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Interleukin-18 in Human Milk

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ABSTRACT

We analyzed IL-18 levels of human milk. Colostrum contained significantly higher levels of IL-18 compared with early milk and mature milk. By stepwise multiple linear regression analysis, preterm delivery and pregnancy complications of mothers significantly correlated with high levels of IL-18 in human milk ($p = 0.0007$ and 0.0018, respectively). There was a significant correlation between the levels of IL-18 and soluble Fas ligand in colostrum ($p = 0.0003$). IL-18 was detected in actively secreting epithelial cells in lactating mammary gland by immunohistochemical staining. These results suggest that IL-18 in colostrum plays an important role in host defense of high-risk neonates. **(*Pediatr Res* 50: 268–272, 2001)**

Abbreviations:
TNF-α, tumor necrosis factor-alpha
sFasL, soluble Fas ligand
IFN-γ, interferon-gamma
TGF-β, transforming growth factor-beta
GAPDH, glyceraldehyde 3-phophate dehydrogenase
Th1, T helper type 1
Th2, T helper type 2

IL-18 is produced by activated macrophages (1), keratinocytes (2), and intestinal epithelial cells (3). IL-18 mediates inflammatory response by inducing the production of chemokines, granulocyte-macrophage colony-stimulating factor, IL-2, and TNF-α by mononuclear cells (4). IL-18 strongly induces IFN-γ and TNF-α production by T and natural killer (NK) cells when costimulated with IL-12, IL-2, mitogen, or microbial agents (4, 5). It also induces Fas ligand (FasL) expression on lymphocytes (6) and enhances their capacity to mediate cytotoxicity against Fas-expressing cells, which may play an important role in the clearance of infected cells (7).

Human milk contains a multitude of enzymes, hormones, growth factors, and immunologic agents (8). Among them, various cytokines, including IL-1β (9), IL-6 (10), IL-12 (11), TNF-α (12), IFN-γ (13), IL-8 (14), IL-10 (15), TGF-β (16), and sFasL (17), may provide both adaptive and innate immune responses in host defense against enteric or respiratory pathogens (18–20).

In this study, we measured IL-18 levels along with other cytokines in human milk from mothers with or without preterm delivery or pregnancy complications. We found that colostrum from mothers with preterm delivery or pregnancy complications contained significantly higher concentrations of IL-18. The significance of IL-18 in human milk, especially for high-risk neonates, is discussed.

MATERIALS AND METHODS

Milk sample collection. Colostrum (within first 72 h postpartum), early milk (between 72 h to 7 d postpartum), and mature milk (1 mo after the delivery) were collected in the maternity and perinatal care units of Kyushu University Hospital and Fukuoka City Hospital. Table 1 summarizes the characteristics of milk sample donors. All mothers received verbal and written information about the aim of this study. Informed consent was obtained from each mother. Milk samples were collected in sterile polypropylene tubes by the mothers with manual breast pumps at the hospital. They were immediately processed. After removal of lipids, they were stored in 1.5-mL polypropylene tubes at −80°C. Cellular components were washed twice with PBS and stored at −80°C in sterile polypropylene tubes until use.

Purification of peripheral blood monocytes. Heparinized blood samples were obtained from four healthy volunteers. Informed consent was obtained from each donor. After mononuclear cells were obtained by density-gradient centrifugation

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Table 1. *Characteristics of milk donors*

	Preterm delivery (n = 45)	Term delivery (n = 71)	Total (n = 116)
No pregnancy complications	5	68	73
Pregnancy complications	40*	3	43
PROM	17	1	18
Chorioamnionitis	12	0	12
Threatened premature delivery	26	0	26
Abruptio placentae	3	0	3
Toxemia of pregnancy	8	2	10
Other infectious complications	0	0	0
Cesarean section	23	6	29

* Twenty of 40 mothers had more than one pregnancy complications. In 20 mothers who had more than one pregnancy complications, 7 had three pregnancy complications (PROM [premature rupture of membrane], threatened premature delivery and chorioamnionitis). Thirteen had two pregnancy complications (5 had PROM and threatened premature delivery, 4 had toxemia of pregnancy and threatened premature delivery or abruptio placentae, 3 had PROM and chorioamnionitis or toxemia of pregnancy, and 1 had threatened premature delivery and chorioamnionitis).

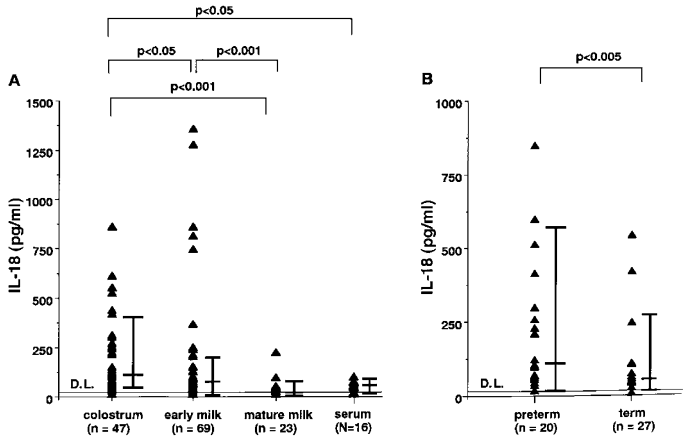

Figure 1. IL-18 levels of human milk. (*A*) IL-18 levels of colostrum, early milk, mature milk, and serum of lactating mothers. (*B*) IL-18 levels of colostrum from mothers with preterm and term delivery. Each bar represents median with a range from 10th percentile to 90th percentile. *D.L.*, detection limit of IL-18.

using Ficoll-Hypaque (Pharmacia, Piscataway, NJ, U.S.A.), $CD14^+$ monocytes were enriched by cell sorting using EPICS ALTRA (Beckman-Coulter, Hialeah, FL, U.S.A.). The purity of $CD14^+$ cells was almost 99%.

Cytokine assays. We measured the cytokine levels of colostrum and serum with ELISA kits according to the manufacturers' instructions (IL-18 and sFasL from MBL, Nagoya, Japan; IL-12, IFN-γ, and TNF-α from Amersham Pharmacia Biotech, Uppsala, Sweden). With respect to IL-18 assay, we used an ELISA kit that detects the biologically active form but not the biologically inactive form of IL-18 (21). The minimum detectable levels of each cytokine were 12.5 pg/mL, 3 pg/mL, 2 pg/mL, 5 pg/mL, and 0.1 ng/mL for IL-18, IL-12, IFN-γ, TNF-α, and sFasL, respectively. To analyze the effects of other properties of human milk on the quantification of IL-18 levels by ELISA, we performed recovery tests by measuring IL-18 levels in 10 milk samples with or without adding specific amounts of recombinant human IL-18. The ratios of the measured and calculated concentrations were 93.3 $\pm$ 12.7% (mean $\pm$ SD). We found that milk components did not greatly influence the quantification of IL-18.

Detection of IL-18 mRNA in cellular components of human milk. As milk cells contained sufficient proportions of monocytes (40–65% in seven milk samples), IL-18 mRNA expression levels of milk cells were compared with those of purified peripheral blood monocytes. Total RNA was isolated from seven milk cell samples and four peripheral blood $CD14^+$ cells using a nucleic acid purification system, MagExtractor MFX-2000 (Toyobo Co., Ltd., Osaka, Japan). Reverse transcription (RT) of RNA was performed by using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). IL-18 mRNA levels were compared among each sample by semiquantitative RT-PCR by setting the same levels of GAPDH mRNA expression. The primer pair for GAPDH was 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. The primer pair for IL-18 was 5'-GCTTGAATCTAAATTATCAGTC-3' and 5'-GAAGATTCAAATTGCATCTTAT-3'. PCR was performed at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension for 5 min at 72°C with a Takara PCR Thermal Cycler PERSONAL (Takara, Otsu, Japan).

Immunohistochemical staining of IL-18. Lactating (n = 7) and nonlactating (n = 2) mammary gland specimens were obtained from nine patients aged 20–29 y with breast tumors (fibroadenoma, fibrocystic disease). Informed consent was obtained from each mother. The paraffin-embedded samples, fixed in buffered formalin, were used. After deparaffinization, each specimen was first incubated for 5 min with 3% hydrogen peroxide, followed by a 5-min incubation with blocking serum. The specimen was then incubated with anti-IL-18 MAb (clone 25–2G, mouse IgG1, MBL, Nagoya, Japan) or control antibody for 10 min. Each incubation was followed by a wash with PBS. The specimen was incubated with biotinylated second antibody for 10 min and with peroxidase-labeled streptavidin for 10 min. Staining was completed after a 10-min incubation with 3% 3-amimo-9-ethylcarbazole.

Statistical analysis. Kruskal-Wallis test and multiple comparison (Bonferroni method) were used to compare IL-18 levels among each group and Mann-Whitney U test was used to compare between two groups (colostrum and maternal serum, and colostrum from mother with term or preterm delivery). Stepwise multiple linear regression analysis was performed to analyze factors that influenced IL-18 levels (22). The correlation between the maternal events of pregnancy complications and premature delivery was analyzed by χ^2 test.

Ethics. This study was approved by the Regional Ethics of Committee for Human Research at the Faculty of Medicine of Kyushu University.

RESULTS

IL-18 levels of human milk. We analyzed IL-18 levels of human milk from mothers with preterm and term delivery by ELISA, which detects the biologically active form of IL-18. Forty-three of 47 samples (91%) in colostrum, 56 of 69 samples (81%) in early milk, and 11 of 23 samples (48%) in mature milk were beyond the detection limit by ELISA for

Table 2. *Factors influencing IL-18 concentration in colostrum (stepwise multiple linear regression analysis)*

Variable	Coefficient	SE	*F* Value	*p* Value
Analysis A ($n = 116$)*				
Preterm delivery	0.51	0.1463	12.29	0.0007
Analysis B ($n = 116$)†				
Pregnancy complications	0.4686	0.1469	10.17	0.0018

* Preterm delivery, each pregnancy complication (toxemia of pregnancy, chorioamnionitis, threatened premature delivery, abruptio placentae, and premature rupture of membrane) and cesarean section were included as variables in analysis A.

† Gestational weeks at delivery, pregnancy complications, and cesarean section were included as variables in analysis B.

IL-18. Colostrum contained significantly higher levels of IL-18 compared with early milk (Bonferroni method, $p < 0.05$) or mature milk (Bonferroni method, $p < 0.001$) (Fig. 1*A*). In addition, colostrum contained higher levels of IL-18 compared with maternal serum (Mann-Whitney U test, $p < 0.05$). Colostrum from mothers with preterm delivery contained significantly higher levels of IL-18 compared with those with delivery at term (Mann-Whitney U test, $p < 0.005$) (Fig. 1*B*). There were no correlations between colostrum and serum IL-18 levels from same mothers (data not shown).

We performed stepwise multiple linear regression analysis to further access the factors that contribute to the high levels of IL-18 in colostrum. As shown in Analysis A of Table 2, when preterm delivery and pregnancy complications (toxemia of pregnancy, threatened premature delivery, abruptio placentae, chorioamnionitis, and premature rupture of membrane) and cesarean section were included as variables, preterm delivery was significantly associated with high levels of IL-18. On the other hand, when gestational weeks at delivery were included as variables in addition to the pregnancy complications and cesarean section (Analysis B of Table 2), high levels of IL-18 were significantly associated with pregnancy complications but not with gestational weeks of delivery, although each pregnancy complication by itself did not significantly affected the levels of IL-18 (data not shown). A χ^2 test revealed that pregnancy complications and preterm delivery were significantly associated ($p < 0.0001$, data not shown). These results suggested that high levels of IL-18 were associated with preterm delivery, which was closely associated with pregnancy complications.

IL-18, IFN-γ, TNF-α, IL-12, and sFasL concentrations in colostrum. As shown in Figure 2, colostrum contained TNF-α, IL-12, IFN-γ, and sFasL, as reported previously (10, 11). TNF-α levels in colostrum were higher in mothers with preterm delivery than in those with delivery at term, whereas IL-12, IFN-γ, and sFasL levels showed no significant differences between those with preterm and term delivery. Then, we analyzed associations between each cytokine and IL-18 in colostrum. By stepwise multiple linear regression analysis, we found that only sFasL was significantly correlated with high concentration of IL-18 in colostrum (coefficient: 1.155, SE: 0.3042, *F* value: 14.41, $p = 0.0003$).

Semiquantitative PCR analysis of IL-18 mRNA from cellular components of colostrum. As milk cells contained sufficient proportions of monocytes (40–65% in seven milk samples), IL-18 mRNA levels of cellular components in colostrum were examined in seven samples and compared

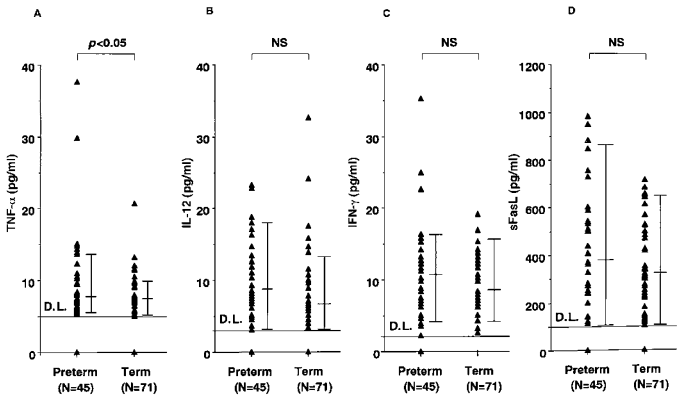

Figure 2. TNF-α (*A*), IL-12 (*B*), IFN-γ (*C*), and sFasL (*D*) levels in colostrum from mothers with preterm and term delivery. Each bar represents median with a range from 10th percentile to 90th percentile. Colostrum samples from mothers with preterm delivery and term delivery were positive (beyond the detection limit by ELISA) for TNF-α in 97% and 77%, for IL-12 in 91% and 86%, for IFN-γ in 97% and 100%, and for sFasL in 94% and 95%, respectively. *D.L.*, detection limit.

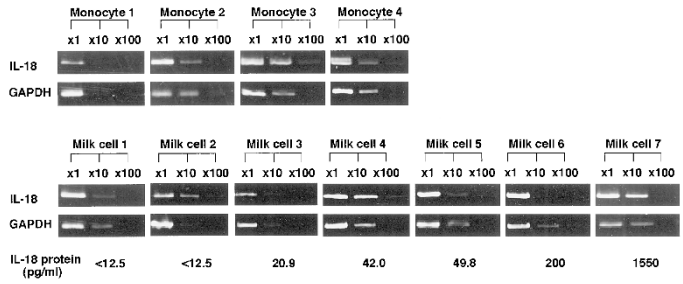

Figure 3. IL-18 mRNA levels in milk cells. IL-18 mRNA levels of milk cells were compared with peripheral blood monocytes from healthy controls. PCR was performed on 10-fold serially diluted samples of cDNA for the comparison of IL-18 mRNA expression. GAPDH mRNA level was used as an internal control. IL-18 protein concentrations of each human milk sample were also indicated.

with those of purified monocytes of peripheral blood from four healthy controls to determine the contribution of milk cells to the high levels of IL-18 in colostrum. IL-18 mRNA levels in human milk cells showed no clear correlation with IL-18 protein concentrations in human milk or with IL-18 mRNA levels in monocytes of peripheral blood, suggesting that cellular components of colostrum did not always make a major contribution to IL-18 levels in colostrum (Fig. 3).

Immunohistochemical identification of IL-18 in mammary gland epithelial cells. Two of seven samples in lactating mammary gland were positive for IL-18 by immunohisto-

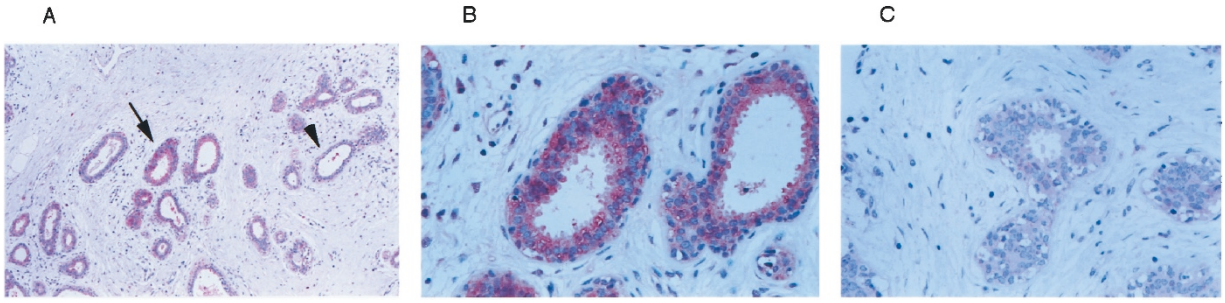

Figure 4. Immunohistochemical staining of IL-18 in mammary gland. (*A*) IL-18 was detected in lactating mammary gland epithelial cells with active secretion (*arrow*) but not in those with inactive secretion (*arrow head*) (×50 original magnification). (*B*) IL-18 producing epithelial cells of lactating mammary gland (×100 original magnification). (*C*) IL-18 nonproducing epithelial cells of nonlactating mammary gland (×100 original magnification).

chemical staining (Fig. 4, *A* and *B*). However, IL-18 was not detected in nonlactating mammary gland (Fig. 4*C*).

DISCUSSION

Th2 dominance in local maternal uterine environment persists during pregnancy, whereas Th1 dominance occurs during abortion-prone pregnancy (23–25). The present finding that the production of IL-18, one of the Th1-inducing cytokines, in human milk was associated with maternal pregnancy complications leading to termination of pregnancy suggested that there might be a common pathophysiology between these two. Pregnancy complications include those with infectious and noninfectious causes; the former was actually associated with an increase in Th1 cytokines in some cases (26). Noninfectious as well as infectious complications affect the production of hormones such as progesterone and β-estradiol, which suppress Th1 response and enhance Th2 reaction (27). In addition, a recent report has shown that preeclampsia induced Th1 cytokine production (28, 29). Thus, it is possible that even noninfectious complications affect local cytokine balance directly or through hormones, especially at the hormone-target organs such as uterus and mammary gland.

Although protective roles of IL-18 are suggested in some bacterial (30, 31) and viral infections (32), physiologic and pathologic roles of IL-18 *in vivo* have not been fully investigated. The functional significance of cytokines in breast milk for neonates was demonstrated by experiments in newborn mice disrupted for TGF-β1 gene, showing that they were rescued by milk TGF-β1 to survive and develop normally (33), although in humans, there is no direct evidence that the cytokines in milk affect the recipient infants. In humans, breast-fed infants showed efficient protection during lactation against several different forms of infections (34–39), higher proliferative response against purified protein derivative (40), and higher IFN-γ production when vaccinated against measles-mumps-rubella (41). Thus, it is possible that IL-18 in colostrum plays important roles in the induction of systemic Th1 response as well as in the local host defense in neonates.

In addition, it is likely that IL-18 in colostrum affects its cellular components. IL-18, in the presence of IL-12, effectively elicits macrophage activation, which provides local host defense by inducing the production of other inflammatory cytokines (42), and more directly, by enhancing phagocytosis and intracellular killing of microorganisms. IL-18 may be one of the cytokines, such as TNF-α, monocyte chemoattractant protein-1, and RANTES (regulated upon activation, normal T cell expressed and secreted), that plays a role in inducing activation and differentiation of T cells, which account for 4% of milk leukocytes and bear CD45RO antigen (43, 44). The presence of radiolabeled milk lymphocytes was demonstrated in the intestinal mucosa (45) and mesenteric lymph nodes of neonatal rats and lambs (46). Maternal T cells, which had been primed by antigens of microorganisms in maternal intestine, further differentiated to Th1 cells by IL-18 in colostrum, may provide cellular immunity in neonates (47).

IL-18 seems to be produced or secreted mainly by epithelial cells of lactating mammary gland (Fig. 4), similar to macrophage colony-stimulating factor reported previously (48), although it might be partially produced by cellular components of human milk. The significant correlations between the IL-18 and sFasL levels suggested the possibilities that 1) these cytokines were derived from the same origin, 2) IL-18 induced sFasL production by other cells in colostrum (4), and 3) sFasL enhanced IL-18 production by activated macrophages, as Tsutsui *et al.* (49) reported that IL-18 was induced in macrophages with the stimulation with sFasL. In addition, it is possible that the effect of IL-18 in human milk could be enhanced in the presence of IL-12 and sFasL (4).

These results suggested that human milk contained various cytokines in the context of complicated network. Thus, IL-18, through the interaction with other cytokines in human milk, might offer immunologic signal for the host defense against microorganisms through NK cell and macrophage activation as well as Th1 cytokine induction in high-risk neonates.

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