

## The Concentration of Epidermal Growth Factor in Japanese Mother's Milk

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**Summary** The concentration of epidermal growth factor (EGF) in human milk was measured by radioreceptor assay (RRA). Human milk samples collected from healthy Japanese mothers who delivered at full term were divided into 30 groups according to differences in the duration of lactation, seasons of the year and place of residence. Human milk collected from 3 to 5 days after delivery in winter and summer contained 15.03  $\mu\text{g}/100\text{ ml}$  and 15.46  $\mu\text{g}/100\text{ ml}$  of EGF, respectively. The concentrations decreased rapidly to 8.04  $\mu\text{g}/100\text{ ml}$  and 8.12  $\mu\text{g}/100\text{ ml}$  in milk from 31 to 60 days, and to 5.10  $\mu\text{g}/100\text{ ml}$  and 5.44  $\mu\text{g}/100\text{ ml}$  in milk from 241 to 481 days. Seasonal and regional differences in EGF concentration were observed to some extent, but no clear tendency could be defined. These results suggest that EGF is an essential and fundamental factor for the growth of infants in the very early stages after delivery, because it was high only at the beginning of lactation and then it decreased.

**Key Words** epidermal growth factor, human milk, full term, Japanese mother, concentration, radioreceptor assay (RRA), radioimmune assay (RIA), lactation, season, region

Human milk secreted from healthy mothers taking balanced diets is the ideal nutrition for newborn infants. Human milk contains some components which protect infants against infection, or are responsible for physiological regulation, as well as essential nutrients for growth. Among these components, several hormones were reported as growth stimulators for the maturation of intestinal mucosal cells in newborn infants. Epidermal growth factor (EGF) is one of those potent growth factors found in human milk (1). EGF, a peptide hormone of 53 amino acids (MW=6,000 Da), was first identified in mouse salivary gland by Cohen in 1969 (2). EGF is widely distributed in mammalian organs and fluids, such as kidneys, liver, blood, urine and milk. Among its biological functions, EGF was reported to suppress gastric acid secretion (3), stimulate growth of epithelial cells (4) and

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stimulate digestive enzyme activity (5-7). These findings suggest that EGF is fundamental for the regulation of pH in the stomach and maturation of gastrointestinal epithelial cells in newborn infants. Therefore, it is expected that human milk EGF participates in the maturation of digestive functions, and shows anti-infection and anti-allergic effects in infants (8-10).

Several researchers have determined the content of EGF in human milk, however, the results varied greatly (11-18). In these studies, a small number of samples was investigated. It is important for infant growth that clarifying the standard concentration of EGF in normal milk secreted from healthy mothers. To know the average concentration of EGF in normal milk, we should measure it in a large population.

In this study, we measured the concentration of EGF in Japanese mother's milk by radioreceptor assay (RRA) to clarify lactational, seasonal and regional differences. We collected samples of human milk under restricted conditions: i.e. mother's age, nutritional conditions, number of children, gestation weeks, days after delivery, milking method and seasonal and regional factors.

#### MATERIALS AND METHODS

*Milk samples.* Milk samples were obtained from 2,434 mothers, (17 to 41 years old, average 28.2), who visited maternity hospitals in 48 towns in Japan (Fig. 1) for regular check ups of their infants. The samples were collected in winter and summer (winter milk and summer milk, respectively). Of them, 2,779 samples which fulfilled the criteria described in Table 1 were selected, divided into 8 lactational stages, two seasons and 7 sampling regions, and pooled as described in Tables 2 and 3 (19). All the samples were stored under  $-80^{\circ}\text{C}$  until analyzed.

*Sample treatment.* The samples were centrifuged at  $2,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and the intermediate layer was centrifuged again at  $43,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The resulting intermediate layer was filtrated through a syringe filter unit (pore size:  $0.22 \mu\text{m}$ ; Kurabo, Osaka, Japan).

*Preparation of the EGF receptor fraction.* The EGF receptor fraction was prepared from human placenta obtained after a cesarean operation according to the method of Hirata et al (20). The amnion and chorion were trimmed off immediately and washed with cold saline. The tissue was homogenized for 30 s in 2 ml/g wet tissue of 0.25 M sucrose with a Waring blender and further homogenized for 60 s with a polytron (type P7-10, Kinematica A.G. Luzern, Switzerland). The homogenate was centrifuged at  $12,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  and the supernatant was recentrifuged at  $40,000 \times g$  for 30 min. The resulting pellet was the EGF receptor fraction and was stored in  $100 \mu\text{l}$  aliquots at  $-80^{\circ}\text{C}$  until analyzed.

*Radioreceptor assay (RRA).* EGF content was measured by RRA as already described (20). Briefly, samples or purified mouse EGF (mEGF, Boehringer Mannheim, Mannheim, Germany) as the standard were diluted to  $1-20 \mu\text{g}$  EGF/100 ml with standard diluent, which was 50 mM Tris-HCl buffer (pH 7.5) contain-



Fig. 1. Sampling areas (●).

Table 1. Conditions and collecting method of human milk.

Mother and infant	
Mother:	healthy, balanced diet, without vitamin supplements normal delivery
Infant:	healthy, normal growth from delivery to the time of sampling in 3-97 percentile of the standard body weight growth curve, breast fed
Collection	
Sampling time:	10:00-18:00 JMT
Device:	manual milking apparatus
Sampling milk:	intermediate milk fore and hind milk
Treatment:	samples were frozen immediately, transported to our laboratory, thawed at 30°C, then stirred, dispensed in 5-ml aliquots and stored at -40 to -80°C until assayed

ing 0.25% bovine serum albumin (BSA). A 100- $\mu$ l aliquot of sample or mEGF was mixed with 100  $\mu$ l of the standard diluent and 100  $\mu$ l of the receptor fraction (250  $\mu$ g protein/ml) and preincubated for 10 min at 25°C. Then 100  $\mu$ l of 19 kBq/ml <sup>125</sup>I-labelled mEGF (Amersham, Buckinghamshire, England) was added to each tube and the mixture was further incubated for 40 min at 25°C. The reacted mixture was centrifuged at 6,000  $\times$  g for 10 min at 4°C and the supernatant was discarded.

Table 2. Properties of human milk samples in lactational stages.

Days after delivery	3-5	6-10	11-15	16-30	31-60	61-120	121-240	241-482
Total number of samples	110	181	177	351	562	314	280	149
In winter (winter milk)								
Number of samples [male]	59 [28]	103 [54]	67 [38]	159 [83]	243 [124]	194 [110]	189 [98]	84 [47]
[female]	[31]	[49]	[29]	[76]	[119]	[84]	[91]	[37]
Days after delivery [mean $\pm$ SE]	4.4 $\pm$ 0.8	6.9 $\pm$ 1.3	13.3 $\pm$ 1.4	24.7 $\pm$ 4.7	40.0 $\pm$ 9.2	95.6 $\pm$ 16.6	176 $\pm$ 35	302 $\pm$ 43
Mother's age	27.4 $\pm$ 4.6	27.5 $\pm$ 3.7	28.0 $\pm$ 3.5	28.3 $\pm$ 4.1	28.1 $\pm$ 3.6	28.5 $\pm$ 3.8	28.4 $\pm$ 3.6	29.4 $\pm$ 4.0
Birth weight [male]	3,139 $\pm$ 387	3,266 $\pm$ 343	3,201 $\pm$ 339	3,170 $\pm$ 315	3,147 $\pm$ 289	3,208 $\pm$ 317	3,216 $\pm$ 312	3,214 $\pm$ 301
[female]	3,023 $\pm$ 196	3,122 $\pm$ 330	3,193 $\pm$ 374	3,105 $\pm$ 289	3,157 $\pm$ 302	3,158 $\pm$ 309	3,204 $\pm$ 288	3,191 $\pm$ 306
Number of deliveries [mean $\pm$ SE]	1.8 $\pm$ 0.8	1.7 $\pm$ 0.8	1.7 $\pm$ 0.8	1.8 $\pm$ 0.9	1.6 $\pm$ 0.7	1.7 $\pm$ 0.9	1.6 $\pm$ 0.8	1.8 $\pm$ 0.8
In summer (summer milk)								
Number of samples [male]	51 [24]	78 [37]	110 [59]	192 [98]	319 [143]	120 [70]	91 [52]	65 [27]
[female]	[27]	[41]	[51]	[94]	[176]	[50]	[39]	[28]
Days after delivery [mean $\pm$ SE]	4.3 $\pm$ 0.8	7.1 $\pm$ 1.3	13.0 $\pm$ 1.4	24.0 $\pm$ 5.1	37.4 $\pm$ 7.5	87.4 $\pm$ 17.5	172 $\pm$ 33	310 $\pm$ 53
Mother's age	28.1 $\pm$ 4.7	28.1 $\pm$ 3.9	28.5 $\pm$ 4.4	28.2 $\pm$ 3.2	27.8 $\pm$ 3.9	27.6 $\pm$ 3.6	28.7 $\pm$ 3.9	28.5 $\pm$ 3.5
Birth weight [male]	3,095 $\pm$ 311	3,292 $\pm$ 262	3,242 $\pm$ 362	3,136 $\pm$ 277	3,212 $\pm$ 342	3,151 $\pm$ 296	3,183 $\pm$ 325	3,340 $\pm$ 311
[female]	3,178 $\pm$ 356	3,189 $\pm$ 336	3,132 $\pm$ 276	3,081 $\pm$ 305	3,124 $\pm$ 312	3,110 $\pm$ 284	3,113 $\pm$ 323	3,175 $\pm$ 389
Number of deliveries [mean $\pm$ SE]	1.8 $\pm$ 0.7	1.8 $\pm$ 0.7	1.8 $\pm$ 0.8	1.8 $\pm$ 0.7	1.7 $\pm$ 0.8	1.7 $\pm$ 0.8	1.7 $\pm$ 0.8	1.6 $\pm$ 0.7

Table 3. Properties of human milk samples in various districts.

Days after delivery	Kyushu Okinawa	Chugoku Shikoku	Kinki	Tokai	Kanto Koshin-etsu	Tohoku	Hokkaido
Total number of samples	155	117	60	109	110	91	72
<b>In winter (winter milk)</b>							
Number of samples [male]	74 [42]	62 [33]	30 [17]	50 [17]	52 [27]	47 [25]	42 [23]
[female]	[32]	[29]	[13]	[33]	[25]	[22]	[19]
Days after delivery [mean±SE]	36.0±17.2	38.1±14.8	43.4±19.0	34.3±12.5	41.1±20.8	38.0±14.3	42.0±19.5
Mother's age	28.1±4.1	28.4±3.7	27.6±3.3	27.9±3.7	28.6±4.4	28.5±3.6	27.8±4.0
Birth weight [male]	3,150±316	3,100±270	3,144±312	3,109±306	3,147±296	3,214±338	3,166±329
[female]	3,130±252	3,054±308	3,208±365	3,098±278	3,248±288	3,097±369	3,175±249
Number of deliveries [mean±SE]	1.7±0.8	1.8±1.0	1.7±0.8	1.6±0.7	1.7±0.8	1.9±0.9	1.6±0.6
<b>In summer (summer milk)</b>							
Number of samples [male]	81 [33]	55 [29]	30 [18]	59 [26]	58 [21]	44 [22]	30 [13]
[female]	[48]	[26]	[12]	[33]	[37]	[22]	[17]
Days after delivery [mean±SE]	36.3±13.8	35.3±12.9	43.8±23.9	33.9±11.7	34.6±14.7	45.9±19.2	41.0±16.8
Mother's age	28.0±3.6	28.7±3.7	28.4±3.4	27.7±3.3	27.3±4.2	28.5±3.6	27.1±3.4
Birth weight [male]	3,084±278	3,128±295	3,220±318	3,123±350	3,289±299	3,196±340	3,051±338
[female]	3,074±336	3,021±301	3,094±233	3,053±303	3,078±335	3,087±317	3,208±279
Number of deliveries [mean±SE]	1.6±0.7	1.7±0.8	1.6±0.6	1.7±0.7	1.9±0.7	1.8±0.8	1.7±0.8

The pellet was suspended in 1 ml of ice cold standard diluent and recentrifuged at  $6,000 \times g$  to discard the supernatant. The radioactivity of the resulting pellet was counted in a gamma counter (Auto-gamma 5110, Packard, Meriden, CT) for 3 min. Specific binding was defined as the difference in radioactivity between the sample tube and the "non-specific binding" tube which contained 10 ng of unlabelled EGF. EGF content was estimated from the mEGF standard curve for specific binding. All analyses were performed in duplicate.

## RESULTS

### Curve fitting for RRA

Using human placental membrane as the EGF receptor, we were able to measure the concentration of EGF in human milk by RRA accurately. The shape of the dilution curve for human milk samples (6–10 days after delivery, pooled summer milk) was similar to a standard curve for the assay (Fig. 2). The EGF concentration of the standard curve ranged from 0.5 to 20  $\mu\text{g}/100\text{ml}$  and the

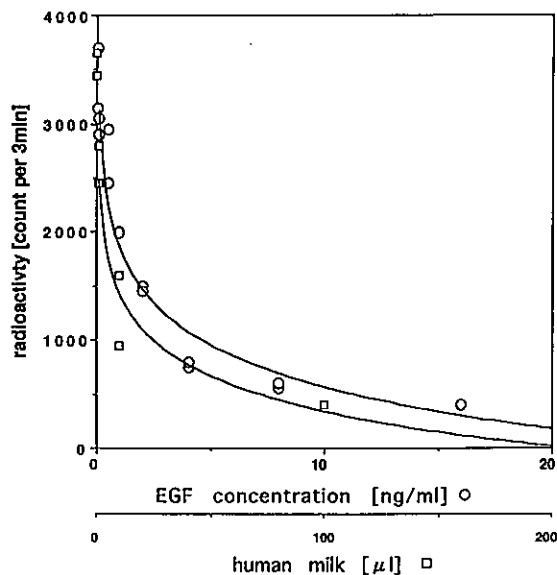


Fig. 2. Comparison of a standard EGF curve and a dilution curve of human milk. A standard curve (○) for the assay was obtained as described in MATERIALS AND METHODS by incubating the receptor fraction with increasing concentrations (0.5–16 ng/ml) of mouse EGF. A dilution curve of human milk (□) was obtained by incubating the receptor fraction with human milk diluted with standard diluent (dilution factor: 1, 10, 100, 1,000). ○, std. curve  $y = 1,856.1 - 1,288.7 \times \log(x)$ ,  $R_2 = 0.945$ ; □, human milk  $y = 1,422.5 - 1,080.0 \times \log(x)$ ,  $R_2 = 0.969$ .

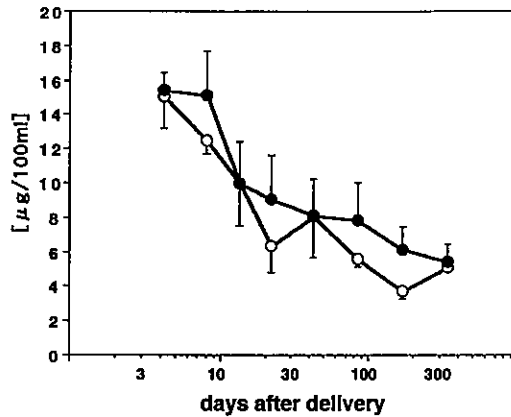


Fig. 3. EGF concentration in human milk during lactation. —○—: winter milk, —●—: summer milk.

coefficient of variation which was defined as the standard error divided by the mean was 0.108.

#### *Lactational and seasonal differences in EGF concentration*

Pooled human milk samples were analyzed (Table 2). The concentration of EGF decreased during lactation. The value was plotted on days after delivery as logarithmic abscissa (Fig. 3). The average concentration of EGF in winter milk was slightly lower than that of summer milk, and there were no significant differences between them. In the first month, the concentration of EGF decreased rapidly to 58% in winter milk and to 41% in summer milk. Thereafter, it continued to decrease gradually. The ratio of EGF concentration in milk collected from 241 to 481 days after delivery to colostrum (from 3 to 5 days after delivery) was 0.34 in winter and 0.35 in summer, approximately.

#### *EGF/protein ratio in human milk*

As shown in Table 4, the concentration ratio of EGF to protein was slightly higher in colostrum (from 3 to 10 days) than during the rest of lactation period and it remained at approximately 5 ng/g protein after 11 days. There was no clear seasonal difference between winter and summer milk.

#### *Regional differences in EGF concentration*

The EGF concentration was measured in pooled samples collected from 33 to 46 days after delivery from healthy mothers who lived in various regions of Japan (Table 3). In winter, the highest EGF concentration was observed in Hokkaido, in the northern part of Japan, and the lowest was seen in Kinki, the middle west part of Japan. On the other hand, in summer, the highest concentration was found in Kyushu-Okinawa, the south-west part of Japan, and the lowest in Tokai, the

Table 4. EGF/protein ratio in human milk.

Days after delivery	Crude protein g/dl (19)		EGF ng/g protein	
	in winter	in summer	in winter	in summer
3-5	2.04	2.21	7.38	6.90
6-10	1.94	1.93	6.42	7.14
11-15	1.68	1.63	5.97	6.15
16-30	1.53	1.46	4.14	5.28
31-60	1.36	1.33	5.92	6.08
61-120	1.17	1.18	4.74	5.67
121-240	1.09	1.13	3.39	4.35
241-481	1.13	1.11	4.51	4.75

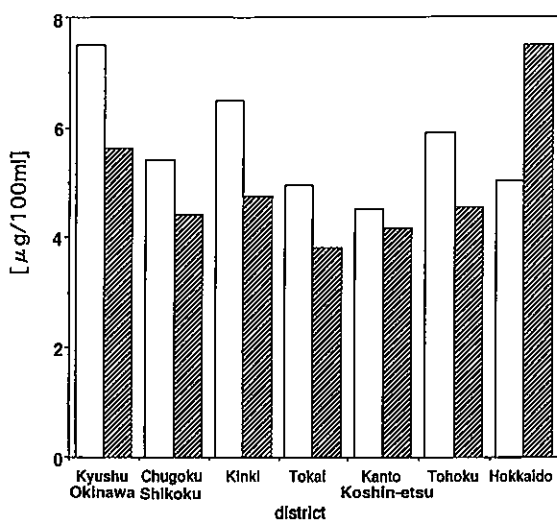


Fig. 4. EGF concentration in human milk from mother's living in various districts of Japan. The location of these districts is shown in Fig. 1. □, winter milk; ▨, summer milk.

central part of Japan (Fig. 4). At this stage of lactation, the concentration of EGF in winter milk ( $5.7 \mu\text{g}/100 \text{ml}$ ) was slightly higher than that found in summer milk ( $5.0 \mu\text{g}/100 \text{ml}$ ).

#### DISCUSSION

The composition of human milk depends on several factors such as the mother's age, the mother's nutritional condition, lactational stage, geographic location, gestation age, milking time in a day, milking method and milking volume among others. In this study, we standardized conditions to collect human milk



samples to avoid biases in quality of the samples.

Milk samples were collected systematically in summer and in winter from 2,434 mothers living in various regions of Japan at different stages of lactation (3–482 days after delivery). Of them, 2,279 milk samples which fulfilled the criteria employed (19) were used for analysis. The samples were divided into 8 lactational stages and 7 regions in 2 seasons as described in Table 2.

Many researchers have measured the concentration of EGF in human milk, bovine milk and bovine milk-based infant formulae (11–18). Human milk contains a high concentration of EGF in comparison with other tissues or body fluids. However the concentration of EGF in human milk varied among the various studies because of differences in measuring methods: radioreceptor assay (RRA, 15, 16, 17, 18, 20) and radioimmune assay (RIA, 11, 12, 14). Even by RRA, Iacoppta et al. reported that when placental plasma membrane is used for RRA the concentration of EGF might be overestimated, whereas intact A431 cells are used this does not happen. However in our study, we showed that the dilution curve of human milk fitted the standard curve of human EGF for RRA by placental plasma membrane (Fig. 2). This indicates that RRA by placental membrane is an accurate method to measure the concentration in human milk.

Azuma et al. (21) reported that the EGF in human milk has size heterogeneity using ELISA, the DNA synthesis stimulatory activity of the high molecular weight EGF complex was 5.5% when compared with that of low molecular weight (6 kDa) EGF and these EGF fractions may have been in an apparent equilibrium state. Since it is possible that high and low molecular weight EGF are detected in both RIA and RRA, it is difficult to distinguish the concentration of high and low

Table 5. Reported concentration of EGF in human milk [ $\mu\text{g}/\text{dl}$ ].

Days after delivery	3–5	6–10	11–15	16–30	31–60	61–120	121–240	241–481
<b>RRA</b>								
Suzuki et al. [1984]	4.6 (2–4)		4.9 (8–10)					
Yagi et al. [1985]	14.5 (2–4)	12.1 (5–7)	16.1 (8–10)					
Read et al. [1986]	33.0 (colostrum)			3.0 (20)				
This study	15.3	13.8	10.0	7.7	8.1	6.7	4.9	5.3
<b>RIA</b>								
Beardmore et al. [1983]	3.5–43.8 (colostrum)				2.0–11.1 (mature milk)			
Jasson et al. [1985]	2.5–3.8 (colostrum)				0.5–1.6 (28–56)			
Oguchi [1988]	19.6 (3)	7.2 (7)		3.8 (30)				

( ): days after delivery.

molecular weight EGF, and it is not clear yet what the biological significance is of the size heterogeneity of EGF. If the significance is different, it seems necessary to determine the concentration of EGF in each size.

Although RIA using anti-EGF antibody has shown high sensitivity for EGF, the antibody's reactivity does not always parallel biological activity (20,21). On the other hand, in RRA, the EGF bound to EGF receptor can be measured and indicates biological activity such as growth-promoting activity more accurately than that detected by immunoassay.

Table 5 summarizes the data reported in the literature regarding the concentration of EGF as determined by RRA and RIA. Because some researchers reported that the concentration of EGF varies among individual samples, we measured it in pooled human milk as the representative for each stage of lactation and each area in Japan. The number of samples varied from 110 to 562 per lactational stage.

Suzuki et al. (15) and Yagi et al. (16) reported no lactational changes in the concentration of EGF, whereas other authors suggested the concentration of EGF in human milk decreased during lactation.

Our results show a decrease in EGF concentration during lactation, which is in agreement with the data reported by Oguchi (18). The ratio of EGF to protein in human milk was also higher in colostrum than in mature milk (Table 4), which suggests that EGF is an important factor for the maturation of intestine in infants, especially in the newborn period.

We found slight seasonal and regional differences in the concentration of EGF in human milk. The highest EGF concentration was observed in Hokkaido in the north of Japan in winter, and the lowest was observed in Kyushu and Okinawa in the south-west of Japan in summer. There was no clear correlations between the EGF concentration and atmospheric temperature or other environmental factors inherent to the geographical location.

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