Granulocyte Colony-Stimulating Factor Is Present in Human Milk and Its Receptor Is Present in Human Fetal Intestine

Darlene A. Calhoun, DO; Mathilde Lunøe; Yan Du, MD; and Robert D. Christensen, MD

ABSTRACT. Objective. Human milk provides neonates with a meaningful degree of protection from infection, but the responsible mechanisms are not well understood. Discovering these mechanisms is important, because of the possibility of supplementing infant formulas with factors that simulate human milk’s protective capacity. We postulated that granulocyte colony-stimulating factor (G-CSF), a cytokine known to augment antibacterial defenses through its salutary effect on neutrophil production, might be one such factor. To test this hypothesis, we quantified G-CSF in milk of healthy women and those with intraamniotic infection, and sought the presence of functional G-CSF receptors (G-CSF-R) in fetal/neonatal intestinal villi.

Study Design. G-CSF was measured by enzyme-linked immunoassay in 126 milk samples obtained from breastfeeding women, and the concentrations were analyzed according to gestational age, postpartum day of collection (first 2 days vs greater 2 days), and the presence versus absence of intraamniotic infection. G-CSF-R messenger ribonucleic acid transcripts were sought from fetal/neonatal intestine using reverse transcriptase-polymerase chain reaction, and localized using in situ RT-PCR. G-CSF-R protein, and specific intracellular signaling proteins (Janus tyrosine kinase-1, Janus tyrosine kinase-2, and tyrosine kinase-2), were sought by immunohistochemistry.

Results. All milk samples contained G-CSF, and significantly more G-CSF was contained in milk collected during the first 2 postpartum days than during subsequent days. Milk from women who delivered prematurely had less G-CSF during the first 2 postpartum days than milk from women who delivered at term. When intraamniotic infection was present, the concentration of G-CSF in milk was elevated significantly compared with concentrations in milk of noninfected women. G-CSF concentrations were also higher in milk collected during the first 2 postpartum days from women who had received intrapartum recombinant G-CSF treatment, compared with milk obtained from women with intraamniotic infection, regardless if they delivered prematurely or at term. G-CSF-R messenger ribonucleic acid and protein were expressed on fetal villus enterocytes, and Janus tyrosine kinase-1, Janus tyrosine kinase-2, and tyrosine kinase-2 were present within the cytoplasm of these cells.

Conclusions. Human milk contains substantial quantities of G-CSF. G-CSF-R are abundantly expressed on villus enterocytes, and specific proteins associated with G-CSF-R signaling are present in these cells. Pediatrics 2000;105(1). URL: http://www.pediatrics.org/cgi/content/full/105/1/e7; growth factors, cytokines, hematopoiesis, immunity.

ABBREVIATIONS. G-CSF, granulocyte colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-polymerase chain reaction; G-CSF-R, granulocyte colony-stimulating factor receptor; JAK-1, Janus tyrosine kinase-1; JAK-2, Janus tyrosine kinase-2; Tyk-2, tyrosine kinase-2.
women have perinatal infections or deliver prematurely. We also sought to determine whether receptors for G-CSF were present in fetal intestine, and if so, whether these were functional. We considered these studies to be essential steps toward testing the hypothesis that adding physiologic concentrations of recombinant G-CSF to infant formulas would improve their ability to reduce infection.

METHODS

Participants

Human milk was obtained from breast-feeding mothers who delivered at term (n = 59) or prematurely (n = 67) and included those with intraamniotic infection (n = 12), defined by Gistrap et al. Milk also was obtained either as a single or multiple-sample collection, the latter over a period of up to 4 weeks (n = 24). To assess potential variabilities in G-CSF concentrations associated with the postpartum day of collection, 2 additional subgroups were created: 1) milk collected within postpartum days 1 and 2, and 2) milk collected after this time. Four mothers, who delivered at ≤30 weeks’ gestation, received a single dose (25 μg/kg) of recombinant G-CSF (Neupogen; Amgen, Thousand Oaks, CA) during labor as part of a clinical trial to assess the transplacental passage of G-CSF. In these patients, serial milk samples were collected daily for 5 days after delivery. Human milk was collected into sterile containers using a low-pressure electric pump (Medela, McHenry, IL). The institutional review board of the University of Florida approved all studies, and informed consent was obtained from all study participants.

G-CSF ELISA Assay

The aqueous phase of human milk was aliquoted and stored at −80°C until analysis. G-CSF was quantified by ELISA (Quantikine Human G-CSF Immunoassy; R & D Systems, Minneapolis, MN; lower limit of sensitivity 7 pg/mL). The assay has no measurable cross-reactivity with other cytokines and recognizes both natural and recombinant G-CSF. Before the analysis of any human milk samples, we performed validation studies of the ELISA using human milk. These included: linearity, intrassay precision, inter-assay precision, reproducibility, and percent recovery using the aqueous phase of human milk.

Concentration of G-CSF in Human Milk

The concentration of G-CSF in the aqueous phase of human milk was examined. Samples were centrifuged at 1 × 1000 g for 30 minutes, and the aqueous fractions were separated from the lipids and solid precipitate. Samples were frozen at −80°C until assayed.

Statistics for G-CSF Determinations

Initial descriptive statistics were calculated and analysis of variance were used to evaluate the relationship between G-CSF concentrations in milk type (colostrom or mature), birth status (premature or term), and intraamniotic infection status (present or absent). A priori between-group contrasts were then performed with the α level set to .05. The preliminary statistical power to assess these variables was .99. All statistics were computed using the statistical software package SAS Version 6.12 (SAS, Cary, NC).

Tissue Preparation

Tissues were obtained from human fetuses at 8 to 10, 16 to 18, and 22 to 24 weeks’ postconceptual age. Only fetuses that were normal by ultrasound examination and underwent elective pregnancy termination were studied. Pregnancy terminations were conducted by suction curettage (8–16 weeks) or by cervical dilatation and extraction curettage (17–24 weeks). The investigators were not the physicians caring for the women and had no input on their decision to terminate pregnancy.

Some tissues were immediately frozen for RNA extraction. Other tissues were fixed in 4% paraformaldehyde (for in situ reverse transcriptase polymerase chain reaction [RT-PCR]) or Bouin’s solution (for immunohistochemistry).

RT-PCR

RT-PCR was performed to determine whether intestinal tissue contained mRNA for the G-CSF receptor (G-CSF-R). Total cellular RNA was extracted using the method described by Chomczynski and Sacchi, and the extracted RNA was stored at −80°C until analysis. For RT-PCR analysis, total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gathersburg, MD) to synthesize first strand complementary DNA. Amplifications of the complementary DNA were performed using specific primer pairs, and the amplified bands visualized by ethidium bromide staining. The G-CSF-R cDNA 340-bp fragment was detected by PCR of 35 cycles at 94°C for 1 minute, 51°C for 1 minute, and 72°C for 1 minute with 5’-AAG ACC CCC CTT ACC CAC TAC ACC ATC TT-3’ (forward primer), and 5’-TGC TGT GAG CTT GGT CTG GGA CAC TT-3’ (reverse primer) which amplify a segment located between nucleotides 1863 and 2149 of the human G-CSF-R. Healthy term human placenta was the positive control.

In Situ RT-PCR

To determine whether the G-CSF-R mRNA signal in the RT-PCR was the result of contaminating blood in the fetal tissues, detection of G-CSF-R mRNA also was performed by in situ RT-PCR according to the modified method described by Nuovo et al. After fixation, frozen sections were digested with proteinase K, RNA was extracted overnight using the method described by Nuovo et al. (Perkin-Elmer, Norwalk, CT). The tissues were then incubated with the primers and reverse transcriptase. The sequences of the primers were described in the previous section, and digoxigenin dUTP was used as the reporter molecule. The positive control (purple-red) for in situ RT-PCR eliminated the DNAase digestive reaction. An intense signal was generated from the target-specific detection of DNA. The control intact DNA did not hybridize. If no definitively positive signals were seen on the tissues, then the results were defined as negative. Negative and positive controls were performed on the same glass slide along with the experimental analysis using serial sections of tissue.

Immunohistochemistry

Immunohistochemistry was performed to determine whether the mRNA for G-CSF-R was translated into protein and to identify the specific cellular location of this protein and its associated signaling proteins, Janus tyrosine kinase-1 (JAK-1), Janus tyrosine kinase-2 (JAK-2), and tyrosine kinase-2 (Tyk-2). Tissue sections were deparaffinized, rehydrated, and processed using an enzyme-labeled biotin-streptavidin system (Ventana; NexES, Tucson, AZ). The chromagen used was diaminobenzidine tetrahydrochloride (DAB). After blocking of nonspecific binding of immunoglobulin G, a primary antibody (G-CSF-R, 1:10; JAK-1, 1:10; JAK-2, 1:10; or Tyk-2, 1:50) was applied and counterstaining was performed. All primary antibodies were prepared commercially (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Each antibody is specific for human G-CSF-R, JAK-1, JAK-2, or Tyk-2, respectively. There is no known cross-reactivity with other molecules.

Specificity of the staining was verified by negative and positive controls. Immunostaining was absent when rabbit immune serum was substituted for the primary antibody or when specific blocking peptide (Santa Cruz Biotechnology) was used in the case of G-CSF-R. Staining was considered positive when present in tissue sections of term placenta, a tissue with abundant G-CSF-R. Staining was considered positive for JAK-1, JAK-2, and Tyk-2 when present in preparations of purified neutrophils.

RESULTS

Performance Characteristics of the ELISA

Linearity of the assay in human milk was observed over the concentration range 7 to 1000 pg/mL. The correlation coefficients of 2 separate spiking studies were .998 and .990, respectively. Interassay and in-
tra assay precision were within ranges established by the manufacturer. The recovery of recombinant G-CSF spiked at 5 different levels in 3 separate samples was 71% to 114%, a range similar to that given by the manufacturer for other matrices (75%–117%).

**Patient Results**

Concentrations of G-CSF were found in all samples of milk. The concentration of G-CSF did not vary during a single collection when fore-, mid-, and hind-milk samples were compared and was measurable in samples \( n = 88 \) collected longitudinally during the month after delivery. In milk collected during the first 2 postpartum days of women who delivered at term \( (n = 19; \text{Table 1}) \), concentrations of G-CSF were significantly greater compared with milk from women who delivered prematurely \( (P = .007; n = 19) \). Milk collected during the first 2 postpartum days from women with intraamniotic infection \( (n = 12) \) had G-CSF concentrations considerably higher than those without infection, regardless of whether the mother delivered prematurely or at term \( (P = .0001 \text{ and } .03) \). The concentration of G-CSF in milk obtained during the first 2 postpartum days from women who received intrapartum recombinant G-CSF \( (n = 4) \) was significantly greater than the concentration in milk from either women with or without intraamniotic infection who had delivered prematurely. In milk collected after postpartum day 2, concentrations of G-CSF were significantly less from women delivering prematurely \( (32 \pm 18 \text{ pg/mL}; n = 48) \), compared with women delivering at term \( (37 \pm 21 \text{ pg/mL}; P = .01; n = 40) \).

**RT-PCR and In Situ RT-PCR**

PCR products of the predicted size for G-CSF-R \( (340 \text{ bp}; \text{Fig 1}) \) were obtained from various segments of fetal intestine \( (n = 20 \text{ fetuses}; \text{tissue included duodenum, jejunum, ileum, colon, and stomach}) \) at each gestation examined. A representative section of intestine at 22 weeks' postconceptual age for G-CSF-R transcripts as determined by in situ RT-PCR, as well as controls, are shown in Fig 2A–C. The G-CSF-R mRNA was localized to the apical surface of villi within enterocytes (Fig 2A). Nonspecific staining resulted when the tissue was not subjected to DNase treatment (Fig 2B), and absence of staining occurred when reverse transcription was not performed (Fig 2C).

**Table 1.** G-CSF Concentrations* \((\text{pg/mL})\) in Milk Collected During the First Two Postpartum Days From Women With and Without Intraamniotic Infection and From Women Who Received Recombinant G-CSF

<table>
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<tr>
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<th>Without Intraamniotic Infection</th>
<th>With Intraamniotic Infection</th>
<th>Women Who Received Recombinant G-CSF</th>
<th>( P-e^{d} ) Values</th>
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<tr>
<td><strong>Term</strong></td>
<td>156 ± 112 ((n = 11))</td>
<td>221 ± 165 ((n = 8))</td>
<td>ND</td>
<td>.03**</td>
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<tr>
<td><strong>Preterm</strong></td>
<td>80 ± 41 ((n = 11))</td>
<td>118 ± 62 ((n = 4))</td>
<td>392 ± 625 ((n = 4))</td>
<td>.0001(^{c}), .0005(^{c}), .01(^{d})</td>
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<tr>
<td>( P-e^{d}) value</td>
<td>( **.007)</td>
<td>( **.77)</td>
<td>ND</td>
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ND indicates not done. Only women who were about to deliver prematurely received recombinant G-CSF. 

\( P-e^{d}\) values are comparisons of term versus preterm.

\( P-e^{d}\) values are comparisons of \( a) \) term women without intraamniotic infection versus term women with infection, \( b) \) preterm women without intraamniotic infection versus preterm women with infection, \( c) \) preterm women without intraamniotic infection versus preterm women who received intrapartum recombinant G-CSF, and \( d) \) preterm women with intraamniotic infection versus preterm women who received intrapartum recombinant G-CSF.

* Mean ± standard deviation.

**Immunohistochemistry**

Representative sections of intestines from fetuses \( (n = 15) \) at 8 to 10, 16 to 18, and 22 to 24 weeks' postconceptual age demonstrated the presence of G-CSF-R (Fig 3A–C). At 8 to 10 weeks' postconceptual age, immunoreactivity for G-CSF-R was present in nuclei within the mucosal epithelium and was occasionally prominent at the base of the crypts and in fibroblast nuclei of the lamina propria and submucosa. At 16 to 18 weeks, only the smooth muscle blush was evident. By 24 weeks, reactions were distinctly cytoplasmic and located at the cell apex of the mucosal epithelium in developing enterocytes, and within the basally located nucleus. At 22 to 24 weeks, staining of protein representing JAK-1, JAK-2, and Tyk-2 occurred within the cytoplasm of enterocytes of developing villi (Fig 3D–F).

**DISCUSSION**

Human milk contains a variety of cellular and soluble components that protect infants from infection. Indeed, feeding with human milk is among the most effective methods known for protecting preterm neonates from infection. In the present study, we investigated a specific cytokine in human milk, G-CSF, and the expression of its receptor, G-CSF-R, on fetal intestinal villi. Significant quantities of G-CSF were found in milk, and these remained measurable during the first 4 weeks of lactation. These findings are similar to concentrations reported by Gilmore and colleagues\(^{10}\) and Wallace et al.\(^{11}\) G-CSF was higher in milk collected during the first 2 postpartum days than in milk collected later and was
significantly elevated when intraamniotic infection was present. No difference in concentration was observed in fore-, mid-, and hind-milk. G-CSF concentrations in milk collected during the first 2 postpartum days were significantly increased in mothers who received an intravenous dose of recombinant G-CSF immediately before delivery.

Although mechanisms are not known to exist in breast tissue for concentrating cytokines from the blood, we observed that G-CSF concentrations in milk are generally much higher than the serum G-CSF concentrations of postpartum women, as reported by Bailie et al. With respect to other cytokines in milk, local production is common, and it is conceivable that other cellular elements, like milk macrophages, are responsible for G-CSF production, in a manner similar to that of peripheral blood monocytes. Macrophages are prominent in milk, and cytokine production can be altered dramatically by their presence. Still, other cytokines,

Fig 2. Representative in situ RT-PCR of intestine. Panel A contains a section of fetal intestine at 22 weeks’ postconceptual age subjected to in situ RT-PCR using the same primer pairs as for RT-PCR (×100). Digoxigenin labeling reveals the receptor within enterocytes. Panel B is the positive control, the result of nonspecific staining when the tissue is not subjected to DNAse treatment. Panel C is the negative control.

Fig 3. A–F, Representative immunohistochemistry of G-CSF-R, JAK-1, JAK-2, and Tyk-2 in intestine. The panels illustrate representative sections (magnification ×100) of human fetal intestines obtained at 8 to 10 (A), 16 to 18 (B), and 22 to 24 (C) weeks’ postconceptual age. The sections were treated with antibodies to the G-CSF-R using an enzyme labeled biotin streptavidin system. At 22 to 24 weeks (C), immunolocalization of the receptor is within the developing enterocytes and cells of the villus crypt. Panels D–F are representative sections of fetal intestine at 22 to 24 weeks’ postconceptual age incubated with antibodies specific for JAK-1, JAK-2, and Tyk-2, respectively (×200). The corresponding proteins can be seen within the cytoplasm of the enterocytes.
such as macrophage colony-stimulating factor, interleukin 6 and interleukin 8, are produced by mammmary gland epithelial cells. \(^3\) Much remains to be learned about the origins of G-CSF in human milk and how that production is regulated.

We observed G-CSF-R in fetal intestine and its anatomic location changed with development. In fetal villi at 22 to 24 weeks, the receptor was localized to the enterocyte, and the signaling proteins JAK-1, JAK-2, and Tyk-2 were present within the cytoplasm. If signaling by the G-CSF-R within the developing intestine occurs in a manner similar to that proposed in neutrophils, the presence of these proteins suggests that the G-CSF-R in the fetal intestine at 22 to 24 weeks is functional. However, testing function is clearly more complicated than measuring signaling proteins. Further, whether G-CSF in human milk specifically activates the G-CSF-R on enterocytes cannot be concluded from this study.

Recently, we observed that significant concentrations of G-CSF in human milk are measurable after in vitro simulations of digestion. \(^3\) Milk increases the hydrogen ion concentration of the stomach, and thus ingested proteins resist gastric digestion by proteolytic enzymes, which require low hydrogen ion concentrations for activation. \(^3\)

In early postnatal life, the balance of factors affecting luminal proteolysis favors limited protein digestion. \(^3\) G-CSF may be protected from degradation by the presence of antiproteolytic agents known to be present in milk. \(^3\) Other studies are needed to determine whether the G-CSF in human milk is biologically active.

It remains to be determined what role, if any, G-CSF in human milk has on fetal enterocytes or systemically on neutrophil proliferation, differentiation, or survival. Moreover, it is not clear whether the beneficial effect of human milk on reducing infection rates is, in part, a consequence of its G-CSF content. Understanding these issues will be important in the efforts to improve the antiinfection properties of infant formulas, for those infants for whom no human milk is available.

**ACKNOWLEDGMENTS**

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