Human breast milk suppresses the transcriptional regulation of IL-1β-induced NF-κB signaling in human intestinal cells

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Neonatology has made great advances in recent years, but many diseases remain whose mechanisms have not been fully clarified. Neonatal necrotizing enterocolitis (NEC) is one such disease and is the most serious and frequent gastrointestinal disease of the low-birth-weight infant. Its clinical prognosis is still poor despite progress in neonatal surgery and neonatal intensive care management. NEC is considered to be caused by the coincidence of intestinal ischemia-reperfusion injury and systemic inflammation due to the colonization of pathogenic bacteria. Interleukin (IL)-8, a proinflammatory cytokine, plays an important role in the pathophysiology of NEC. It was recently reported that IL-1β activates the IL-8 gene by regulating the transcriptional nuclear factor κB (NF-κB) signaling pathways in intestinal cells. The protective role of maternal milk in NEC pathogenesis has been reported in both human and animal studies. In this study, we show that human breast milk dramatically suppressed the IL-1β-induced activation of the IL-8 gene promoter by inhibiting the activation pathway of NF-κB. Moreover, we also show that human breast milk induced the production of IkBα. These results suggest that human breast milk could be protective and therapeutic in neonates with NEC by inhibiting the activation pathway of NF-κB.

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heat-sensitive proteins and peptides in human breast milk, we boiled the aqueous layer of human breast milk for 5 min, centrifuged it once (12,000 rpm for 10 min at 4°C), and obtained the supernatant.

**IL-8 ELISA.** Cells were seeded onto six-well plates at a density of 1 × 10⁵ cells/well. The culture medium was then changed, and the cells were pretreated with a 5% volume of human breast milk obtained as described above or without any additive. Subsequently, 5 ng/ml of IL-1β (Sigma-Aldrich, St. Louis, MO) was added to the culture medium, and 6 h after addition of this cytokine, the supernatants of the culture medium were collected and the concentration of secreted IL-8 was determined using ELISA. Cells were also pretreated with or without a 5% volume of breast milk and then treated with 10 µg/µl of LPS (Sigma-Aldrich) for 24 h as described previously (22), and the IL-8 concentration in the culture medium was measured by performing ELISA. In other experiments, cells were pretreated for 24 h with either a 5% volume of breast milk or boiled breast milk obtained as described above, and IL-8 secretion into the culture medium of cells that were then supplemented with 5 ng/ml IL-1β for 6 h was measured using ELISA. Furthermore, cells were pretreated with breast milk in the presence of 1 ng/µl of anti-IL-10 antibody (Techne, Minneapolis, MN), 0.5 µg/µl of anti-human EGF antibody (R&D Systems, Minneapolis, MN), or 5 ng/µl of anti-Epo antibody (R&D Systems) and subsequently stimulated with 5 ng/ml of IL-1β. The concentration of IL-8 secretion into the culture medium was determined using ELISA. A human IL-8 ELISA kit (R&D Systems) was used to quantify cytokine levels as recommended by the manufacturer. All experiments were repeated three times, and each sample was assayed in duplicate. The data are presented as the concentration of IL-8 in picograms per 10⁵ cells (means ± SD).

**Plasmids and plasmid construction.** The IL-8 promoter luciferase plasmid [(wt)Luc] and IL-8 promoter luciferase plasmids with site-directed mutations of activator protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and NF-κB binding elements [(mAP-1)Luc, (mC/EBP)Luc, and (mNF-κB)Luc, respectively] were provided to us by Dr. T. Okamoto (Dept. of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan) (24). The structures of these luciferase constructs are illustrated in Fig. 1.

**DNA transfection and luciferase assays.** Caco-2 cells were plated at a density of 4 × 10⁴ cells/cm² in 12-well plates. DNA transfection was performed by the Lipofectamine Plus reagent-mediated transfection procedure (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. In each experiment, 1 µg of reporter plasmid and 0.5 µg of pSV40 LacZ (as an internal control for transfection efficiency) were used. Twenty-four hours after transfection, either a 5% volume of human breast milk obtained as described above or nothing was added to the culture medium, and the culturing was continued for 24 h until analysis. Furthermore, 5 ng/ml of IL-1β was added to the culture medium, and 6 h after addition of this cytokine, cell extracts were prepared and assayed for luciferase activity using PicoGene (Toyo B-Net, Tokyo, Japan). All experiments were performed in triplicate; the mean of three replicates for each experiment was adopted as the result, and each result was expressed as the luciferase activity (luciferase activity/Luc) of reporter plasmid [(wt)Luc] and the corresponding mutant reporter plasmid [(mAP-1)Luc] and [(mNF-κB)Luc], respectively. The data are presented as the concentration of IL-8 in picograms per 10⁵ cells (means ± SD).

**SDS-PAGE and Western blot analysis.** Caco-2 cells were cultured in 10-cm dishes with complete medium (20% FBS) until 90% confluence, either a 5% volume of human breast milk or nothing was added to the culture medium, and then the cell cultures were further incubated for 24 h. During this additional incubation, the cells were stimulated with IL-1β (5 ng/ml) for various periods as indicated. After stimulation, the cells were harvested and lysed by incubation for 60 min in 100 µl of lysis buffer as described previously (36). These protein samples were separated by performing SDS-PAGE (25 µg/lane) and analyzed by blotting with anti-IL-1 soluble receptor type 1 antibody (Sigma-Aldrich), anti-phospho-IκBα antibody, anti-IκBα antibody, anti- IkBα antibody, anti-cIκBα antibody, anti-phospho-IκBα antibody (Cell Signaling Technology, Beverly, MA), or anti-β-actin antibody (Sigma-Aldrich). Subsequently, the proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and rehybridization was performed using a Reblot Western blot recycling kit (Chemicon International, Temecula, CA).

**Statistical analysis.** Statistical analysis was performed using the Kruskal-Wallis test, and P < 0.05 was accepted as statistically significant.

**RESULTS**

**Human breast milk suppresses IL-1β-induced IL-8 secretion in Caco-2 cells.** As shown in previous studies by Claud et al. (9), IL-8 secretion by Caco-2 cells was increased by IL-1β stimulation. When the cells were pretreated with human breast milk, the increase of IL-8 secretion induced by IL-1β was suppressed as shown by ELISA (Fig. 2A). IL-8 secretion by Caco-2 cells induced by LPS under the same pretreatment conditions was also suppressed by human breast milk (Fig. 2B).

**Suppressive effect of human breast milk on IL-1β-induced IL-8 secretion is heat sensitive, and the main effect is not due to IL-10, EGF, or Epo.** To examine which type of substance in human breast milk, such as a heat-sensitive substance (and thus presumably a protein), is involved in this inhibitory effect, we assayed IL-1β-induced IL-8 secretion in the culture medium of...
Caco-2 cells by performing ELISA after pretreatment with either breast milk or boiled breast milk. Boiled breast milk was less effective than nonboiled breast milk in suppressing the induction of IL-8 secretion by IL-1β (Fig. 3). These results suggest that some heat-sensitive proteins or peptides are associated with the inhibitory effect of human breast milk on IL-1β-induced IL-8 secretion. Furthermore, to identify the factors in breast milk that mediate its suppressive effects on NF-κB activation, we performed IL-8 ELISA on the supernatants obtained from Caco-2 cells pretreated with breast milk in the presence of neutralizing antibodies for IL-10, EGF, or Epo (Fig. 3). In the presence of these antibodies, the suppressive effect of breast milk on IL-1β-induced IL-8 secretion was reduced. There was no significant difference between the IL-8 secretion, induced by IL-1β only, and that induced by the combination of IL-1β, human breast milk, and the above-noted neutralizing antibodies. Thus we hypothesized that the suppressive effect of breast milk on IL-1β-induced IL-8 secretion was partially due to the effect of IL-10, EGF, or Epo, but that the main effect was due to something other than those factors.

Human breast milk suppresses IL-1β-induced IL-8 promoter activation by inhibiting the NF-κB pathway. Previous studies by Wu et al. (39) showed that IL-1β activated the IL-8 promoter via the regulation of NF-κB, C/EBP, and AP-1. When cells were treated with human breast milk, the induction of the activity of the IL-8 promoter by stimulation with IL-1β was significantly suppressed (Fig. 4A). To determine which transcriptional factor is associated with this inhibitory effect of human breast milk on IL-1β-induced IL-8 promoter activation, promoters with point mutations in the AP-1, C/EBP, and NF-κB response elements (Fig. 4, B–D) were transfected into Caco-2 cells. In cells pretreated with human breast milk for 24 h and stimulated with IL-1β for 6 h, mutation in the AP-1 or C/EBP response element did not influence the inhibitory effect of human breast milk on IL-8 promoter activity, although this inhibitory effect disappeared with mutation of the NF-κB response element. These results suggest that of these three transcriptional factors, NF-κB is associated with the inhibitory effect of human breast milk on IL-1β-induced IL-8 promoter activation.

Human breast milk can suppress NF-κB-dependent promoter activity. Subsequently, to directly confirm that the suppressive effect of human breast milk on IL-1β-induced IL-8 promoter activation is exerted via inhibition of the NF-κB pathway, we performed luciferase assays in Caco-2 cells transfected with two different reporter constructs consisting of either four repeats of the NF-κB-responsive element (4×κBw-LUC) or a mutant thereof (4×κBm-LUC). Human breast milk exerted an inhibitory effect on the IL-1β-induced stimulation of expression directed by the 4×κBw-LUC plasmid, but not on that directed by the mutant plasmid, 4×κBm-LUC (Fig. 5). These results demonstrate that human breast milk exerts an inhibitory effect on IL-1β-induced IL-8 promoter activation by suppressing the activation pathway involving NF-κB rather than pathways involving AP-1 or C/EBP.

Human breast milk does not affect the expression of IL-1 receptor proteins. Next, we analyzed which part of the IL-1β-NF-κB signal transduction pathway was disturbed in breast milk-treated cells. For this purpose, first we examined whether breast milk affected the expression of IL-1 receptor (IL-1R)
protein (Fig. 6). Western blot analysis showed that human breast milk did not change the level of expression of IL-1R protein. This result demonstrated that the disturbance of the IL-1β-NF-κB pathway in breast milk-treated cells was not due to a decrease in IL-1R.

**Human breast milk does not affect the phosphorylation of IKK.** Subsequently, we examined whether human breast milk repressed IL-1β-induced phosphorylation of IKKα/IKKβ (Fig. 7). Western blot analysis showed that human breast milk did not affect IL-1β-induced phosphorylation of IKKα/IKKβ, while nonphosphorylated IKKα and IKKβ were present at constant levels with or without breast milk or IL-1β. This result demonstrated that breast milk did not disturb IKK activation in the IL-1β-NF-κB pathway.

**Human breast milk inhibits IL-1β-induced degradation and phosphorylation of IκBα.** As shown in a previous analysis by Su et al. (36), IκBα protein in Caco-2 cells was rapidly degraded within 45 min after stimulation with IL-1β. Densitometric analysis showed that the protein level at 45 min was significantly decreased to about one-fifth the level in the unstimulated state (Fig. 8A, bottom). In contrast, IκBα was resistant to IL-1β-induced degradation in breast milk-pre-treated cells (Fig. 8B). This result indicated that the IL-1β-induced degradation of IκBα protein was suppressed by human breast milk.

Next, by comparing the protein level of phospho-IκBα with and without breast milk treatment, we analyzed whether breast milk repressed the IL-1β-induced phosphorylation of IκBα (Fig. 8, C and D). Without treatment with breast milk, phospho-IκBα proteins accumulated in Caco-2 cells within 45 min after stimulation with IL-1β (Fig. 8C, top). Quantification of this accumulation by densitometry revealed that the protein level of phospho-IκBα at 45 min was 1.7-fold that in the unstimulated state (Fig. 8C, bottom). This accumulation was attenuated by pretreatment with human breast milk (Fig. 8D, top). Densitometric analysis revealed that the level of phospho-IκBα protein in breast milk-treated cells at 45 min was one-half that in the unstimulated state (Fig. 8D, bottom). These
BM does not affect the expression of IL-1 receptor (IL-1R) protein in Caco-2 cells. Total cell extracts isolated from Caco-2 cells that were untreated or treated with BM (5% for 24 h) and then stimulated with IL-1β (5 ng/ml) for 20 min were subjected to Western blotting using anti-phospho-IKKα/IKKβ antibody (top). The blots were then stripped and reprobed with anti-IKKα-antibody (middle) or anti-IKKβ-antibody (bottom).

breast milk exerts its suppressive effect on the NF-κB signaling pathway.

**DISCUSSION**

Breastfeeding, as numerous investigators have reported, has many advantages for the development of neonates, especially that of premature infants (10), including a decrease in the incidence of retinopathy of prematurity (23), an increase in intelligence quotient (29), and a decrease in the incidence of respiratory infection (5, 8). It has been reported in several studies that the incidence of NEC was higher in formula-fed babies than in babies fed breast milk alone (6, 28), and even higher than in infants who were fed formula plus breast milk. It appears that the course of the disease is less severe in the case of breastfeeding (27), and this fact has also been confirmed in animal experiments using rats with chemically induced colitis (17). However, in vitro research into this protective effect of human breast milk against NEC has been rare until now.

IL-8 has been a good model of the immunological response to inflammation in many previous studies, especially in intestinal epithelial cells (25). Also, IL-1β is considered to be useful in these studies as an endogenous inflammatory stimulant that upregulates IL-8 secretion as an innate response of enterocytes (15, 33). In other studies, in vivo evidence that NEC is caused by the coincidence of bacterial infection, intestinal ischemia-reperfusion injury, and systemic inflammation has been obtained using animal models (21). As shown in recent studies by Claud et al. (9), IL-8 secretion from intestinal epithelial cells stimulated by IL-1β was decreased by the addition of several factors found in human breast milk (16, 18), such as TGF-β, Epo, IL-10, and EGF. However, whether human breast milk transcriptionally suppresses the IL-8 gene has never been examined, and the in vitro mechanism of suppression remains unclear. Thus our present study is the first demonstration of the molecular mechanism of the anti-inflammatory effect of human breast milk in vitro on human intestinal epithelial cells. Our findings show that the suppression of the IL-8 promoter by human breast milk occurs via the inhibition of the activation pathway of NF-κB.

TNFα and LPS are also well known as physiological inflammatory stimulators and potent NF-κB activators. Consistent with findings in two previous studies (22, 32), Caco-2 cells responded to IL-1β and LPS in our experiments. We have
shown that human breast milk suppressed both IL-1β- and LPS-induced IL-8 secretion (Fig. 2) as well as IL-8 promoter activation (Fig. 4 and unpublished data). However, as also previously reported by Eckmann et al. (13), we have shown in luciferase assays using IL-8 promoter that Caco-2 cells did not respond to TNFα (our unpublished data).

As reviewed in a previous report (7), platelet-activating factor (PAF) is another factor that is known to play an important role in neonatal intestinal injury. PAF, TNFα, and LPS act synergistically to amplify inflammation (21), and PAF enhances the DNA binding activity of NF-κB in the intestine, predominantly as p50 subunits (12). Human breast milk may also exert an inhibitory effect on PAF-induced intestinal inflammation by suppressing the activation pathway involving NF-κB. Further examination is needed to test this possibility.

Our findings suggest that there may be two different mechanisms by which human breast milk exerts its inhibitory effect on NF-κB activity, namely, through the regulation of both the production and phosphorylation of IκBα proteins. One possibility is that an increased basal quantity of IκBα protein caused by human breast milk may lead to increased interaction between IκBα and NF-κB, which may in turn prevent the activation of NF-κB protein. We can also suggest another possibility, namely, that human breast milk exerts its inhibitory effect by directly suppressing the phosphorylation of IκBα protein. As shown in our study, human breast milk did not decrease the protein level of IL-1R (Fig. 6), which occurs in the first step of the IL-1β-induced NF-κB signaling pathway. Furthermore, we also have shown that the IL-1β-induced phosphorylation of IKKα/IKKβ was not affected by human breast milk (Fig. 7). Therefore, the target of the inhibitory effect of human breast milk on NF-κB signaling is downstream of IKK.

It is well known that phosphorylation of IκBα leads to its ubiquitination and degradation by the 26S proteasome, thus leading to NF-κB nuclear translocation (34). Hypoxia-inducible factor-1α (HIF-1α) is also a well-known transcriptional factor that is regulated by the ubiquitin-proteasome degradation pathway (26). HIF-1α is activated under conditions of reduced oxygen and regulates the transcription of several genes that are responsive to a lack of oxygen, such as Epo, vascular endothelial growth factor, and glucose transporter 1. In a previous report (14), the parallel induction of HIF-1α and intestinal trefoil factor (ITF) was observed under hypoxic conditions in the Caco-2 cell line. ITF is a protein that has a protective role against reduced blood flow in the intestine and works to preserve the barrier function of the intestine against outer stimuli, and thus HIF-1α may have a protective role...
against the ischemic changes that occur in the neonatal intestine in disease conditions such as NEC. In our previous studies, we proved that human breast milk caused significant induction of HIF-1α proteins in the nuclei of Caco-2 cells (our unpublished data). The inhibitory effect of human breast milk may be associated with suppression of the ubiquitination and degradation pathway and may cause an increased level of proteins with a protective role against intestinal ischemia-reperfusion injury and systemic inflammation, such as HIF-1α or IκBα.

Glucocorticoids are among the most potent agents whose effects of anti-inflammation and immunosuppression are widely accepted, and they are commonly used for the treatment of inflammatory bowel diseases such as Crohn’s disease or ulcerative colitis. They are considered to inhibit the synthesis of cytokines necessary for the immune response, and as proved in several previous studies (2, 6), glucocorticoids exert their inhibitory effect against the activation of NF-κB by suppressing the induction of IκBα. Human breast milk contains glucocorticoids, and they are among the candidate breast milk components that showed an inhibitory effect in our experiments. As shown by our findings, the repressive effect of human breast milk was diminished when the milk was boiled (Fig. 3). Thus some unknown substance in human breast milk that is sensitive to heat, like most proteins, exerts this inhibitory effect. We also have shown that the suppressive effect of human breast milk on IL-1β-induced IL-8 secretion was reduced in the presence of neutralizing antibodies to IL-10, EGF, or Epo (Fig. 3). However, the suppressive effect of breast milk was not totally abrogated; instead, the effect of boiling on these neutralizing antibodies was partial. We are now attempting to identify this unknown factor with the hope that it may be therapeutic for NEC in the future.

As reported in previous studies (4, 11), longer breastfeeding may also have a protective effect against childhood acute leukemia and lymphoma. The detailed mechanism of this phenomenon has not been fully clarified, but it was suggested in another report that NF-κB may play a determining role in the sensitivity or resistance to the progression of anaplastic large cell lymphoma or Hodgkin disease (HD) (30). In that study, HD cells were sensitized by ectopic overexpression of IκBα. Therefore, our present findings can explain the mechanism by which human breast milk exerts its protective effect against childhood acute leukemia or lymphoma, and the unknown proteins in human breast milk that suppress NF-κB signaling may also be therapeutic agents for these diseases. In conclusion, further studies are needed before applying the inhibitory factors in human breast milk as therapeutic agents in humans for the prevention of inflammatory bowel diseases such as NEC or for the treatment of childhood lymphoma.

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