Milk-Borne Growth Factors and Gut Development

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The gastrointestinal tract undergoes substantial changes during the period of development. Profound growth, morphological changes, and functional maturation are observed during this developmental period in the small intestine. Differentiation of the gastrointestinal tract during the late gestation period prepares it for its many extrauterine tasks. Birth represents possibly the most critical period in gastrointestinal tract development, when placental supply of macro- and micronutrients is replaced by enteral nutrition. Enteral nutrition initiates changes in mucosal structure and function required for utilization of milk feeds. Finally, this process is concluded by the weaning phase, when the transition from milk to solid food occurs. Physiological changes in digestive and absorptive processes are well described (1), but regulation of these changes is still not clearly understood. For example, the intestinal mucosal surface undergoes nearly complete turnover of the epithelial population every 24 to 96 hours in man and other species. The processes of constitutive growth and proliferation of the intestinal mucosa occur in a highly organized spatial context of proliferation, followed by differentiation and maturation of epithelial cells. Cell production in each crypt is maintained by a few stem cells, and the organization of this process is vertical, from the intestinal crypt to the villus.

Understanding of the mechanisms whereby intestinal growth and epithelial turnover are regulated remains fragmentary. However, it is clear that biologically active peptides play a critical role. The number of biologically active peptides important for regulation of intestinal growth is still increasing. Research studies have documented the essential role of peptide growth factors in intestinal development. These factors include both growth factors that are produced locally within the small intestine and exogenously produced growth factors delivered into the intestinal lumen.

In this review we will summarize recent understanding of the role of milk-borne epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) on the developing gut.
EGF and TGFα IN THE GASTROINTESTINAL TRACT DURING PERINATAL DEVELOPMENT

EGF and TGFα belong to a group of diverse low-molecular-weight polypeptide growth factors (2). EGF was first characterized by its mitogenic activity (3) and inhibition of gastric acid secretion (4). TGFα, described originally as a sarcoma-derived growth factor, was found to be structurally related to EGF and is now accepted as an integral physiological regulator of growth in normal tissues (5). Both EGF and TGFα share the same EGF receptor (2,5).

Expression of EGF receptor has been demonstrated in the intestine of developing rats (6–9), mice (10–12), pigs (13,14), and human fetus (15,16). Whereas in fetal rats EGF receptors are detectable, in suckling rats they were not detected (8,17). This could be due to the potential binding of the milk-borne EGF to this receptor.

Transcript levels of EGF and TGFα in developing small intestine have not been well characterized. Originally, the presence of TGFα mRNA transcripts was found in the gastrointestinal tract from adult rodents only, and the expression of EGF mRNA was not detected at all in either adult (18–21) or suckling gastrointestinal tract (22). Miettinen (23) detected the presence of TGFα and EGF mRNAs in human fetal intestine. Her observation suggested a higher abundance of TGFα transcripts in human fetal intestine in comparison with EGF mRNA. Previous studies from our laboratory have shown the presence and cellular localization of both EGF and TGFα mRNAs in the small intestine of suckling and adult rats (24). Using an in situ hybridization technique, we have shown that the intestinal crypt epithelium is the major site of EGF and TGFα transcripts in both suckling and adult rats. In suckling rats, however, the EGF mRNA signal was very low or absent, whereas TGFα mRNA signal was markedly higher. Since gene expression of growth factors in developing small intestine is, in general, very low, the reverse transcription (RT) competitive polymerase chain reaction (PCR) assay was established to measure mRNA levels quantitatively (25). In suckling rats, the intestinal TGFα mRNA level was about 10-fold higher than that of EGF mRNA. The expression of EGF-R mRNA in suckling rat jejunum was the highest, about sixfold more than TGFα mRNA, and about 60-fold higher than the EGF mRNA level (26).

The presence of EGF peptide in the stomach and duodenum of the human fetus, newborn, and child was demonstrated first by immunohistochemistry (27). Later, EGF and TGFα immunoreactivity was detected after the sixteenth gestational week in the human fetal small intestine (28,29). By radioreceptor assay, the fetal intestine contained 10 times more EGF receptor binding substance than EGF, as measured by immunofluorometric assay. Chromatographic analysis suggests that TGFα-like peptides account for at least part of this activity (28). EGF levels in the small intestine were several times higher in suckling rats than in adults; however, the intestinal concentrations of TGFα in suckling and adult rats were similar (30). The large amount of EGF present in the small intestinal mucosa and lumen of suckling rats depends on the intake of milk-borne EGF (31,32), whereas TGFα content is influenced much less (30). Furthermore, EGF peptide was demonstrated in the small intestine of fetal and neonatal mice (12). Presence of TGFα peptide was also detected in the small
intestine of suckling pigs at 1, 2, and 3 weeks of postnatal age (33) and in a high density in duodenum of fetal mice (34).

EGF AND TGFα IN MILKS

Numerous papers have reported the presence of EGF in human milk as well as in milk of other species (13,35). However, in infant formulas the EGF concentration is very low or undetectable (36). Human milk does contain high-molecular-weight EGF complexes, but these are unstable at neutral and acidic pH levels (37). Rat milk-borne EGF is present in several forms (38,39). In contrast to this, TGFα peptide was detected in low amounts in human milk (40,41) but could not be detected in rat milk (30).

EFFECTS OF ADMINISTRATION OF EGF AND TGFα

The effect of the administration of EGF and TGFα on the developing gastrointestinal tract has been studied using either in vitro organ cultures or in vivo. The administration of these growth factors to animals was performed either parenterally or oro-gastrically. With few exceptions, all the doses used must be considered pharmacological. The question of physiological doses is discussed in the review of the oro-gastric effects of these peptides.

In organ culture studies, addition of EGF to fetal jejunal explants (11-14 weeks of gestation) increased lactase activity, but surprisingly repressed the normally occurring increase of sucrase activity. Furthermore, EGF inhibited hydrocortisone-stimulated DNA synthesis (42). In fetal colon (14–17 weeks of gestation), EGF evoked a significant decrease of [3H]-thymidine incorporation into DNA and a decrease of sucrase and activities digesting maltose (called maltase) (43). In rodent studies, Beaulieu and Calvert (44) have shown that addition of EGF to the medium accelerated differentiation of rough endoplasmic reticulum in duodenal explants from fetal mice. EGF added to mouse duodenal explants (17 days of gestation, 48 hours' cultivation) increased levels of alkaline phosphatase, maltase, and trehalase activities, whereas sucrase activity and DNA content were unchanged (44). In the jejunum of 8-day-old mice (48 hours of incubation), no effect of EGF addition was seen in activities of microvillus disaccharidases and DNA synthesis. It is not clear if the differences between these two experiments from the same laboratory are due to the age of donors or to the intestinal segments used. No EGF effect was seen on DNA and protein synthesis in duodenojejunal explants obtained from rat fetuses taken 5 and 3 days before birth as well as on postnatal days 4 and 7. Interestingly, in 1-day prenatal and 1-day postnatal rats, an EGF effect on DNA and protein synthesis was demonstrated (45).

Parenteral Effects

In the postnatal period, parenteral administration of EGF increased intestinal proliferation in human infants with a congenital microvillus atrophy (46) or necrotizing
enteritis (47) who were treated intravenously with recombinant EGF. Suckling rats injected with EGF showed increased weight of the whole stomach; increased DNA, RNA, and protein content of the oxyntic gland mucosa; and higher rates of basal acid secretion and pentagastrin stimulated acid secretion (48). Odaka et al. (49) reported increased small intestinal weight in suckling rats injected with EGF for 4 days. Administration of EGF caused intestinal hypertrophy in fetal rhesus monkeys (50).

Injections of EGF into newborn rats also caused a large decline in lactase activity in the colon (51) but did not affect the incorporation rate of [$^3$H]-thymidine (52). Arsenault and Menard (53) reported an increase in thymidine incorporation into DNA of the small intestine of 3- to 8-day-old suckling mice treated with high doses of EGF. Hormi and Lehy (54) found that the proliferative effect of EGF and TGFα differed quantitatively in various segments of the gastrointestinal tract of suckling rats. Oka et al. (55) have shown that EGF given for 3 days subcutaneously in high doses inhibited body weight gains by 13% but increased the protein content of the duodenum by 32% in the brush border, increased the concentration of calbindin by 35% to 65%, and increased sucrase activity two- to sixfold.

With regard to absorptive processes, Greene et al. (56) reported an increase in calcium transport in rats treated in the same way as in the study by Oka et al. (55). Harada et al. (57) have shown that giving EGF subcutaneously to suckling rats suppressed intestinal absorption of IgG. Giving mouse EGF (mEGF) intraperitoneally to rabbits from postnatal day 3 to day 17 upregulated intestinal absorption of H$_2$O, Na$^+$, and glucose and caused alterations in the membranes of the microvilli (58). Parenteral administration of EGF into 3-day-old piglets increased sucrase and maltase activities in the small intestine, whereas alanine uptake was not affected (59). Subcutaneous administration of EGF inhibits gastric evacuation and intestinal propulsive motility acutely in suckling rats; interestingly, administration of antibodies against EGF in amounts sufficient to block the estimated amount of EGF present in the suckling rats accelerated intestinal propulsive motility (60). Similarly, TGFα affected the gastric evacuation and intestinal propulsive motility in suckling rats (Shinohara H, unpublished results).

Lastly, several other studies have explored the EGF effect on disaccharidases in more depth. Harada et al. (57) found no effect on sucrase activity, whereas Foltzer-Jourdainne and Raul (52), Foltzer-Jourdainne et al. (61), and Odaka et al. (49) showed an increase of intestinal sucrase activity in suckling rats after administration of EGF. Since this effect was not inhibited by adrenalectomy or co-administration of RU 38486 (a glucocorticoid antagonist), Foltzer-Jourdainne et al. (61) concluded that EGF acts in a glucocorticoid-independent manner. Later from the same laboratory the interaction between EGF and other maturational factors was analyzed by Emvo et al. (62) in suckling rats, starting on day 12. EGF evoked an increase of sucrase activity and sucrase mRNA in both normal and adrenalectomized rats.

Orogastric Effects

The impetus for all studies related to the role of milk-borne EGF was the work of Cohen and Taylor (63), who reported that oral administration of mEGF caused
precocious eyelid opening in newborn mice. Various laboratories were able to
demonstrate effects of orogastrically administered hormones on the suckling using
high pharmacological doses. This led us to define the physiological dose of a milk-
borne hormone as the amount (DD = daily dose) that corresponds to that taken in
milk by the suckling per day, as calculated from the daily milk intake (64) and the
known concentration of the hormone in milk. We have calculated for the suckling rat
that the dose of EGF is about 2 μg/100 g body weight. Another important fact to re-
alize is that under normal conditions (i.e., suckling), milk-borne hormones are pre-
sented to the suckling as a cocktail containing agonistic, antagonistic, and neutral
hormones, growth factors, and binding proteins. Before discussing the orogastric ef-
fect, the gastrointestinal handling of EGF in suckling should be mentioned.

If an ingested peptide hormone is to function within the gastrointestinal tract and
beyond, its survival in the gastrointestinal tract (i.e., its resistance to proteolytic
degradation) is necessary. Britton et al. (65) have shown that in vitro degradation of
$^{[125]}$I-EGF by the gastric juices of preterm infants is negligible. Interestingly, degra-
dation of EGF was found to be greater in gastric and intestinal juices from adult sub-
jects (66). Similar animal studies show low degradation in suckling and a decrease in
degradation after weaning (65,67).

$^{[125]}$I-EGF given orogastrically to suckling rats, using doses in the range of calcu-
lated daily intake, was degraded very little in the stomach and small intestinal lumen
(68,69); similar results were seen in suckling mice (22,70) and lambs (71). $^{[125]}$I-
EGF given orogastrically or into the lumen of the small intestine was detected in the
gastric and small intestinal wall by biochemical methods and by autoradiography
(68,69,72,73). In lambs, EGF (71) is absorbed into the bloodstream, but not into
lymph. Intravenously administered $^{[125]}$I-EGF appears in bile of suckling rats, thus
suggesting the possibility of reabsorption of orogastrically delivered EGF (74). The
significance of an intake of milk-borne EGF was strongly suggested in experiments
where suckling rats were fed pooled rat milk to which antibody against EGF was
added; the intestine of these rats had lower wet weight, lower DNA synthesis and
content, and lower RNA content (75). In vitro studies showing the presence of in-
hibitors protecting EGF from degradation by luminal contents in the suckling gas-
trointestinal tract indicate enhanced survival of milk-borne as well orogastrically de-
livered EGF in the presence of milk (73). Absorption of $^{[125]}$I-EGF from the
gastrointestinal tract of suckling animals was demonstrated in rats (68,69,72,73) and
mice (22). Importantly, in another study, luminally added EGF stimulated rapid ty-
osine phosphorylation of the EGF receptor in the jejunum of suckling rats; the re-
sponse was rapid (within several minutes), and EGF doses used were within the phys-
iological range (8).

Newborn rats fed between 0 and 39 hours of age with an artificial milk that con-
tained high doses of EGF (25 to 120 × DD) showed an increase in DNA synthesis
and content in the small intestine (75) and an increase of DNA synthesis in the liver
(76). Stomach wet weights of newborn rats fed artificial formula to which EGF (10
× DD) was added were greater than in those fed formula only (77). Orogastrically
instilled EGF (3 × DD) given to suckling rats between day 11 and day 13 increased
the cell-labeling indices of fundic, antral, and ileal mucosa, and of the exocrine
pancreas (78). In suckling rats fed rat milk substitute (RMS) from 11 to 14 days of age with added EGF (1.6 × DD), the protein content of the colon was significantly lower and the DNA content was significantly higher than that in rats fed RMS only. Supplementation of RMS with a similar amount of EGF normalized the development of Kupffer cell functions in suckling rats (79). Orogastric administration of EGF (8 × DD) to suckling rabbits for 2 weeks evoked an increase in wet weight of the stomach and pancreas, and of DNA content in the ileum, and an increase in sucrase activity concomitant with a decrease in lactase activity in the proximal segments of the small intestine (80). Absorption of H2O, Na+, and glucose was increased owing to the intestinal mucosal hyperplasia (58). Similar treatment caused precocious maturation of liver functions—that is, the bile salt pool and the secretion and activity of glucokinase (81). Luminally administered EGF did not affect gastric acid secretion in suckling rats (82), although EGF-specific receptors were detected in the stomach mucosal membranes from 8- to 30-day-old rats (82).

The effect of milk-borne hormones during the suckling period, both in experimental mammals and in human neonates, might be not only physiological (i.e., enabling normal development) but also protective against noxious factors (cytoprotection), a role previously demonstrated for EGF (83). In this respect, we may speculate about the significance of growth factors such as EGF as protectors and healers in the case of necrotizing enterocolitis (35).

**CONCLUSIONS**

We hypothesize that EGF may play multiple roles during postnatal development. During the suckling period, when suckling EGF production is low, milk-borne EGF provides important maintenance. After weaning, when the offspring’s EGF production dramatically increases, endogenous EGF is likely to be involved with induction of developmental changes in the gastrointestinal tract. Moreover, we hypothesize that EGF and other growth factors present in milk are important for gastrointestinal development and for the development of specific organs such as liver, muscle, and skin. Studies on the EGF effects (after both parenteral and enteral administration) have direct relevance not only to a general understanding of neonatal growth, but also to specific problems relating to feeding of low-birthweight infants in intensive-care nursery settings. Recent advances in technology and respiratory physiology have allowed a marked improvement in survival of the premature neonate, but the ability to feed low-birthweight babies enterally has been drastically limited owing to the extreme immaturity of their gastrointestinal tracts. Studies in experimental animals are important to gain a more complete understanding of the effects of milk-borne EGF for suckling mammals before recommending trials of altered formulas for human premature newborns.

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DISCUSSION

Prof. Haschke: How mature is the gut of the suckling rat compared with humans?
Prof. Dvorak: It corresponds to a highly premature baby, but I could not tell you how many weeks.

Prof. Haschke: Has anybody studied the interaction between growth factors in breast milk?
Prof. Dvorak: There have been studies showing interaction between IGF-1 and EGF. Injection of EGF to neonatal rats regulates IGF-1 serum levels, hepatic gene expression of IGF-binding proteins and IGF BPs serum levels [1,2].

Dr. Debauche: You said that EGF in milk is stable in an acidic environment, but you also said that milk EGF has a high molecular weight that would be unstable at acidic pH. Can you comment on that?
Prof. Dvorak: It is true that there is partial degradation, but in suckling rats, about 80% to 90% survives passage through the stomach.

Dr. Walker: As you pointed out, the preweaned rat is comparable to the quite premature human infant. In the late 1980s an investigator named Menard from Sherbrook looked at the effect of EGF on 12-week gestation and 19- to 22-week-gestation fetal intestinal cells from the human and found striking differences [3]. My question is, “Do you see similar results in comparing, let’s say, 2-day-old rats with 14-day-old? Growth factors have different effects at different times in development, and that is a very important area to study if we are going to be able to apply the data to humans.

Prof. Dvorak: Though we did not specifically do developmental studies, I measured the expression of mRNA in 4-day-old, 10-day-old, 12-day-old, and 14-day-old animals, and did not see significant changes. There were differences, but they were not sufficient to allow me to say there was a trend to increase or decrease.

Dr. Sedaghatian: Have you investigated EGF in premature mother’s milk and compared it with full-term mother’s milk?
Prof. Dvorak: We are comparing these term rat babies with premature human infants, so it’s a difficult comparison. The milk we used in our experiments was mature rat milk, harvested from day 8 to day 12 of lactation. We were not using colostrum or early milk. We were also using animals between 8 and 12 days old, but we believe there may be a time window when it is most important to have this growth factor. We are now going to see whether EGF has a greater effect if it is added between, say, day 0 and day 4, instead of day 8 to 12. Of course there are many growth factors and other biologically active substances in milk, and we are trying to target them one by one. Another option is to develop antibodies against individual growth factors and knock them out of the milk, and then compare the effects.

Dr. Walker: There is a large amount of EGF in preterm milk, and there is a fairly high level in term colostrum, but the concentration falls off with time.

Prof. Lucas: Presumably, growth factors are present in cow’s milk and have biological effects on the calf. How species-specific are growth factors of this nature in their activity, and how well preserved or not preserved are they in the production of an infant formula? Could you preserve them, and would they have an effect on the infant?

Prof. Dvorak: It would be difficult to preserve them in a formula. There are differences in the level of these growth factors from species to species. TGFβs, for example, is present in human milk but we couldn’t detect it in rat milk, while EGF is present in both. Insulin-like growth factor is present attached to a binding protein, so it is protected. But to my knowledge, there are no formulas containing growth factors in comparable amounts to human milk.

Dr. Guesry: I don’t know whether TGFβ2 is comparable to other growth factors, but it is very well preserved during processing and large amounts are present in infant formula.
**Prof. Dvorak:** We have focused more on the EGF and IGF families, and those growth factors are not very well preserved during processing.

**Prof. Haschke:** As far as I know, growth factors from cow’s milk colostrum are commercially available. They are not species-specific, because they also work in the rat. So even if there is a difference in binding or whatever, it seems that growth factors from cow’s milk could be effective in the human infant.

**Prof. Dvorak:** I think that is true. We actually used human EGF for a long time, since it was cheaper than rat EGF. We switched to rat EGF to be on the safe side, but there were no significant differences in activity. There are changes in structure—there are two different amino acids, and the human EGF molecule is a little longer than the rat molecule, but biologically there are no significant differences in activity.

**Dr. Walker:** In relation to the structure, the receptor may be the same, but I’m not sure that the programming is the same. We need to do a lot more studies before we can infer that the TGF present in cow’s milk has an appropriate effect on human intestine.

**Prof. Dvorak:** It needs a very high level of biochemical expertise to do such studies, and then convert them into animal models.

**Prof. Moro:** Do you know if heat treatment of human milk can have any effect on these growth hormones?

**Prof. Dvorak:** It reduces the biological activity of these growth factors significantly.

**Prof. Endres:** If IGF-1 is destroyed by hydrolysis, will EGF and TGF also be destroyed by this procedure?

**Prof. Dvorak:** Yes, I believe so.

**Prof. Berger:** Over the last few years there has been increasing interest in parathyroid related protein, which apparently has protean effects—on cell maturation, immunity, surfactant induction in the lung, and so on. I am intrigued by the fact that the concentration in human milk is apparently 1,000 times higher than in the maternal plasma. Do you know anything about this in relation to gut maturation? I know people have been looking at that in relation to calcium absorption.

**Prof. Dvorak:** I am unable to comment on this.

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