90	68

* Values are means ± SD of six dishes of two separate experiments. I, insulin.

† Residual activity is expressed in terms of the activity of milk-1 with no treatment as 100%.

Mr of the activity in milk-1. Fractions were divided into five groups (F1-F5) and lyophilized. The ability to stimulate DNA synthesis at a dose equivalent to 7.5% of the original milk was tested in neonatal rat hepatocytes.

Neutralization of milk with anti-hEGF MAb. Human milk and hEGF was treated with anti-hEGF antibody at 37°C for 1 h, added to the culture medium at a final concentration of 7.5% milk and 75 mg/L anti-hEGF. The stimulation of DNA synthesis by anti-hEGF treated and untreated milk was compared in milk-1 and milk-2.

Isolation and primary culture of hepatocytes. Neonatal rats (d 5, Wistar) were anesthetized with ether and hepatocytes were isolated by the method of Nakamura *et al.* (21). For digestion of neonatal liver tissue, 0.42 g/L collagenase, 0.1 g/L trypsin inhibitor, and 5 mmol/L CaCl₂ in Hanks-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer was used and the liver was perfused with the collagenase solution *in situ* for 5 min. The cell viability of the final preparation, as determined by the ability to exclude trypan blue, ranged from 93 to 97%. The isolated cells were plated at a density of 0.8×10^3 cells/mm² in the wells of plastic dishes (Corning Glass Works, Corning, NY) precoated with highly diluted collagen gel (type I, Nitta Gelatin Co., Yao, Japan). The cells were grown in Williams' E medium with 5% FCS, 1.0 nmol/L insulin, and 1.0 nmol/L dexamethasone and incubated at 37°C in an atmosphere of 95% air and 5% CO₂.

Fig. 3. Gel filtration of human milk. The supernatant (10 mL) of milk-1 was chromatographed on Sephadex G-75. Fractions (4.3 mL/tube) were monitored for absorbance at 280 nm, divided into five groups (F1–F5), frozen, and lyophilized. They were added to the culture medium with 0.1 μ mol/L insulin and the ability to stimulate DNA synthesis was tested in neonatal rat hepatocytes. Values of [3 H]thymidine incorporation are the means of three dishes. *The incorporation of controls only with 0.1 μ mol/L insulin. The following Mr markers were used: catalase (250 kD), human albumin (hAlb, 68 kD), ovalbumin (ovAlb, 45 kD), chymotrypsinogen (Chymo, 25 kD), cytochrome c (Cyt c, 12.5 kD), blue dextran (Vo), and dinitrophenyl-alanine (Vt).

Table 4. Effects of hEGF and human milk with or without anti-hEGF antibody on DNA synthesis of neonatal rat hepatocytes*

Additions to primary culture	[3 H]thymidine incorporation (dpm/h/mg protein $\times 10^{-4}$)	
	Without anti-hEGF	With anti-hEGF (75 mg/L)
None	1.22 $\pm$ 0.14	
I (0.1 μ mol/L)	1.83 $\pm$ 0.26	2.10 $\pm$ 0.21
hEGF (5 μ g/L) + I	4.31 $\pm$ 0.35	2.14 $\pm$ 0.44
hEGF (20 μ g/L) + I	4.91 $\pm$ 0.48	2.27 $\pm$ 0.60
Milk-1 (7.5%, vol/vol) + I	4.60 $\pm$ 0.49	2.67 $\pm$ 0.39†
Milk-2 (7.5%, vol/vol) + I	5.17 $\pm$ 0.35‡	3.16 $\pm$ 0.29§

* Values are means $\pm$ SD of six dishes of two separate experiments. I, insulin.

† $p < 0.05$ compared with I plus anti-hEGF.

‡ $p < 0.05$ compared with milk-1 plus I with and without anti-hEGF.

§ $p < 0.01$ compared with I plus anti-hEGF.

After 3 h, the medium was replaced with serum-and hormone free-medium containing 0.12 mg/L aprotinin (22).

Assay of DNA synthesis. The medium was replaced again and insulin, hEGF, or human milk were added 24 h after plating. The cells were then cultured for 36 h with [3 H]thymidine present for the last 20 h. Replicative DNA synthesis was assayed by measuring incorporation of [3 H]thymidine into DNA with or without 10 mg/L aphidicolin (23). Cellular proteins were measured by the method of Lowry *et al.* (19).

Materials. The following compounds were purchased from Sigma Chemical Co., St. Louis, MO: insulin, aprotinin, trypsin, trypsin inhibitor, and BSA. FCS was from Flow Laboratories, Rockville, MD and Williams' E medium was from Kyokutou Pharmaceutical Co., Tokyo, Japan. Human EGF and the MAb to hEGF were from Wakunaga Pharmaceutical Co., Hiroshima, Japan. [3 H]thymidine (84.8 μ Ci/mmol) was from DuPont Co., Wilmington, DE. Other chemicals of reagent grade were all from Wako Pure Chemical Industries, Osaka, Japan.

Statistical analysis. All values are reported as means $\pm$ SD. Comparisons of the results were made with unpaired *t* test and two-way analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS

Table 1 shows that the concentrations of both total protein and hEGF in milk-1 were the same as those in milk-2. The concentration of hEGF in milk-3, which had been collected at an earlier stage of lactation, was higher than that in milk-1.

Neonatal rat hepatocytes in culture showed the ability to synthesize DNA without the stimulation of any growth factors and that this ability was enhanced by several factors as shown in Table 2. Insulin at a concentration of 0.1 μ mol/L stimulated DNA synthesis about 1.5 times the rate of control cells and 20 μ g/L hEGF stimulated DNA synthesis about five times that of controls. Milk-1 at a concentration of 7.5% (vol/vol) plus 0.1 μ mol/L insulin stimulated DNA synthesis of neonatal hepatocytes as actively as did 20 μ g/L hEGF plus insulin. Human milk and hEGF with insulin showed their maximum effects on DNA synthesis at the concentrations of 7.5% and 20 μ g/L respectively, as shown in Figure 1. However, the simultaneous addition of milk-1, insulin, and hEGF markedly stimulated DNA synthesis of neonatal hepatocytes (Table 2). Moreover DNA synthesis was significantly higher than that of insulin plus hEGF ($p < 0.01$). The same results were obtained using milk-2 and milk-3.

Figure 1A shows that hEGF (0–20 μ g/L) stimulated DNA synthesis in a concentration-dependent manner. Human milk also stimulated DNA synthesis in a concentration-dependent manner from 0 to 7.5% as shown in Figure 1B. The effect of 10% milk was the same as that of 7.5% milk. Although the concentration of hEGF in milk-3 was higher than that in milk-1, the DNA synthesis-stimulating activity of milk-3 was the same as that of milk-1. In contrast, the concentration of hEGF was the same in milk-1 and milk-2, yet the stimulating activity of 7.5% milk-2, which was from mothers of infants with the breast-milk jaundice, was significantly higher than that of milk-1 from mothers of healthy infants ($p < 0.01$).

Furthermore, the effect of human milk on DNA synthesis was compared in younger neonatal rat (d 2) hepatocytes and adult rat (8 wk, male) hepatocytes to determine whether age is a factor in this response to milk. The methods of isolation and culture of the adult rat hepatocytes were the same as those for neonatal hepatocytes, except that a 0.5 g/L collagenous solution was used. The stimulation of DNA synthesis by milk-1 was tested in hepatocytes cultured at high (at an initial density of 1.3×10^3 cells/mm 2) and low (0.65×10^3 cells/mm 2) cell densities. The effect of human milk on DNA synthesis in adult rat hepatocytes was significantly suppressed when hepatocytes had been cultured at a high density as shown in Figure 2 ($p < 0.01$). At a relatively low density, the effect of 7.5% milk-1 on DNA synthesis of adult hepatocytes was about four times that of controls, which was similar to the response to 20 μ g/L hEGF. The effect on DNA synthesis of neonatal (d 2 and 5) rat hepatocytes was not suppressed even at a high density.

We also tried to characterize the mitogenic substance in milk. Table 3 shows the stability of the activity in milk-1 after various treatments. The effect of milk on DNA synthesis of neonatal hepatocytes decreased markedly after heat treatment and was lost completely after reduction with DTT. The stimulation of DNA synthesis was maintained after treatment with trypsin and with 1 mol/L acetic acid (pH 4.5), which precipitated casein in milk. Thus the growth-promoting substance in human milk was heat-labile but acid-stable and resistant to digestion, most likely a protein containing disulfide bridges.

To estimate the Mr of the growth-promoting factor in human milk, the supernatant of milk-1 was subjected to gel filtration on a Sephadex G-75 column. The ability to stimulate DNA synthesis of five subfractions (F1–F5) from gel filtration is shown in Figure 3. F3 (Mr 36–76 kD) showed the strongest stimulation of DNA synthesis of neonatal hepatocytes and the activity was five times as high as that of controls. The activity in F4 (12.5–36 kD) was nearly equal to that of F3. We also recognized the activity in F5 (less than 12.5 kD). We found no ability to stimulate DNA

synthesis in F1 (more than 250 kD) and F2 (76–250 kD). In further experiments, the activity was found in three Mr ranges, which were between human albumin (68 kD) and ovalbumin (45 kD), between chymotrypsinogen (25 kD) and cytochrome c (12.5 kD), and less than cytochrome c.

Subsequently, we examined immunologically whether the mitogenic factor of human milk for neonatal hepatocytes was hEGF. Table 4 shows the effect on DNA synthesis of milk-1 and milk-2 treated with anti-hEGF MAb. Anti-hEGF antibody at a concentration of 75 mg/L completely inhibited the activity of 5 μ g/L hEGF. It also inhibited the activity of 20 μ g/L hEGF at the same concentration. In contrast, the addition of 75 mg/L anti-hEGF antibody did not completely inhibit the activity of human milk and more than 20% of original activity remained after treatment with the antibody. The concentration of hEGF in 7.5% milk was only 1.25 μ g/L, so 75 mg/L anti-hEGF represented a large excess of antibody to the hEGF of 7.5% milk. The effect of human milk treated with anti-hEGF antibody was lower than that of untreated milk. However, the activity of the treated milk was significantly higher than that of the control with insulin and anti-hEGF ($p < 0.05$, $p < 0.01$). Moreover, after the treatment with anti-hEGF, the activity of 7.5% milk-2 was higher than that of 7.5% milk-1 ($p < 0.05$).

DISCUSSION

We demonstrated that human milk stimulates DNA synthesis of neonatal rat hepatocytes in primary culture. The presence of a growth-promoting activity in human milk has already been demonstrated in several cell lines such as fibroblasts (3), glia (5), and myoblasts (4). Shing *et al.* (24) purified the growth factor of human milk for cultured fibroblasts and reported that the major growth factor has a Mr of 6000 and an isoelectric pH value of 4.5, and is probably a form of hEGF. In addition, it is known that human milk contains a very high concentration of hEGF (1, 2). However, the effect of human milk on neonatal hepatocytes had not yet been studied.

In our study, the differences in DNA synthesis-stimulating activity did not coincide with differences in the concentration of hEGF in milk. The effect of 7.5% human milk was additive with the maximum effect of insulin plus hEGF. The mitogenic substance of milk has heat-instability characteristics that are different from those of hEGF previously reported (25). Three peaks of the activity were detected in milk by gel filtration and the major peak was not in the fraction of Mr less than 12 500. Carpenter (5) showed that the addition of antibody to hEGF reduces the stimulatory effect of milk by more than 90% in cultured fibroblasts. In neonatal hepatocytes, more than 20% of the original activity was residual after the addition of antibody. These results indicate that human milk has growth-promoting activity for neonatal hepatocytes in primary culture and that hEGF could be one of the major factors, but other unknown mitogens are also present in human milk.

The DNA synthesis stimulatory activity of the milk from mothers of infants with breast-milk jaundice was significantly greater than that of the milk from control mothers. The difference in mitogenic ability of two groups was due to the presence of mitogenic factors other than hEGF because the concentration of hEGF in the two groups was the same. Several growth factors are known to affect the specific functions of liver as well as growth (9, 14–17). Orally administered mEGF can induce precocious development of glucokinase (EC 2.7.1.2) activity in the liver and increase bile salt secretion (9). Nakamura *et al.* (17) have found a reciprocal relation between growth and differentiation of certain enzymatic activities in neonatal hepatocytes *in vitro*. Thus the growth of neonatal hepatocytes may regulate the expression of specific liver functions. Therefore, we speculated that the unknown growth-promoting substance in milk from mothers of infants with breast-milk jaundice may have inhibitory effects on maturation of liver functions of bilirubin metabolism.

These include the process of uptake, conjugation, and excretion of bilirubin in neonatal hepatocytes. Moreover, a reciprocal relationship between growth and maturation of bilirubin metabolism in liver might be present. A study that reported elevation of serum α -fetoprotein in breast-fed infants with jaundice coincides with this speculation (26).

In adult hepatocytes, human milk showed a growth-promoting effect only at a low density as well as in the presence of other growth factors such as insulin, hEGF, and hepatic growth factor (27, 28). The hepatocytes cultured at a low density correspond to the liver in regeneration after partial hepatectomy *in vivo* (28). In contrast, inasmuch as the effect on neonatal hepatocytes was not density-dependent, the trophic effect of milk could be active in normal neonatal liver. The role of growth factors in milk on the growth of the liver might be more important during the neonatal period.

To be active in infants, growth factors in milk would have to survive passage through the stomach, which has a pH of about 2 to 3 (29) and the gastrointestinal tract, which contains proteolytic enzymes (30). The DNA synthesis stimulating activity contained in milk was stable after treatment with trypsin and with acetic acid (pH 4.5). Britton *et al.* (31) observed intact survival of hEGF in gastric juice of preterm infants. Orally ingested mEGF absorbed by the intestinal tract can be localized to various somatic organs and it is associated with hepatic and somatic growth in neonatal rats (7–10, 32). Therefore, it is possible that the growth-promoting factors in milk that stimulate DNA synthesis of neonatal hepatocytes *in vitro* could be absorbed in an active form and play a role in the immature liver. In addition, the activity of human milk may be involved in growth and development of the liver in infants.

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