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Evidence for physiological function of epidermal growth factor: Pregestational sialoadenectomy of mice decreases milk production and increases offspring mortality during lactation period

(salivary gland/mammary gland/growth and differentiation/lactogenesis)

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ABSTRACT **Female virgin mice, whose submandibular glands were removed, underwent normal pregnancy and delivery. During the nursing period, however, a substantial number of pups born to and nursed by sialoadenectomized mothers died within 5 days of birth, whereas this did not occur among pups born to normal mothers. Cross-foster nursing experiments indicated that the cause of death of pups was to be found in sialoadenectomized mothers, not in the pups. The capacity of the sialoadenectomized mothers to nurse pups was much less than that of normal mothers, as shown by experiments involving alterations in the number of pups nursed by both sialoadenectomized and normal mothers. The mammary gland of lactating sialoadenectomized mice was smaller in size and produced less milk compared with that of normal mice. No apparent qualitative difference in milk proteins was found in the milk produced by the two groups of mothers. The decreased growth of the mammary gland of sialoadenectomized mice was also manifested during the second half of pregnancy, and mammary explants from those mice synthesized less casein in response to lactogenic stimuli, insulin, cortisol, and prolactin, in an organ culture system, when compared with mammary explants from normal pregnant mice. When epidermal growth factor, a polypeptide hormone that is synthesized and secreted by the submandibular gland, was injected daily at a dose of 5 μg into sialoadenectomized pregnant mice, the survival rate of the pups nursed by their mothers increased to the value obtained with normal mothers. The results were discussed in terms of a possible role of the submandibular gland and epidermal growth factor in the development of the mammary gland.**

The development of the mammary gland remains arrested in the virgin female and resumes at the onset of pregnancy, culminating in production of milk proteins after parturition. During pregnancy, mammary epithelial cells proliferate extensively, giving rise to daughter cells that form the lobuloalveolar structure and synthesize various components of milk during lactation (1). Classical procedures involving ablation of endocrine organs and hormone replacement therapies have made a great contribution to the understanding of mammary gland development, which requires the interplay of pituitary, ovarian, and adrenocortical hormones (2–5). In addition, studies using cell and organ cultures of mammary epithelium have provided much important information concerning the complex nature of the regulation of mammary gland development (6, 7). It has been established (6–8) that insulin, cortisol, and prolactin are minimal hormonal requirements for the functional differentiation of mammary epithelium in culture. Although both estrogen and progesterone have been thought to promote the growth of the mammary gland *in vivo*, they elicited little stimulation of mammary epithelial cell proliferation *in vitro* (3). Recently, it was shown (9, 10) that the physiological concentrations of epidermal growth factor (EGF), a polypeptide hormone produced by the submandibular gland (11), stimulates mammary cell proliferation in culture. The mitogenic action of EGF is specific in the sense that other growth factors such as nerve growth factor, multiplication-stimulating activity, insulin, platelet-derived growth factor, and fibroblast growth factor are either ineffective or much less potent in the stimulation of mammary cell proliferation (9, 10). Based on these and other findings (9, 10, 12–15), it was proposed that EGF is involved in the growth of the mammary gland during pregnancy.

The mouse submandibular gland is a rich source of various biologically active polypeptides such as EGF and nerve growth factor (16). These polypeptides have been found in the blood of mouse (17) and man (18). Moreover, hormonal stimulation of the submandibular gland has been shown to increase the production and secretion of EGF into circulation (11). These results suggest that the submandibular gland functions as an endocrine organ, but its specific physiological role has not been established.

Based on our previous findings of the effect of EGF on mammary cells in culture (9, 10), we began a series of studies to assess a possible role of the submandibular gland in the development of the mammary gland during pregnancy and lactation. In the present study, we performed sialoadenectomy of virgin mice to examine its subsequent effect during pregnancy and lactation in terms of mammary gland development, milk production, and nursing capacity of mothers. The data presented here showed that pregestational sialoadenectomy decreases the growth of the mammary gland and its capacity to synthesize milk, resulting in an increase in offspring mortality. EGF administration to sialoadenectomized pregnant mice, however, increases survival rate of offspring to almost a normal level.

MATERIALS AND METHODS

Materials were obtained as follows: ^{3}H-labeled amino acid mixture (1 mCi/ml; 1 Ci = 37 GBq) and Protosol (tissue solubilizer) were from New England Nuclear; medium 199 (Hanks' salts) was from GIBCO; oxytocin was from Calbiochem–Behring; bovine serum albumin was from Bio-Rad; bovine prolactin (NIH lot B5) was from the Hormone Distribution Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases; crystalline porcine zinc insulin was from Eli Lilly Research Laboratories; and EGF was from Collaborative Research (Waltham, MA).

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Abbreviation: EGF, epidermal growth factor.

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C3H/HeN virgin mice were obtained from the Animal Breeding Facility (National Institutes of Health). At 50–60 days of age, the submandibular and/or sublingual glands or parotid glands were bilaterally removed under ether anesthesia. Sham operation was also performed using age-matched virgin mice. At 80–90 days of age, all of the mice were mated with males of the same strain, and pregnancy was checked by examining vaginal plug. Pregnant mice were caged individually and fed Purina Laboratory Chow *ad lib*. The experimental results obtained with the mice whose submandibular glands were removed were essentially the same as those obtained with the mice whose submandibular and sublingual glands were removed; thus, these data were pooled; these animals were collectively referred to as sialoadenectomized mice, whereas sham-operated mice were referred to as control or normal.

Milk was collected from lactating mice according to the method of Anderson and Turner (19) with a slight modification. Nursing mice were separated from their pups 14 hr before injection of oxytocin (2 IU per mouse, s.c.), and milk was collected by manually squeezing the mammary gland, while the mice were under ether anesthesia. The amount of milk was quantified with a calibrated micropipette. The weight of the mammary gland was measured after removal of the milk. The amount of milk protein was determined by the Bio-Rad protein assay kit using bovine serum albumin as standard.

Milk samples obtained from normal and sialoadenectomized mothers were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis to assess qualitative and quantitative differences in major milk proteins. Milk proteins were denatured and electrophoresed under conditions described by Laemmli (20) using a 3% stacking gel and a 12.5% resolving gel.

Explants of mammary glands from pregnant mice (day 14) were cultured as described (21). The concentrations of hormones used were as follows: insulin and prolactin at 5 μg/ml and cortisol at 3 μM. Casein synthesis was determined by allowing mammary explants to incorporate ^{3}H-labeled L-amino acid mixture (10 μCi/ml) for 48 hr. The amount of ^{3}H-labeled casein was measured by an indirect immunoprecipitation method (8).

EGF administration was given daily to sialoadenectomized mice during days 11–17 of gestation. EGF was injected subcutaneously at a dose of 5 μg per mouse in 0.1 ml of 0.9% NaCl. The dose and timing of EGF injection was determined by several preliminary experiments to give optimal results. After parturition, the number of pups to be nursed was standardized to eight pups per mother, using those born to normal mothers.

RESULTS

Preliminary experiments indicated that sialoadenectomy caused 5%–11% loss in body weight within a couple of days after the operation, but in ≈2 weeks, the sialoadenectomized mice regained their body weight to a normal level: at the time of the operation, the body weight of mice was ≈19.0 ± 0.64 g; at the time of mating, the body weights of control and sialoadenectomized mice were 22.4 ± 0.21 g and 20.8 ± 0.18 g, respectively. No apparent signs of infertility or abnormality during the pregnancy were observed in sialoadenectomized animals.

The mean numbers of pups born to control and sialoadenectomized mothers were 7.1 and 6.1, respectively (Fig. 1). During the first 15 days of the lactation period, ≈37% of the pups nursed by sialoadenectomized mothers died, whereas pups nursed by control mothers showed >90% survival rate. The death of pups nursed by sialoadenectomized mothers occurred mostly within 5 days of lactation. In all cases examined, death was preceded by a lack of increase in body weight and a progressive decrease in vitality of pups.

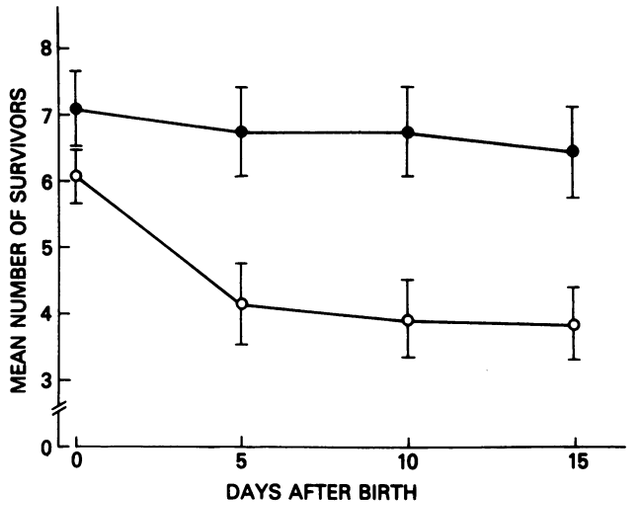

FIG. 1. The mean number of surviving pups born to and nursed by control (●) or sialoadenectomized (○) mice during the period of lactation. Results are expressed as the mean number ± SEM of surviving pups per mother. The numbers of control and sialoadenectomized mice were 11 and 14, respectively. Significant differences were found between the two groups on days 5, 10, and 15 ($P < 0.01$) by Student's t test.

On the other hand, the mean number of pups born to parotidectomized mothers ($n = 6$) was 6.0; pups nursed by their mothers showed a 94% survival rate during 15 days of lactation. These results indicated that parotidectomy had no effect on survival of the offspring.

To determine whether the cause of death of pups born to and nursed by sialoadenectomized mothers lay with the mothers or the pups, cross-fostering experiments were carried out. As shown in Fig. 2, the survival rate of pups born to sialoadenectomized mothers and then nursed by normal mothers was nearly 100%—i.e., 60 of 61 pups, as was the case with pups that were born to and nursed by normal mothers (Fig. 1). By contrast, the survival rate of normal pups nursed by sialoadenectomized mothers was ≈70%–i.e.,

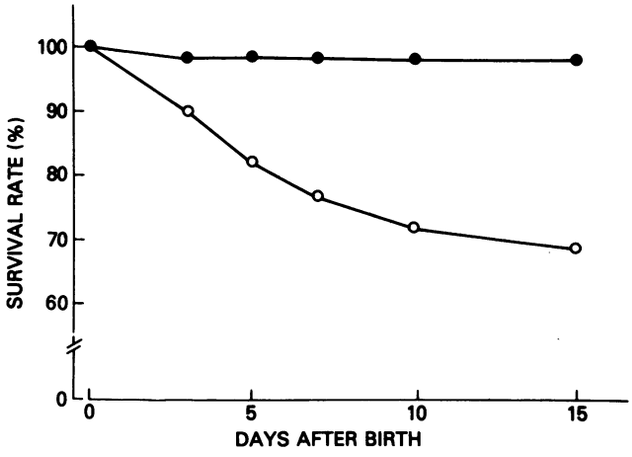

FIG. 2. Cross-fostering of pups and mothers and offspring mortality during the period of lactation. Eight paired sialoadenectomized and normal mothers that produced 7–8 pups each were exchanged within 12 hr after delivery. Offspring mortality was assessed for 15 days, using ≈60 pups per experimental group. ●, Survival rate of pups born to sialoadenectomized mothers and nursed by control mothers; ○, survival rate of pups born to control mothers and nursed by sialoadenectomized mothers.

42 of 61 pups, during the 15-day nursing period. These results indicated that sialoadenectomized mothers were unable to sustain the normal size litter by their own nursing, whereas pups born to sialoadenectomized mice were able to grow normally when nursed by normal mothers.

The data presented above showed that, although sialoadenectomized mothers lost one-third of their pups during the nursing period, ≈4 pups per mother were able to survive. This indicated that sialoadenectomized mothers had a limited capacity to nurse pups, and thus the number of pups to be nursed was an important factor determining their survival rate. This view was tested by changing the number of pups to be nursed by each mother from 4 to 12, using normal pups and normal and sialoadenectomized mothers. As shown in Fig. 3, the number of surviving pups changed in an inverse relation to the number of pups used for nursing. Both sialoadenectomized and control mothers were able to maintain all 4 pups; the body weights of pups were 8.01 ± 0.21 g and 8.87 ± 0.15 g, respectively at 15 days after birth. When the number of pups was increased to 8, only normal mothers could maintain them. When the number of pups was increased to 12, both sialoadenectomized and normal mothers lost a substantial number of pups: however, the loss was greater with sialoadenectomized mothers.

The observed difference in the nursing capacity of normal and sialoadenectomized mothers suggested that sialoadenectomized lactating mice were unable to supply adequate amounts of milk, the single most important source of nutrients for the pups. As shown in Fig. 4, analysis of the milk proteins produced by normal and sialoadenectomized mothers on days 5, 10, and 15 of the lactation period revealed no

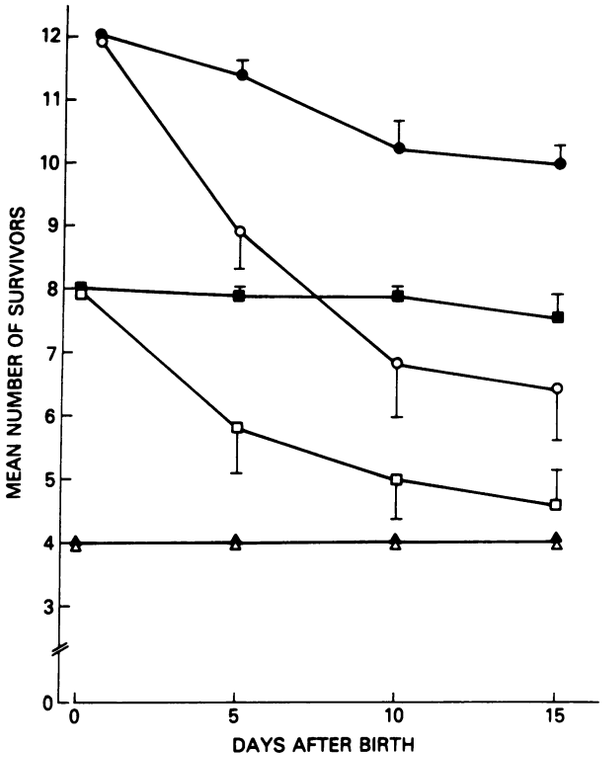

FIG. 3. Effect of litter size on survival of pups nursed by control or sialoadenectomized mothers during the period of lactation. The litter size was adjusted to 4 (▲, △), 8 (■, □), or 12 (●, ○) within 12 hr of delivery, using pups born to normal mothers. Results are expressed as the mean number ± SEM of surviving pups per mother. Six to 10 control (closed symbols) and sialoadenectomized (open symbols) mice were used. Significant differences were found on days 5, 10, and 15 ($P < 0.01$) in reference to day 0, when the number of pups used was 8 or 12, by Student's t-test.

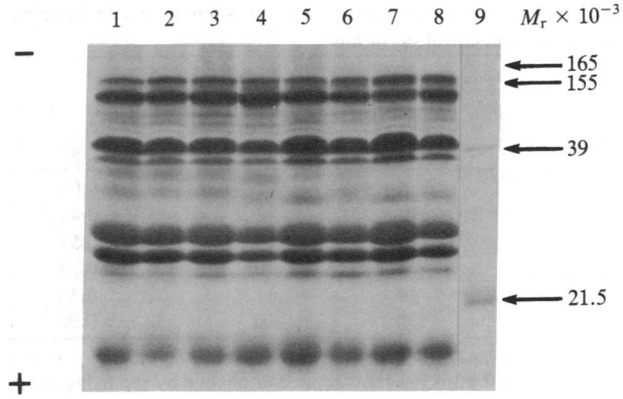

FIG. 4. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of milk proteins produced by normal and sialoadenectomized mothers. Samples were applied at an amount of 40 μg of protein per lane and were stained with Coomassie brilliant blue after electrophoresis. Lanes 1 and 2, milk sample from normal mothers on day 5 of lactation; lanes 3 and 4, milk sample from sialoadenectomized mothers on day 5 of lactation; lane 5, normal mothers on day 10; lane 6, sialoadenectomized mothers on day 10; lane 7, normal mothers on day 15; lane 8, sialoadenectomized mothers on day 15; lane 9, molecular weight standards (RNA polymerase α, -β, and -β', M_r, 39,000, 155,000, and 165,000, respectively; trypsin inhibitor, M_r, 21,500).

qualitative and quantitative differences in those identifiable species of milk proteins, such as caseins (M_r, 43,200, 37,000, 27,700, and 25,900), a whey acidic protein (M_r, 14,000), α-lactalbumin (M_r, 14,700), lactoferrin (M_r, 88,000), and serum albumin (M_r, 64,000) as well as other unknown species. Thus, milk proteins produced by normal and sialoadenectomized lactating mice appeared to be qualitatively similar, and their concentrations per milliliter of milk were virtually the same, as judged by these results.

The data given in Table 1 show that the mammary gland of lactating sialoadenectomized mice was considerably smaller and produced about 50% less milk than the gland of normal lactating mice on day 5. These differences were also found in the glands of mice on days 10 and 15 of lactation. By contrast, the concentration of milk protein was about the same between sialoadenectomized and normal lactating mice. This agrees with the results given in Fig. 4.

Previously, it was established that the combination of insulin, cortisol, and prolactin induced casein synthesis in cultured mammary glands (6–8). Since the data given in Table 1 indicated that the mammary gland of sialoadenectomized lactating mice was smaller and produced less milk, it was of interest to examine the ability of the mammary gland of pregnant sialoadenectomized mice to synthesize casein in organ culture. The results presented in Table 2 show that mammary explants from sialoadenectomized mice, like those from normal pregnant mice, produced casein in response to insulin, cortisol, and prolactin during 3 days of culture. However, the extent of casein production in cultured mammary tissue from sialoadenectomized mice was considerably less than that in tissue from normal mice. In addition, the mammary gland of sialoadenectomized mice was 20%–23% smaller than that of normal mice at this stage of pregnancy. The abdominal glands of the former and the latter were 70–80 mg and ≈100 mg, respectively.

The above data indicated that pregestational sialoadenectomy of mice decreases milk production and increases offspring mortality during the lactation period. Since mouse submandibular glands synthesize and secrete EGF, which has been implicated in the growth and development of the mammary gland (9, 10), it was of interest to examine whether administration of EGF to pregnant mice could abbreviate

Table 1. Comparison of normal and sialoadenectomized lactating mice in terms of body weight, mammary gland weight, milk production, and protein concentration in milk

Mother	Days of lactation	No. of mice	Body weight, g	Mammary gland		Milk production, ml	Protein concentration in milk, mg/ml
				Weight, g	Weight/body weight, %		
Normal	5	7	30.5 ± 0.94	3.35 ± 0.21	11.1 ± 0.29	0.35 ± 0.04	64.1 ± 2.1
	10	8	33.0 ± 0.41	4.31 ± 0.14	13.1 ± 0.42	0.67 ± 0.06	61.2 ± 2.8
	15	11	34.0 ± 0.53	5.32 ± 0.17	15.7 ± 0.40	0.72 ± 0.06	67.0 ± 3.3
Sialoadenectomized	5	10	26.8 ± 0.73	2.53 ± 0.21	9.3 ± 0.50	0.20 ± 0.02	63.6 ± 3.1
	10	11	29.0 ± 0.39	3.49 ± 0.14	12.0 ± 0.43	0.52 ± 0.03	54.6 ± 2.6
	15	10	30.1 ± 0.90	4.45 ± 0.37	14.7 ± 0.96	0.55 ± 0.05	57.6 ± 4.8

Both normal and sialoadenectomized mothers on days 5, 10, and 15 of the lactation period were examined. The number of pups per mother was a constant 7 pups per mother, accomplished by replacing expired pups with new ones throughout 15 days. Results are expressed as mean ± SEM. Significant differences ($P < 0.01$) were found between normal and sialoadenectomized mice in all parameters except protein concentration in milk (tested by analysis of variance).

the nursing problems associated with sialoadenectomized mothers. As shown in Table 3, EGF administration to sialoadenectomized mice during pregnancy markedly enhanced the survival rate of the pups to a level comparable to those nursed by normal mothers. It was further observed that EGF-treated sialoadenectomized mothers had a mammary gland of normal size and weight and produced an amount of milk comparable to that of control mothers. Thus, it appears that EGF replacement therapy during pregnancy can largely overcome the lactational problems of sialoadenectomized mothers.

DISCUSSION

In recent years, a number of studies have reported that the growth factors such as EGF derived from the submandibular gland exert a profound effect on the growth and differentiation of mammalian cells in culture (9–11). However, the physiological function of the gland with respect to these factors has not been well understood. In the present study, we showed that pregestational sialoadenectomy of virgin mice resulted in decreased growth of the mammary gland and its ability to produce milk and an increase in offspring mortality during lactation. About one-third of the pups born to and nursed by sialoadenectomized mothers died within 5 days of the nursing period. The data obtained from cross-fostering experiments indicated that the nursing sialoadenectomized mothers, but not their pups, were responsible for the acute death of pups. Moreover, experiments involving alterations of the litter size showed that sialoadenectomized mothers have substantially less capacity to nurse pups than normal mothers. This is most likely a result of insufficient supply of milk to the pups. The sialoadenectomized lactating mice were shown to have smaller mammary glands that produced less milk as compared to normal mothers. This was also the case with mice during pregnancy, as shown by organ culture studies. In addition, EGF administration to sialoadenectomized pregnant mice was shown to be effective in enhancing the survival rate of their pups during the nursing period.

The data presented here indicate that the submandibular gland is important for lactogenesis. In view of its known ability to serve as part of the digestive system and to produce various biologically active polypeptides, several points can be raised in assessing our present findings. It can be argued that the removal of the submandibular gland causes a decrease in salivation, leading to insufficient food intake and malnutrition during pregnancy and lactation. However, according to Hall and Schneyer (22), compensatory enlargement of the parotid gland occurs after sialoadenectomy, within 4 weeks after the operation, and compensates for partial desalivation. In this study, mating took place at least 30 days after sialoadenectomy, during which time the compensatory mechanism involving the parotid gland had taken place. In addition, there was no apparent malnutrition of sialoadenectomized mice, as judged by their body weight and the size of newborn pups. Furthermore, experiments involving the removal of the parotid gland, instead of the submandibular gland, indicated that the operation did not affect the nursing capacity of the mothers.

The second point relates to the ability of the submandibular gland to form various biologically active polypeptides such as EGF. Previous studies of EGF in cultured mammary cells showed that it acts as a potent mitogen and also inhibits casein production, whereas nerve growth factor had no effect (9, 10). The stimulatory effect of EGF on mammary cells was also reported by other investigators (13, 14). In addition, it was reported (23) that the circulating level of EGF was increased during pregnancy, whereas its level was low in

Table 2. Effect of insulin, cortisol, and prolactin on casein production in cultured mammary explants from normal and sialoadenectomized mice in pregnancy

Culture condition	Casein production, 10^3 × cpm per mg of tissue		
	24 hr	48 hr	72 hr
Normal mice			
Insulin/cortisol	12.5	10.8	10.0
Insulin/cortisol/ prolactin	32.7	70.0	93.0
Sialoadenectomized mice			
Insulin/cortisol	5.0	3.8	3.6
Insulin/cortisol/ prolactin	15.0	44.5	68.9

Mammary explants from pregnant (day 14) normal or sialoadenectomized mice were cultured for the indicated period, and the accumulation of casein was determined. Each value represents the average of duplicate determinations, which varied <5%.

Table 3. Survival rate of pups nursed with sialoadenectomized mothers that were treated with EGF during pregnancy

Mother	Total no. of pups used	Surviving pups 15 days after birth	
		Body weight, g	Survival rate, %
Normal	80	5.45 ± 0.13	73.8*
Sialoadenectomized	72	5.01 ± 0.22	26.4†
Sialoadenectomized and EGF treatment	56	5.72 ± 0.12	64.3

*$P > 0.05$

†$P < 0.01$ were obtained by χ^2 test, comparing pups to those nursed by sialoadenectomized mothers who were given injections of EGF during pregnancy.

nonpregnant states. These observations led us to propose that EGF is involved in the development of the mammary gland that takes place during pregnancy, by stimulating growth and inhibiting precocious differentiation (9). During pregnancy, the cytostructure of the female submandibular gland has been shown to undergo masculinization (24). This is probably because the circulating level of androgens increases during pregnancy (25). In view of the potent effect of androgen in stimulating the production of EGF in the submandibular gland (11), it is possible that the increase in the circulating level of EGF during pregnancy results from the increase in circulating androgens derived from the ovary or adrenal gland. Based on the proposed role of EGF in the development of the mammary gland (9, 10), our present findings can be accounted for as follows: pregestational sialoadenectomy decreases the level of circulating EGF during pregnancy and causes less growth of the mammary gland, which produces a limited amount of milk that is not enough for sustaining a normal sized litter. Our findings that EGF administration to sialoadenectomized mice during pregnancy was able to decrease the mortality of pups nursed by their mothers provides additional support for this view. This view is also in line with our preliminary data, which indicate that the removal of the submandibular gland results in a marked decrease in the circulating level of EGF during pregnancy and lactation: the level of EGF in these instances was too low to be detected by the radioimmunoassay, which has the sensitivity of detecting as little as 0.2–0.5 ng of EGF (unpublished observations).

Another alternative possibility to explain our present data is to postulate that the increase in offspring mortality is due to some critical substance in milk, which is lacking in sialoadenectomized mothers during lactation. Although electrophoretic analysis of milk proteins produced by normal and sialoadenectomized mothers did not reveal any apparent differences, it still remains possible that a minute amount of some substance that is not detectable by the present analysis is critical for the growth of pups during pregnancy. Such a substance can be made by the submandibular gland and eventually appears in milk, or can be made by the mammary gland by itself. At present, no evidence is available to support the presence of such a substance.

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