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Epidermal Growth Factor in Mouse Milk during Early Lactation: Lack of Dependency on Submandibular Glands

A. GRUETERS, J. ALM, J. LAKSHMANAN, AND D. A. FISHER

Department of Pediatrics, Harbor-UCLA Medical Center, Torrance, California 90509

ABSTRACT. Using a specific and sensitive radioimmunoassay, we found epidermal growth factor (EGF) in mouse milk during early lactation in normal and sialoadenectomized mice. Levels of EGF peaked around the 6th day postpartum and decreased thereafter up to day 12. Sephadex G-50 column chromatography of milk from normal and sialoadenectomized mice showed a single immunoreactive component comigrating with purified 6045 dalton EGF from the mouse submandibular gland. The concentrations of EGF were similar in milk collected from the breast and the stomach of the offspring immediately after feeding. The molecular profiles, concentrations, and ontogeny of EGF in milk of control and sialoadenectomized mice were also similar, suggesting that the submandibular gland is

not the major source of EGF in mouse breast milk. (*Pediatr Res* 19: 853-856, 1985)

Abbreviations

EGF, epidermal growth factor
SMG, submandibular gland
RIA, radioimmunoassay
PBS, phosphate-buffered saline

EGF is present in high concentrations in mouse SMG and in lesser concentrations in many mouse and human tissues and body fluids (1-5). EGF stimulates accelerated proliferation and differentiation of skin, corneal epithelium, and lung tissue *in vitro* and induces a cascade of events that results in increased mitogenic activity. *In vivo* EGF accelerates eyelid opening and tooth eruption in newborn mice (6), and promotes functional maturation of the intestine (7) and lungs (8). Functional EGF receptors have been found in many embryonic tissues (9).

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Request for reprints should be addressed to Dr. A. Grueters, University Children's Hospital, Kavh Heubnerweg 6, D-1000 Berlin, FRG.

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EGF is a very energetically stable protein (10) and is biologically active when given orally (11). Human milk has been shown to possess mitogenic activity for cultured cells (12) and EGF is present in high concentrations in both human and mouse milk (13–15). However, the origin of the EGF in milk is not known. The present study was done to characterize the ontogeny and molecular profile of milk EGF during early lactation in normal and sialoadenectomized mice to determine whether the SMG contributes to milk EGF during this period.

MATERIALS AND METHODS

Animals. Adult female Swiss Webster mice (30–35 g) were purchased from Simonsen Laboratories (Gilroy, CA) and were housed individually under controlled temperature (20° C) and lighting (12 h darkness/12 h light) conditions. Animals were provided with regular lab food and water *ad libitum*. In 30 animals the SMG and sublingual glands were removed under general anesthesia with pentobarbital. These animals had previous records of three or four successful pregnancies before the surgery. After a recovery period of 3 wk, sialoadenectomized and control females were mated with normal males; 80% of operated animals became pregnant. The day of birth was considered as day 0, and the litter size of pups was reduced to eight per mother. No differences were observed in litter size or nursing capacity of sialoadenectomized and sham-operated animals. Moreover tooth eruption and eyelid opening occurred at similar times in pups of both groups. Mothers were separated from their pups for 4 h and milk samples were collected by gentle expression and suction of the breasts after intraperitoneal administration of pentobarbital and oxytocin (160 mU). On day 0, 3, 6, 9, and 12 three litters from each group were killed. The stomachs of the pups were opened longitudinally and the contents removed by gentle scraping. These stomach milk and breast milk samples were frozen at -70° C until analysis.

Preparation of milk homogenate supernatant. Milk samples from four mice were pooled and homogenized using a glass Teflon homogenizer in 1 to 9 volumes (v/v) of ice cold 0.05 M (PBS, pH 7.2) containing 0.1% azide. All homogenates were spun at 100,000 × *g* for 1 h. The clear supernatants were used for EGF and protein determinations. In order to adjust concentrations to the EGF-RIA sensitivity range, homogenates were diluted 1:10 with RIA buffer containing 0.5% bovine serum albumin (RIA-grade), 5% normal rabbit serum, and 0.01% azide in 0.05 M PBS. Recovery studies were performed according to Hoath *et al.* (16). EGF was measured using a previously described double antibody method developed in this laboratory (16, 17). Supernatant protein concentrations were determined by the method of Lowry *et al.* (18) using bovine albumin as standard. RIA results were processed using a computerized RIA program with log logit transformation of the data (19). EGF concentrations were expressed per milligram milk homogenate protein collected from the breast or stomach.

Gel exclusion chromatography. Sephadex G-50 fine resin columns (0.9 × 56 cm) were equilibrated and developed with 1) 1 M acetic acid (pH 2.4) containing 10⁻⁴ sodium azide and 2) 0.05 M PBS (pH 7.2) containing 0.1% sodium azide at 4° C. The columns were calibrated with blue dextran and Na¹²⁵I (10,000 counts/min). Unlabeled mouse SMG EGF (6045 daltons), purified by the method of Savage and Cohen (20), was applied to the column and its elution profile characterized. Aliquots of lyophilized milk samples reconstituted in 1 ml acetic acid or 0.05 M phosphate buffer were applied to the column in 0.5 ml volumes and eluted by descending gravity flow (12 ml/h); 0.5 ml fractions were collected. Samples eluted with the acetic acid were evaporated to dryness using a speed-vac concentrator (Savant Instr. Inc., New York, NY), reconstituted in 0.3 ml of assay buffer and assayed directly for EGF, while column fractions eluted with PBS were taken directly for EGF-RIA measurements. Student's *t* test was used for the comparison of mean values.

RESULTS

Studies of displacement of labeled EGF in the RIA by serial dilutions of mouse milk and standard purified mouse SMG EGF (Fig. 1) generated parallel regression lines. The mean (±SEM) recovery of varying amounts of standard mouse EGF added to a pool of mouse milk homogenate supernatant was 100 ± 6% (data not shown). EGF and protein concentrations in breast milk homogenate supernatant at days 1, 3, 6, and 9 of lactation are shown in Table 1. Concentrations in milk samples collected from the stomach were similar. Protein and EGF concentrations both peaked on day 6. Breast milk homogenate supernatant EGF concentrations for control and sialoadenectomized mice are plotted in Figure 2. Values in the two groups of animals are similar at all time points.

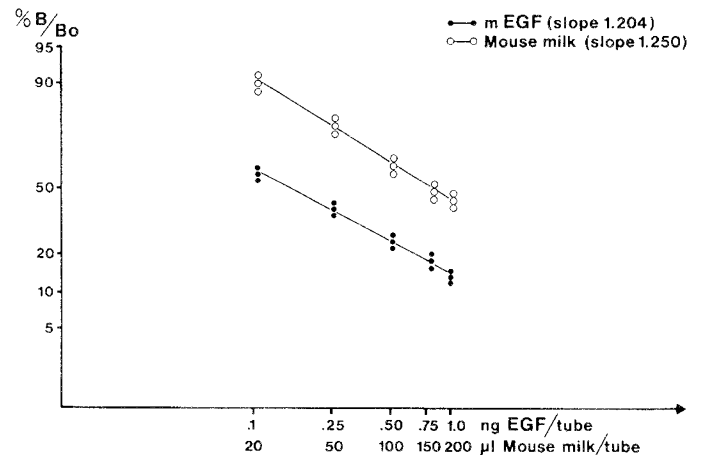


Fig. 1. Competitive binding curves of mouse SMG extract and milk in EGF-RIA.

Table 1. EGF and protein concentrations in mouse breast milk homogenate supernatants during the first 9 days of lactation (mean ± SEM)

| Day of lactation | n | Protein (mg/ml) | EGF (ng/mg protein) |
|------------------|---|-----------------|---------------------|
| 1 | 5 | 11.14 ± 0.22 | 2.42 ± 0.67 |
| 3 | 5 | 13.60 ± 0.34 | 7.44 ± 1.10 |
| 6 | 4 | 20.93 ± 0.82 | 12.65 ± 1.29 |
| 9 | 4 | 9.60 ± 0.01 | 6.69 ± 0.43 |

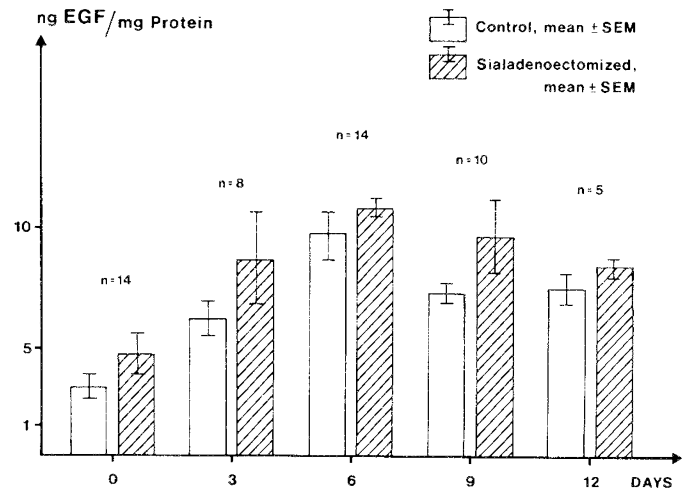


Fig. 2. EGF concentrations in mouse milk during early lactation: comparison of normal and sialoadenectomized animals.

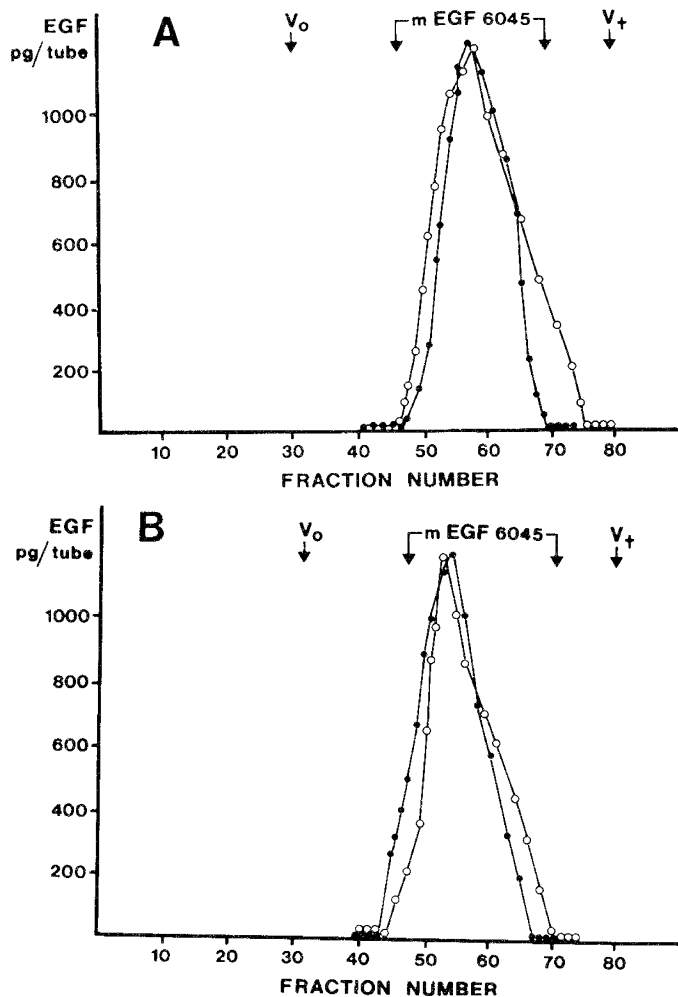


Fig. 3. *A*, Sephadex G-50 fine gel exclusion chromatography in 1 M acetic acid (pH 2.4) of mouse breast milk. *B*, Sephadex G-50 fine gel exclusion chromatography in 0.05 M phosphate buffer (pH 7.2) of mouse breast milk. \circ , sham-operated breast milk; \bullet , sialoadenectomized breast milk.

The chromatographic profiles of milk immunoreactive EGF in control mothers at pH 2.4 and 7.2 are shown in Figure 3. In each instance only one component was observed and this corresponded to standard mouse SMG EGF of 6045 daltons. The profiles of milk immunoreactive EGF from sialoadenectomized mice at pH 2.4 and 7.2 also were studied. Again only one immunoreactive peak was observed in each instance and again this peak comigrated with standard 6045 dalton EGF (data not shown).

DISCUSSION

In the present investigation we found high concentrations of intact EGF in mouse milk with a peak concentration at day 6 postpartum. The concentration of EGF in breast milk was independent of the presence of SMG tissue in the lactating dams. The observed concentration and the pattern of change in mouse milk EGF were found to be in good agreement with earlier observations (15). Our study was restricted to the first 12 days of lactation because this is the time of maximal growth of pups, and most of the EGF-mediated *in vivo* effects occur during this critical period (6, 8). To our knowledge the lack of influence of SMG tissue on breast milk EGF has not been reported earlier. We detected only one 6045 dalton form of EGF in milk from either normal or sialoadenectomized mothers. There was no high

molecular weight EGF, as reported from urine (4). Moreover mouse milk EGF was observed to be acid stable. These results suggest that the EGF in milk of sham-operated and sialoadenectomized mice is quite similar. However, G-50 column chromatography is not adequate to exclude small differences in the EGF molecule.

The origin of EGF in mouse and human milk is not known. Recent studies in humans (1) and goats (21, 22) suggest passive transport of blood EGF to the mammary gland by a transcellular mechanism. Mammary cells have a dependency on EGF *in vitro* (23, 24). The existence of prepro EGF mRNA was reported recently in lactating mouse mammary gland (25). However, it is not clear whether the message is translated ultimately into EGF. Immunocytochemical studies have failed to localize EGF in mammary gland (26) despite high milk EGF content. These results suggest that EGF may not be synthesized in the mammary gland. Alternatively, it may be synthesized, but not stored, in the gland as is the case for kidney tissue (27).

If EGF in milk is derived from blood EGF, it must be concentrated in the mammary gland, presumably by high affinity receptors. Such receptors have not yet been demonstrated. The SMG represents the major pool of EGF in adult mice and SMG EGF concentrations in adult males exceed female levels by 20- to 60-fold (1-3 *versus* 30-70 ng/mg wet weight, respectively) (28). In pregnant females SMG-EGF levels increase to values approximating the male and decline subsequently during lactation (28). Thus it has been suggested that EGF in mouse milk is synthesized in the SMG and transported via blood to the mammary gland. In the present study we demonstrated that the concentrations and profile of breast milk EGF are similar in control and sialoadenectomized mice. This result indicates that SMG is not an important source of breast milk EGF.

During the preparation of this manuscript Okamoto and Oka (29) reported that in virgin mice pregestational sialoadenectomy decreased the growth of the mammary gland and its capacity to synthesize milk leading to an increase in offspring mortality; EGF administration to sialoadenectomized pregnant mice increased survival rate of infants to almost a normal level. Although these authors observed no change in the quality of milk, removal of the submandibular gland decreased maternal serum levels of EGF during pregnancy and lactation. In our studies sialoadenectomy did not seem to impair lactation. The major difference between our studies and those of Okamoto and Oka (29) is that these investigators usually performed sialoadenectomy when the females were relatively young (50-60 days old) and experiencing a first pregnancy. We used older animals (>200 days) having undergone three to four successful pregnancies before the present study. It is our experience that animals with the first two pregnancies are less suitable than more experienced dams for physiological studies involving pregnancy, nursing, and neonatal and infant mouse growth. Wide variations are observed in nesting behavior, litter size, and nursing behavior in mice during the first few pregnancies. These young female mothers often spend less time with the newborn (maternal neglect) and maternal cannibalism is greater than with the older experienced females. In addition, young mothers are very sensitive to frequent handling of pups during routine cage changes.

Other information obtained using adult sialoadenectomized female mice in our laboratory deserves comment. In a previous study (16) we reported a lack of maternal sialoadenectomy on neonatal mouse skin-EGF content. More recently we reported that sialoadenectomy in adult female mice caused no significant decrease in serum-EGF levels (30). In another study (31) we observed that sialoadenectomy of adult female mice increased urinary-EGF levels. In addition we observed a lack of correlation between serum-EGF and SMG-EGF in mice of different age groups (32). These studies indicate that submandibular gland-EGF is not the source of EGF in other tissues and physiological fluids in mice. The decreased milk production and poor nursing ability observed by Okamoto and Oka (29) in younger female

mice may be attributable to poor adaptability to non EGF-related physiological changes brought on by the removal of the SMG. Although the authors claim that all sialoadenectomized animals regained normal weight within 2 wk, observations in our laboratory show a decrease in weight growth velocity caused by submandibular gland excision; this could not be reversed by SMG replacement therapy (30).

The role of milk EGF is not clear. There could be local as well as systemic effects. Besides the classical *in vivo* effects of EGF on eyelid opening and tooth eruption, EGF has been shown to stimulate the maturation and proliferation of mucosal enterocytes (33). Oka *et al.* (7) have shown that EGF increases intestinal weight, intestinal lactase activity, and net calcium transport in 2-wk-old suckling rats, whereas it has no effect in 3-wk-old weanling animals. On the other hand, EGF could be absorbed; EGF is acid stable, trypsin treatment does not inhibit its biological activity (34), and radiolabeled mouse EGF given to immature rats has been shown to be at least partially absorbed unchanged and to bind to specific receptors in skin as well as intestine (35). Thus milk EGF may be important in maturation of intestine as well as other tissues in the newborn mouse.

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