A Physiological Role of Epidermal Growth Factor in Male Reproductive Function

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Epidermal growth factor (EGF) stimulates the proliferation of various mammalian cells in culture, but its physiological role is not well defined. In mature male mice, large amounts of EGF are produced in the submandibular gland; it is present in the circulation at approximately 5 nanograms of EGF per milliliter of plasma. Sialoadenectomy (removal of the submandibular glands) decreased the amount of circulating EGF to an undetectable level but did not affect the circulating levels of testosterone or follicle-stimulating hormone. The number of mature sperm in the epididymis decreased by as much as 55 percent; the number of spermatids in the testis decreased by 40 to 50 percent; and the number of spermatocytes increased by about 20 percent. Administration of EGF to sialoadenectomized mice restored both the sperm content of the epididymis and the number of spermatids in the testis to normal. Thus, EGF may play a role in male reproductive function by stimulating the meiotic phase of spermatogenesis.

THE MOUSE SUBMANDIBULAR GLAND is a rich source of epidermal growth factor (EGF), a polypeptide with a molecular weight of 6045 (*1, 2*); EGF is also present in the circulation (*3, 4*). Hormones such as androgens, progestins, and adrenergic agents can increase the production of EGF in the submandibular gland and its level in the circulation (*3, 5*). Nevertheless, the physiological role of EGF is not clear, even though EGF enhances the proliferation of various mammalian cells in culture and acts as a positive and negative modulator of differentiated functions in certain cultured cells (*6–9*).

Submandibular gland–derived EGF plays a physiological role in the development of the mammary gland and mammary tumorigenesis in female mice (*10, 11*). There is at least ten times as much EGF in the submandibular glands of male mice than in those of female mice (*12*). In male mice the production of EGF in the submandibular gland increases until 7 weeks of age, in parallel with sexual maturation (*13*), and androgens stimulate its production in the gland (*3*). To assess the physiological role of EGF in male mice, we examined the effects of sialoadenectomy and EGF replacement on several parameters of male reproductive functions, including sperm production. Our results indicate a physiological function of the EGF from the submandibular gland in spermatogenesis.

After sialoadenectomy, plasma EGF decreased rapidly and was undetectable by 3 weeks, indicating that the submandibular gland is a major source of circulating EGF (Table 1) (*14*). Sialoadenectomy of male mice also caused a marked decrease (to 45 percent of control) in the sperm content of the epididymis, which stores mature sperm until ejaculation (Table 1). The weight of the epididymis in sialoadenectomized mice was also somewhat less than that in normal mice (Table 1). The daily administration of EGF to mice for 4 weeks after sialoadenectomy reduced the decrease in sperm number; the effect was dose-dependent (Fig. 1). A daily dose of 5 μg of EGF per mouse effectively prevented the decrease in sperm content; lower doses had smaller effects. Treatment of normal mature males with EGF for 4 weeks with the same dose range did not significantly change the sperm content. Administration of nerve growth factor (NGF) (1 μg/day), another polypeptide produced in the submandibular gland, did not increase sperm content in sialoadenectomized mice; thus the effect of EGF was specific.

During the first 2 weeks after sialoadenectomy, the sperm content in the epididymis was normal, but by 3 weeks after surgery it had decreased by 30 percent (Fig. 2). At 4 weeks after surgery the sperm content declined to about 45 percent of control values and remained essentially unchanged thereafter. As described above, daily EGF replacement begun immediately after the operation prevented the decrease of sperm content [Fig. 2, E(0)]. When EGF treatment was started 4 weeks after sialoadenectomy [Fig. 2, E(4)], at which time the sperm content had declined to its lowest level, the sperm content returned to the normal level within 3 weeks.

Sperm in the epididymis originate in the testis where spermatogenesis occurs in three principal phases (*15*): (i) mitotic division of spermatogonia to form primary spermatocytes, (ii) meiosis of primary spermatocytes to form round spermatids, and (iii) transformation of round spermatids into sperm, a process called spermiogenesis. To determine the defective step in spermatogenesis responsible for the decrease in epididymal sperm content in sialoadenectomized mice, the numbers of spermatogenic cells in the testes of normal, sialoadenectomized, and EGF-treated sialoadenectomized mice were compared (Table 2). The numbers of round and condensing spermatids were significant-

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Table 1. Effect of sialoadenectomy on measures of reproductive function in mature male mice. Six 14-week-old male C3H mice were sialoadenectomized and killed 4 weeks later, with age- and weight-matched male mice that had received sham operations. The body weights of sialoadenectomized and sham-operated mice were 28.5 ± 0.9 (SD) and 30.1 ± 2.2 g, respectively. The amounts of EGF in the submandibular gland and plasma were determined by radioimmunoassay as described (*12*). The sensitivity of the assay was 0.01 ng of EGF per assay. The radioimmunoassay of EGF in both plasma and submandibular gland samples was validated by Western blot analysis of immunoreactive materials. In addition, EGF radioreceptor assay with mouse liver cells gave similar values. Finally, we could accurately detect known amounts of EGF added to serum samples. Plasma testosterone was assayed by the method of Collins *et al.* (*19*). Plasma FSH was measured by radioimmunoassay (*20*). The sperm content in epididymis was determined according to the method of Taylor *et al.* (*21*). DNA content of the testis was determined by the diphenylamine reaction (*22*). Results are expressed as mean ± SD.

Treatment	Submandibular gland	Plasma			Epididymis		Testis	
	EGF (μg/mg)	EGF (ng/ml)	Testosterone (ng/ml)	FSH (ng/ml)	Weight (mg)	Sperm content (10^6/epididymis)	Weight (mg)	DNA content (μg/mg)
Control	1.79 ± 0.28	5.11 ± 3.47	0.41 ± 0.11	286 ± 33	75.5 ± 4.2	8.8 ± 2.2	170 ± 12	2.68 ± 0.13
Sialoadenectomized	0	Not detectable	0.45 ± 0.09	253 ± 66	69.6 ± 4.3*	4.0 ± 0.8†	167 ± 12	2.24 ± 0.13†

*$P < 0.05$, significant difference between control and sialoadenectomized mice by Student's t test. †$P < 0.01$, significant difference between control and sialoadenectomized mice by Student's t test.

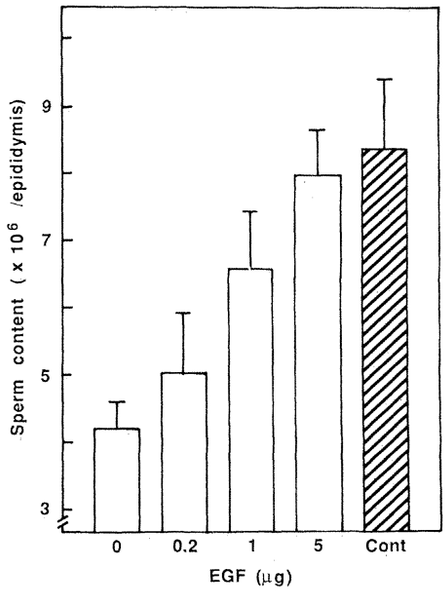

Fig. 1. Dose-response relation between EGF replacement and sperm content in the epididymis of sialoadenectomized mice. Daily EGF injections were given subcutaneously at doses of 0.2, 1, or 5 μg per mouse starting on the day of sialoadenectomy. Mice were killed 4 weeks after the operation, and epididymal sperm contents were determined. The results are expressed as mean ± SD ($n = 3$). Significant differences were found between the untreated sialoadenectomized mouse group and the EGF-treated sialoadenectomized mice or untreated normal controls (Cont) by analysis of variance ($P < 0.01$). The slope of the line calculated by linear regression of sperm content versus log dose of EGF was 1.95 ± 0.414 (SEM) ($P < 0.01$), indicating that higher doses of EGF produce greater sperm content.

ly lower in sialoadenectomized mice than in untreated mice. The reduced number of spermatids could account for the 15 percent decrease of DNA content in the testis of sialoadenectomized mice (Table 1). However, the number of primary spermatocytes in sialoadenectomized mice was significantly increased compared to that in normal mice. In EGF-treated sialoadenectomized mice, the numbers of primary spermatocytes and round and condensing spermatids were about the same as in normal mice.

Our studies show that after sialoadenectomy plasma EGF was undetectable and there was a decrease in the sperm content of the epididymis, as well as in the number of round and condensing spermatids in the testis. These alterations in sperm production were reversed completely by EGF replacement. It took 3 to 4 weeks for the sperm content to decline maximally after sialoadenectomy and 3 to 4 weeks for complete correction of the defect by EGF administration (Fig. 2). The duration of spermatogenesis is approximately 34.5 days: the mitotic phase takes about 8 days, the meiotic phase approximately 13 days, and spermiogenesis about 13.5 days (*15*). On the basis of the duration of each phase in spermatogenesis and the data in Fig. 2, the critical phase in which EGF is involved may be meiosis. This is consistent with our results showing that sialoadenectomy decreased the number of spermatids produced by meiotic division and increased the number of spermatocytes in the testis, and that these imbalances were restored by EGF replacement (Table 2). These results are also consistent with the view that EGF plays a role in male reproductive function by stimulating the meiotic phase of spermatogenesis.

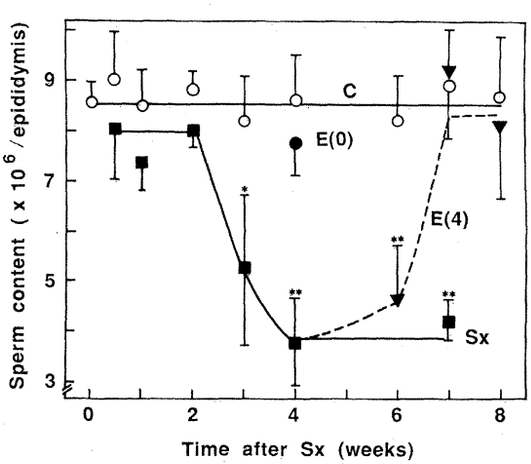

Fig. 2. Effect of sialoadenectomy (Sx) and EGF replacement (E) on sperm content in the epididymis of mature male mice. Sialoadenectomy was performed on 30 mice and epididymal sperm content examined at the indicated times after the operation (Sx, ■). EGF injection, 5 μg/mouse daily, was started on the day of sialoadenectomy [E(0), ●] or 4 weeks later [E(4), ▼]. The sperm contents in three normal mice were examined at the same time (C, ○). Each point represents the mean ± SD ($n = 3$). Student's t test indicated significant differences (*, $P < 0.05$; **, $P < 0.01$) between control mice and sialoadenectomized mice or EGF-treated sialoadenectomized mice.

Spermatogenesis is under the control of various hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and testosterone (*16*). FSH stimulates the proliferation of spermatogenic cells. LH stimulates interstitial cells (Leydig cells) to synthesize testosterone. PRL appears to increase the sensitivity of the testis to LH. Testosterone is important in the meiotic phase and spermiogenesis. Our results indicate that EGF may also regulate spermatogenesis. Since testosterone is known to stimulate EGF production in the submandibular gland, the stimulatory effect of testosterone on spermatogenesis may be, in part, mediated by EGF derived from the submandibular gland. This suggests the presence of a submandibular gland–testis axis in the control of spermatogenesis. However, testosterone or FSH may stimulate spermatogenesis independently of EGF, because about 50 percent of the sperm production was still maintained in sialoadenectomized mice.

The mechanism of action of EGF on spermatogenesis is not known. Since EGF is a potent mitogen in various cells, it may stimulate meiosis of the spermatocyte directly. EGF may also indirectly stimulate spermatogenesis by acting on the Sertoli cells in the testis or on other endocrine tissues. The possibility that EGF stimulates spermatogenesis by enhancing the production of such hormones as FSH and testosterone is unlikely because the circulating levels of FSH and testosterone were unaffected by sialoadenectomy.

The quantity and quality of sperm ejaculated is an important factor in fertility. Thus, sperm count and motility test are used to assess male infertility (*17*). Recently, human seminal fluid has been shown to contain EGF-like immunoreactivity (*18*). Our results raise the question of whether EGF deficiency may be a cause for some cases of male infertility, particularly in unexplained oligospermia.

Table 2. Effect of sialoadenectomy and EGF replacement on the number of primary spermatocytes, round spermatids, and condensing spermatids. Eight mature male mice were sialoadenectomized; four were treated with daily doses of 5 μg of EGF for 4 weeks, whereas the other four received no EGF treatment. Normal males ($n = 4$) were killed at the same time as controls. The testes of each mouse were enzymatically dispersed as described (*23*). The numbers of primary spermatocytes, round spermatids, and condensing spermatids were counted according to their morphological character under a microscope with seminiferous tubule suspensions. Results are expressed as mean ± SD.

Treatment	Primary spermatocytes ($\times 10^6$/testis)	Round spermatid ($\times 10^6$/testis)	Condensing spermatids ($\times 10^6$/testis)
Control	0.88 ± 0.08	4.60 ± 1.22	4.73 ± 0.98
Sialoadenectomized	1.12 ± 0.15*	2.51 ± 0.61*	2.66 ± 0.77*
EGF-treated sialoadenectomized	0.82 ± 0.11	4.60 ± 0.36	5.01 ± 0.88

*$P < 0.05$, significant difference between control and sialoadenectomized mice by Student's t test.

REFERENCES AND NOTES

1. S. Cohen and C. R. Savage, Jr., *Recent Prog. Horm. Res.* **30**, 551 (1974).
2. E. W. Gresik and T. Barka, *J. Histochem. Cytochem.* **25**, 1027 (1977).
3. R. L. Byyny, D. N. Orth, S. Cohen, E. S. Doyne, *Endocrinology* **95**, 776 (1974).
4. R. L. Ladda, L. P. Bullock, T. Gianopoulos, L. McCormick, *Anal. Biochem.* **93**, 286 (1979).
5. L. Bullock, P. L. Barthe, I. Mowszowicz, D. N. Orth, C. W. Bardin, *Endocrinology* **97**, 189 (1975).
6. T. Benvenister *et al.*, *J. Clin. Endocrinol. Metab.* **46**, 169 (1978).
7. I. K. Johnson, J. D. Baxter, I. Vlodavsky, D. Gospodarowicz, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 394 (1980).
8. J. C. Rheinwald and H. Green, *Nature (London)* **265**, 421 (1977).
9. A. J. W. Hsueh, T. H. Welch, T. B. C. Hones, *Endocrinology* **108**, 2002 (1981).
10. S. Okamoto and T. Oka, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6059 (1984).
11. H. Kurachi *et al.*, *ibid.* **82**, 5940 (1985).
12. H. Kurachi and T. Oka, *J. Endocrinol.* **106**, 197 (1985).
13. R. L. Byyny, D. N. Orth, S. Cohen, *Endocrinology* **90**, 1260 (1972).
14. Recent studies also suggest that the submandibular gland is a major source of circulating EGF (*12*). However, our results are apparently not in accord with previous reports in which it was stated that removal of the salivary gland does not alter plasma levels of EGF [(*3*); T. Barka, *J. Histochem. Cytochem.* **28**, 836 (1980)]. This discrepancy may be due to differences in experimental conditions, although details were not available from the earlier studies. In addition, our studies raise the question as to how EGF from the submandibular gland finds its way into the plasma. There are indications that EGF is excreted in an exocrine fashion from the gland (R. A. Murphy, A. Y. Watson, J. Metz, W. G. Forssmann, *ibid.*, p. 890). EGF may be resorbed from the digestive tract into the circulatory system.
15. E. C. Roosen-Runge, *Biol. Rev. Camb. Philos. Soc.* **37**, 343 (1962).
16. W. G. Beamer, M. C. Wilson, E. H. Leiter, in *The Mouse in Biomedical Research*, H. L. Foster, J. D. Small, J. G. Fox, Eds. (Academic Press, New York, 1983), vol. 3, p. 165.
17. H. Mohri and R. Yanagimachi, *Exp. Cell Res.* **127**, 191 (1980).
18. S. D. Elson, C. A. Browne, G. D. Thorburn, *J. Clin. Endocrinol. Metab.* **58**, 589 (1984).
19. W. P. Collins, M. D. Mansfield, N. S. Alladina, I. F. Sommerville, *J. Steroid Biochem.* **3**, 333 (1972).
20. The FSH assay was performed according to the protocol and with the reagents supplied by the National Hormone Pituitary Program, NIADDK, National Institutes of Health.
21. G. T. Taylor, J. Weiss, T. Frechmann, J. Haller, *J. Reprod. Fertil.* **73**, 323 (1985).
22. K. W. Giles and A. Mayer, *Nature (London)* **93**, 4979 (1965).
23. L. J. Romrell, A. R. Bellve, D. W. Fawcett, *Dev. Biol.* **49**, 119 (1976).

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A Plant Flavone, Luteolin, Induces Expression of *Rhizobium meliloti* Nodulation Genes

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The symbiotic interaction of *Rhizobium meliloti* and alfalfa results in the formation of nitrogen-fixing root nodules. *Rhizobium meliloti nodABC* genes are required for the early host responses of cortical cell divisions and root hair curling. The induction of *nodABC* expression by alfalfa exudates demonstrates host-symbiont signaling at an early stage in nodule development. The inducer molecule for *nodABC* expression was isolated from plant exudate by constructing a *nodABC-lacZ* fusion to monitor the inducing activity. From ultraviolet-visible absorption spectra, proton nuclear magnetic resonance, and mass spectrometry, the inducer was determined to be 3′,4′,5,7-tetrahydroxyflavone (luteolin). Luteolin is a normal secondary plant metabolite found throughout the plant kingdom that may serve to control *nodABC* expression during nodule development. This regulatory role for a flavone contrasts with the function of some flavonoids as defense compounds.

MANY SOIL MICROORGANISMS establish nutritionally beneficial symbiotic or parasitic relationships with plants. Bacteria of the genus *Rhizobium* fix nitrogen in root nodules on host plants of the family Leguminosae. This symbiosis develops through a complex and ordered sequence of events, including cell division and differentiation of both partners, which suggests the need for signaling between host and symbiont. A few bacterial and plant genes specifically required for this symbiosis have been identified, including those coding for bacterial nitrogenase and for plant leghemoglobin (*1*). The functions of other genes remain unknown, but can be assigned to specific stages of nodule development (*2*). For example, the *Rhizobium* common nodulation genes (*nodDABC*) are responsible for stimulating the earliest detectable host responses of cortical cell division (*3*) and root hair curling (*4, 5*). These genes are induced by compounds in exudates of plant hosts (*6–8*).

Another example of a bacterial response to plant compounds is the induction of the virulence (*vir*) genes of the opportunistic pathogen *Agrobacterium tumefaciens*. These genes are induced by acetosyringone (*9*) and other phenolic compounds (*10*) whose presence in plant exudates is increased when the plant has been wounded (*9*). In this report, we demonstrate that *R. meliloti nodABC* genes are induced by luteolin, a flavone that is present in exudates of alfalfa seeds.

Our initial observation that expression of *nodC* in *R. meliloti* can be induced by an exudate from alfalfa roots or seeds (*6*) prompted us to isolate and characterize the molecule or molecules responsible for this activity. The inducer was monitored during purification by assaying induction of a *nodABC-lacZ* translational fusion borne on plasmid pRmM57 (*6*). Preliminary tests of alfalfa seed exudate suggested that the inducer molecule was a small aromatic compound. Activity of the plant exudate was not affected by treatment with heat, protease, or nucleases. However, activity was removed from exudates by treatment with activated charcoal or by dialysis through membranes with a molecular weight exclusion limit of 2000 daltons (*11*). Because exposure of exudates to bacteria caused loss of their activity, it was necessary to keep exudates sterile; seeds were routinely used as a source to facilitate aseptic handling of plant material.

The exudate from alfalfa seeds was fractionated by high-pressure liquid chromatography (HPLC) using a reverse-phase C_{18} column. Fractions were assayed for *nodABC-lacZ* inducing activity (Fig. 1A). Activity eluted as a broad peak between 95 and 100% methanol, indicating that the inducing molecule had significant hydrophobic character. The inducer molecule could be partially purified by extracting the inducing factor into diethyl ether. These ether-extracted compounds were separated by HPLC, and fractions containing the inducing activity again eluted between 95 and 100% methanol, along with several ultraviolet (UV)-absorbing compounds (Fig. 1B). To resolve the inducer molecule from these coeluting compounds, we fractionated the ether-extracted materials on a methanol-water gradient of 50 to 100% (Fig. 1C). Under these conditions, the various components were resolved and the majority of inducing activity correlated with a single absorbance peak eluting at 90 to 95% methanol. Other UV-absorbing compounds with little inducing activity eluted between 70 and 75% methanol. Fractions containing the most activity (90 to 95% methanol) were pooled for structural analysis (Fig. 1C,*).

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