0031-3998/04/5505-0802
PEDIATRIC RESEARCH

Vol. 55, No. 5, 2004
Printed in U.S.A.

Effects of Enterally Administering Granulocyte Colony-Stimulating Factor to Suckling Mice

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ABSTRACT

Gastrointestinal (GI) tract development is influenced by multiple growth factors, some of which are delivered directly to the GI lumen, as they are swallowed constituents of amniotic fluid, colostrum, and milk. Granulocyte colony-stimulating factor (G-CSF), traditionally known as a granulocytopoietic growth factor, is an example of one such factor. However, it is not clear whether the large amounts of G-CSF that are normally swallowed by the fetus and neonate have systemic effects on circulating neutrophils or local effects in the developing intestine. To assess this, we administered either active or heat-denatured (control) recombinant human G-CSF to 5- to 7-d-old C57BL/6 × 129SvJ mice. Pups received either a low dose (3 ng) that was calculated to approximate the amount of G-CSF swallowed *in utero* from amniotic fluid or an isovolemic high dose 100 times larger (300 ng). Oral dosing was performed daily for either 3 or 7 d, after which pups were killed and measurements were made on the blood and the GI tract. Absolute blood neutrophil counts and immature to total neutrophil ratios did not differ from controls in any of the test groups. However, intestinal villus area, perimeter, length, crypt depth, and proliferating cell nuclear antigen index increased significantly among those that were treated with active G-CSF. Thus, in suckling mice, enterally administered G-CSF had no effect on the concentration of circulating neutrophils but had trophic effects on the intestine. We speculate that the G-CSF present in amniotic fluid, colostrum, and milk acts as a topical intestinal growth factor and has little or no granulocytopoietic action. **(*Pediatr Res* 55: 802–806, 2004)**

Abbreviations

ANC, absolute neutrophil count
EGF, epidermal growth factor
Epo, erythropoietin
G-CSF, granulocyte colony-stimulating factor
G-CSF-R, granulocyte colony-stimulating factor receptor
GI, gastrointestinal
I:T, immature to total neutrophil ratio
rhEpo, recombinant human erythropoietin
rhG-CSF, recombinant human granulocyte-colony stimulating factor
PCNA, proliferating cell nuclear antigen

The development of the gastrointestinal (GI) tract is a complex process that involves diverse cell types and requires a balance among cellular proliferation, differentiation, and senescence. Growth factors are involved in this regulation (1, 2), and among those factors studied are epidermal growth factor (EGF), insulin-like growth factor (IGF), erythropoietin (Epo), and transforming growth factor (3–14).

Several studies in our laboratory have suggested that granulocyte colony-stimulating factor (G-CSF), generally known as a granulocytopoietic growth factor (15, 16), should be included on this list as a GI trophic factor during fetal and neonatal development. G-CSF is present in significant quantities in amniotic fluid (1612 ± 2100 pg/mL, term; 1708 ± 1763 pg/mL, preterm), colostrum (156 ± 12, term; 80 ± 41 pg/mL, preterm), and milk (37 ± 21 pg/mL, term; 32 ± 18 pg/mL, preterm), each of which is ingested by the fetus and the neonate (17–21). Moreover, the G-CSF in these fluids is stable during simulated digestions, suggesting that intact G-CSF reaches the GI tract *in utero* and postnatally (19, 20). We have demonstrated that the G-CSF receptor (G-CSF-R) is widely expressed in the developing fetal GI tract, where it is localized to the apical surface of enterocytes and the crypts of the intestinal villi (18, 22).

The exact function of the G-CSF swallowed by the fetus and neonate is not known. Potentially, it could be absorbed into the circulation and function as a hematopoietic growth factor, and/or it could have local actions in the developing GI tract. As a step toward understanding the function of the G-CSF swallowed by the fetus and neonate, we fed suckling mice either recombinant human CSF (rhG-CSF) or heat-denatured rhG-

Received June 20, 2003; accepted November 20, 2003.
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Supported by grants HD-01180 (D.A.C.), HD-42326 (D.A.C.), and HL-61798 (R.D.C.) from the National Institutes of Health.

DOI: 10.1203/01.PDR.0000117846.51197.7C

CSF (as a control) for either 3 or 7 d and thereafter measured blood neutrophils and intestinal morphometrics.

METHODS

Animals and rhG-CSF administration. C57BL/6 × 129SvJ mice were housed in a specific pathogen-free environment. The protocols were approved by the University of Florida and the University of South Florida Institutional Animal Care and Use Committees. Two doses of rhG-CSF were tested: 3 and 300 ng. The lower dose was taken as an approximation of a "physiologic" dose, derived from the concentration of G-CSF in human amniotic fluid (17). The higher dose was a "pharmacologic" dose, arbitrarily taken as 100 times the "physiologic" dose.

The rhG-CSF (Neupogen; Amgen, Thousand Oaks, CA, U.S.A.; stock of 300 μg/mL) used in these experiments was diluted with PBS (pH 7.2) to deliver each dose in a total volume of 100 μL. Denatured rhG-CSF was produced, for use as a control, by incubating rhG-CSF for 1 h at 60°C (D.A.C. and Amgen, unpublished observations that the heat treatment of rhG-CSF under these conditions leads to no measurable G-CSF activity).

Pups, between 5 and 7 d of age at the start of the study, received a once-daily oral administration of 100 μL of either heat-treated or untreated rhG-CSF, *via* an animal-feeding blunt needle (Popper and Sons, Inc., New Hyde Park, NY, U.S.A.). This was done for either 3 or 7 consecutive days. The pups were divided into six groups: group 1 received 3 ng of denatured rhG-CSF for 3 d, group 2 received 3 ng of active rhG-CSF for 3 d, groups 3 and 4 were fed 3 ng of either denatured or active rhG-CSF for 7 d, and groups 5 and 6 were fed 300 ng of either denatured or active rhG-CSF for 7 d. After each feeding, pups were returned to the cage with their mother and allowed to suckle *ad libitum* until the completion of the experiment. Sample sizes included a minimum of five animals in each group.

Sample collection and measurements. Twenty-four hours after the final study feeding, the pups were killed after inhalation anesthesia (methoxyflurane; Schering-Plough Animal Division, Union, NJ, U.S.A.). Whole blood from a severed carotid artery was collected directly into heparinized Natelson Blood Collecting Tubes (Fisher Scientific, Pittsburgh, PA, U.S.A.) and used for determination of a complete blood count on an automated hematology cell counter (System 9000; Baker Instruments, Allentown, PA, U.S.A.). Pulled blood films were prepared on coverslips for Wright staining to evaluate differential counts, performed under 100× oil immersion. The absolute blood neutrophil concentration (ANC) and blood immature to total (I:T) neutrophil ratio were determined. ANCs were calculated by multiplication of the total white blood cell count by the percentage of neutrophils in the 100-cell differential count (expressed as the number of cells/μL). The I:T ratio was determined by adding the immature myeloid series cells (band neutrophils, metamyelocytes, and myelocytes) and dividing by the total number of neutrophils (segmented and nonsegmented) (23).

The intestine was removed *en bloc*, and sections of duodenum were washed with saline and placed in Bouin's fixative for 4 h, then transferred to 70% ethanol. Specimens were paraffin embedded and cut at 4- to 6-μm thickness. Slide-mounted sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and processed using an automated enzyme-labeled biotin-streptavidin technique (Ventana NexES; Ventana Medical Systems, Tucson, AZ, U.S.A.) with diaminobenzidine tetrahydrochloride as the chromagen. Duodenal sections from each multiday treatment were stained with an anti-mouse proliferating cell nuclear antigen (PCNA) antibody (20 μg/mL; Santa Cruz Biotechnology), counterstained with hematoxylin and bluing, and dehydrated, and coverslips were applied. Morphometric analysis consisting of villus area, perimeter, length, villi/500 μm, crypt depth, and PCNA staining index [(no. of PCNA-positive cells/no. of crypt cells) × 100] was performed using a standard light microscope and a computer Analysis Imaging Station (Imaging Research Inc., St. Catharines, Ontario, Canada) (24). The individual who performed the morphometric analyses and PCNA index determinations was blinded to the group assignment at the time of analysis.

Statistical analysis. Statistical analysis was performed on the morphometric parameters using SPSS for Windows 9.0 (Chicago, IL, U.S.A.). Using the six subgroups for classification of the animals [variables of duration of feeding, type of G-CSF received (heat treated *versus* rhG-CSF), and dose of G-CSF received], means of the groups were compared using a one-way ANOVA. Data presented in tables and graphically are mean values ± SEM. An $\alpha \leq 0.05$ was used as a measure of significance.

RESULTS

Pup weights did not differ significantly between the animals assigned to each of the treatment groups. Feeding the pups rhG-CSF, at either the low or the high dose, for either 3 or 7 d did not produce any recognized elevation in ANC or I:T ratio (Table 1). Similarly, rhG-CSF feeding did not produce any recognized change in any of the blood parameters examined, including platelet count, Hb concentration, or hematocrit.

Feeding rhG-CSF (3 ng) for 3 d (Table 1, comparing groups 1 and 2) resulted in a larger villus area, longer villus perimeter, longer villus length, increased crypt depth, and increased mitotic activity as estimated by PCNA index (Fig. 1), but no differences were observed in number of villi along 500 μm of intestinal length. When rhG-CSF (3 ng) was fed for 7 d (comparing groups 3 and 4), the intestinal morphometric measurements did not differ between those that were fed heat-inactivated rhG-CSF and active rhG-CSF. Feeding the higher dose of rhG-CSF (300 ng) for 7 d (comparing groups 4 and 6) resulted in a larger villus area, longer villus perimeter, and longer villus length.

DISCUSSION

This study was designed to examine the effects of rhG-CSF administered enterally to suckling mice. We observed that neither a short-term (3 d) nor a longer-term (7 d) administration

Table 1. *Blood counts and morphometrics of experimental groups*

	Group 1 3-day Heat-treated 3 ng		Group 2 3-day rhG-CSF 3 ng		Group 3 7-day Heat-treated 3 ng		Group 4 7-day rhG-CSF 3 ng		Group 5 7-day Heat-treated 300 ng		Group 6 7-day rhG-CSF 300 ng	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Blood count												
ANC/μL	5554	861	5937	1825	2777	1642	1683	305	3534	537	1845	252
I:T Ratio	.265	0.03	.261	0.03	.263	0.04	.201	0.03	.323	0.05	.432	0.05
Villus morphometrics												
Area (μm^2)	9928	310	12357*	397	12796	526	11023	374	13208	501	17594*,**	888
Perimeter (μm)	527	12.50	605*	13.79	600	17.74	574	14.79	550	13.22	706*,**	12.37
Length (μm)	228	5.60	267*	6.33	262	8.49	254	6.97	256	6.46	321*,**	5.49
No. of villi in 500 μm	9.4	0.39	10.1	0.58	8.9	0.40	9.3	0.47	8.7	0.76	8.9	0.39
Crypt depth (μm)	45.0	1.35	49.7*	1.28	49.7	0.93	52.0	1.33	51.0	0.97	50.4	0.68
PCNA index (%)	64.7	2.99	73.9*	1.54	74.0	1.34	70.4	1.74	73.9	0.82	74.9*,**	0.94

Mean and standard error of the mean (SEM) are given for each of the six experimental groups. Data are given for blood counts (Part A) and villus morphometric measurements (Part B).

* $p < 0.05$ vs. heat-treated group.

** $p < 0.05$ Group 6 vs. Group 4.

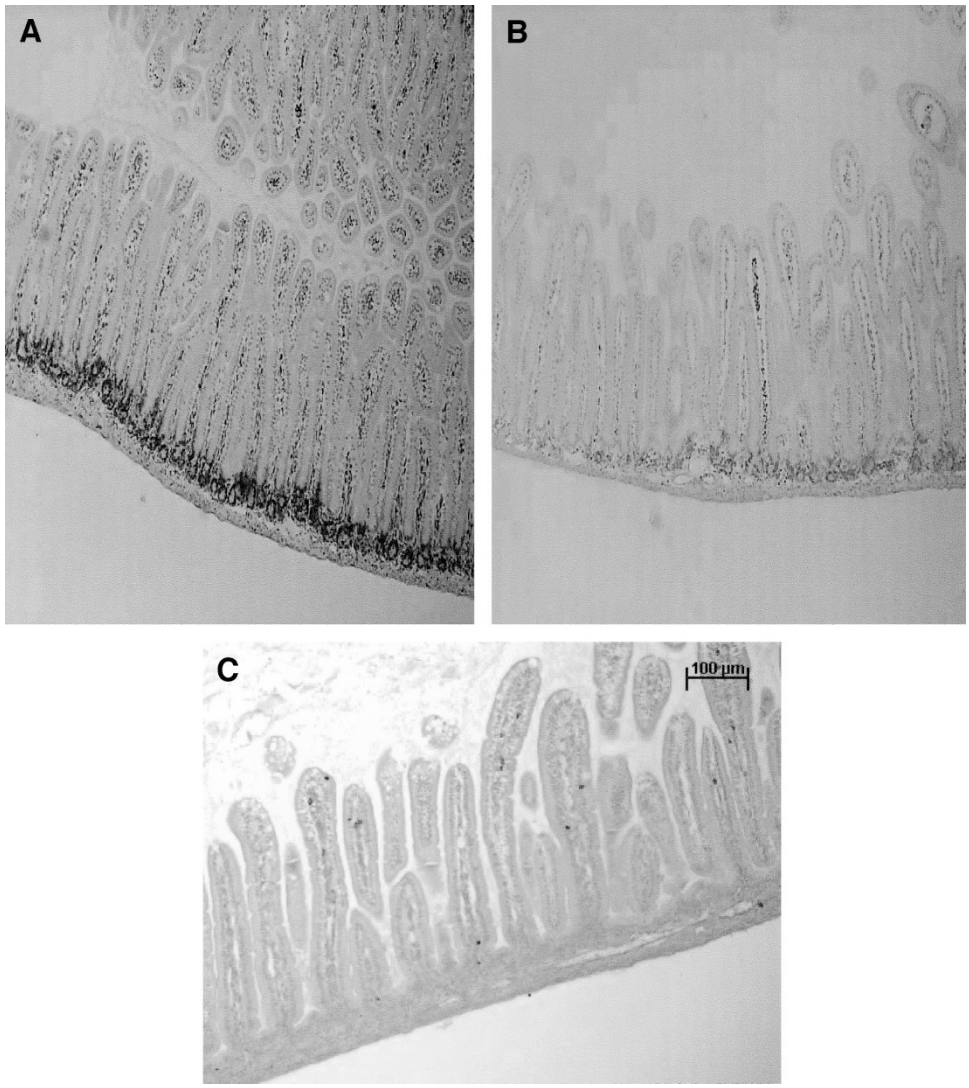

Figure 1. PCNA staining of murine intestine. Representative intestinal sections stained with an anti-PCNA antibody (brown coloration) from mice that received daily enteral rhG-CSF at 3 ng for 3 d (group 1; *A*) and heat-treated rhG-CSF at 3 ng for 3 d (group 2; *B*). (*C*) Negative control. Magnification: ×200.

of either a physiologic or a pharmacologic dose of rhG-CSF produced any recognized change in circulating neutrophil concentration but did have trophic effects on the bowel. In addition, the administration of a pharmacologic dose did not result in any observed adverse effects in these neonatal animals.

Limited data are available on absorption and bioavailability of orally administered rhG-CSF. With a range of 3–4 g body weight for the pups used in this study, the high dose of 300 ng equates to a 75- to 100-μg/kg dose, approximately 10 times the dose used for i.v. or s.c. rhG-CSF administration in human neonates (25, 26). In a study of very high dose (300 or 600 μg/kg) rhG-CSF feeding to adult rats, Takada *et al.* (27) injected rhG-CSF directly into the duodenum and noted a 2-fold increase in the total blood leukocyte count. Also studying adult rats, Jensen-Pippo *et al.* (28) reported that the bioavailability of rhG-CSF was undetectable after duodenal administration of 755 μg/kg rhG-CSF. They did not observe any change in blood leukocytes after enteral rhG-CSF administration. It is not clear why differences in the leukocyte count were observed by Takada *et al.* and not by Jensen-Pippo *et al.* Both studies used much higher doses of rhG-CSF (4–10 times higher) than in the present study, both studied adult rats, and both used a surgically placed duodenal tube for delivery of the rhG-CSF. The surgical stress could have influenced the total neutrophil count observed in one of these studies.

In a separate study, we measured the bioavailability of enterally administered rhG-CSF (3 and 300 ng) to neonatal wild-type mice and neonatal mice with a targeted deletion in the G-CSF-R gene, rendering it nonfunctional (29). In both groups, the bioavailability of G-CSF was $<1\%$ and was not affected by the absence of a functional G-CSF-R. Because the bioavailability of enterally administered rhG-CSF is so low in neonatal animals, one would not expect to observe a significant change in neutrophil count.

We observed that enterally administered rhG-CSF to suckling mice resulted in an increase in certain intestinal morphometric measurements. The increased villus area, perimeter, and length that we observed correspond with the type of intestinal growth observed when neonatal animals were fed Epo, IGF, or EGF (4, 7–9, 11, 13, 30, 31). In addition to the staining of cells located in the crypt, we observed PCNA staining of cells within the core of the lamina propria. To further clarify the nature of these cells, we performed additional staining using hematoxylin and eosin. The cells were primarily reticular cells of the stroma with occasional lymphocytes, macrophages, neutrophils, and eosinophils. No difference in cellular distribution was noted between the groups.

Comparing the measurements of the control subjects (those that were fed heat-inactivated rhG-CSF) after 3 *versus* 7 d reveals the expected fall in ANC and the expected growth in intestinal morphometric indices during this period. Klein *et al.* (32) reported similar increases in growth in the developing rat ileum. We anticipated that increasing the duration of rhG-CSF administration from 3 to 7 d would result in increased indices of growth. However, we speculated that natural growth and proliferation of the developing intestine during this period could mask any effects from administering a low dose of rhG-CSF. Indeed, this might be the reason that the results do not differ between groups 3 and 4.

The use of a pharmacologic dose of rhG-CSF resulted in additional detectable intestinal growth (group 6 *versus* group 5). Comparison of the physiologic (group 4) and pharmacologic doses (group 6) revealed increased villus area, perimeter, length, and PCNA index. Because G-CSF-R are present on the apical surface of enterocytes and crypt cells, perhaps the differences in effect represent different binding affinities of receptors on these cells and/or expression of different isoforms of the G-CSF-R (33).

Although this is the first study to evaluate simultaneously both systemic and local intestinal effects of enteral rhG-CSF in neonatal mice, similar studies have been done using Epo, EGF, and IGF (4, 7–9, 11, 13, 32, 34). After enteral ^{125}I-rhEpo dosing, Miller-Gilbert *et al.* (8) observed that intact ^{125}I-rhEpo was found in gastric and small intestinal walls and lumens. Juul *et al.* (34) found that among rEpo treated pups, *1*) the small bowel was longer, *2*) the intestinal villus surface areas was larger in a dose-dependent manner, and *3*) BrdU uptake was greater.

Shen and Xu found that $>95\%$ of orally administered ^{125}I-EGF in newborn and 5-d-old pigs was in the GI tract (13). Houle et al. (35) reported a 40% increase in jejunal DNA content, greater BrdU incorporation, increased enterocyte proliferation, and a modest effect (20–40% over formula alone) on villus height in piglets that were fed formula that contained 131 nmol/L IGF-I. When pharmacologic concentrations were used (~1.3 mM), significant increases in mucosal protein content, mucosal mass, and villus height occurred (28).

The similarities in effects on intestinal growth noted between Epo, EGF, and our findings using G-CSF could be due to each cytokine's receptor belonging to the same cytokine superfamily (36–38). In hematopoietic cells, Epo and G-CSF bind to their cognate receptor and initiate intracellular signaling *via* the janus kinase and signal transducer and activator of transcription protein pathways (36–38). The specific events triggered by Epo and G-CSF on intestinal epithelial cells are not known. However, G-CSF-R and Epo-R are present on intestinal epithelial cells in human fetuses and neonates (22, 33, 39), and the full-length G-CSF-R (isoform I), which is required for proliferation, differentiation, and enhanced cell viability in myeloid cells (36), is present throughout gestation (33). These findings suggest that it is possible for the G-CSF-R and Epo-R on intestinal epithelial cells to initiate proliferation and differentiation and enhance cell viability as is seen in hematopoietic cells. Further studies are necessary to confirm the specific intracellular signaling pathways used by human intestinal epithelial cells.

In conclusion, this study demonstrates that rhG-CSF administered enterally to suckling mice does not increase the ANC but has trophic effects on the intestine. On this basis, we speculate that the G-CSF normally swallowed by the fetus and neonate has minor if any systemic effects but acts as a trophic factor in the intestinal villi. Future studies on the bioavailability of enterally administered rhG-CSF, the expression of G-CSF-R isoforms on various fetal and neonatal intestinal tissues, and the specific function of G-CSF in the developing intestine will aid our understanding of the nonhematopoietic roles of G-CSF during GI development.

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