Overexpression of Epidermal Growth Factor Induced Hypospermatogenesis in Transgenic Mice

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The in vivo role of epidermal growth factor (EGF) is not well defined even though its effects on culture cells were well studied. To understand the developmental, physiological, and pathological roles of EGF, we have generated transgenic mice widely expressing human EGF with the use of the β-actin promoter. EGF and transforming growth factor α (TGFα) bind with equal affinity to the EGF receptor, a transmembrane tyrosine kinase, to trigger various biological responses. EGF and TGFα signaling are implicated in the development of the reproductive system. EGF also plays a physiological role in reproduction. Removal of the salivary gland in rodents, which reduces circulating EGF, reduces spermatogenesis, which can be corrected by EGF replacement. Here we show that in our transgenic males, only few post-metosis II gametes were found, and the mice were sterile. This resembles a common cause of infertility in humans. Furthermore, the transgenic males had reduced serum testosterone. Our findings contrast the previous report on transgenic mice overexpressing a reduced form of human EGF precursor (hEGF) was biologically active in transforming NIH 3T3 cells (13). Because little is known about the in vivo role of EGF, we generated transgenic mice widely expressing hEGF in the present study. The β-actin promoter was used because it had been shown to drive widespread transgene expression (14). The eight EGF-like repeats in the EGF precursor were deleted to give hEGF, leaving the active EGF domain in the transmembrane form. Transgenic males expressing hEGF were sterile with hypospermatogenesis. This is in contrast to transgenic mice overexpressing TGFα in the testis, which were reported to have normal testis morphology and spermatogenesis (7). Together with the expression studies described above, we suggest that EGF instead of TGFα is likely to be the physiological ligand for EGF receptor in spermatogenesis.

Epidermal growth factor (EGF) was identified by Cohen (1) in the 1960s. Surprisingly, injection of crude salivary gland extract into newborn mice induced precocious eyelid opening and incisor eruption. Mature EGF purified from mouse salivary gland is composed of 53 amino acids but is derived from a much larger transmembrane precursor of 1217 amino acids (2, 3). The physiological role of EGF in reproduction is suggested by various studies on the effects of sialoadenectomy (removal of salivary gland), which depleted circulating EGF. In male mice, the production of EGF in submandibular gland increases until sexual maturation (4). Studies showed that after sialoadenectomy, serum testosterone either remained unchanged in adults (5) or increased in peripubertal mice (6). Nevertheless, both studies reported reduced sperm count, which returned to normal with EGF replacement. So, EGF deficiency was linked to some unexplained cases of male infertility, in particular oligospermia.

Transforming growth factor α (TGFα) is biologically and structurally related to EGF. From the expression patterns of EGF, TGFα, and their common receptor, TGFα was suggested to act during early pubertal stages to support the active somatic cell growth in testis. However, the role of TGFα in spermatogenesis is not evident (7, 8). EGF is involved in differentiation of the male reproductive system through modulation of androgen receptor activity (9). Various in vitro studies also showed that EGF affects the functions of Sertoli cells (10) and Leydig cells (11). Furthermore, expression of EGF protein in specific cell types of the testis suggests that it may act in a paracrine/autocrine fashion in spermatogenesis. Mature EGF was found in Sertoli cells, pachytene spermatocytes, and round spermatids in mice. In contrast, EGF precursor immunostaining was limited to pachytene spermatocytes and round spermatids (12). The transcript (7) and protein (8) for EGF receptor was identified specifically in all the above testicular cell types.

We have recently reported that both full-length and a shortened form of human EGF precursor (hEGF) was biologically active in transforming NIH 3T3 cells (13). Because little is known about the in vivo role of EGF, we generated transgenic mice widely expressing hEGF in the present study. The β-actin promoter was used because it had been shown to drive widespread transgene expression (14). The eight EGF-like repeats in the EGF precursor were deleted to give hEGF, leaving the active EGF domain in the transmembrane form. Transgenic males expressing hEGF were sterile with hypospermatogenesis. This is in contrast to transgenic mice overexpressing TGFα in the testis, which were reported to have normal testis morphology and spermatogenesis (7). Together with the expression studies described above, we suggest that EGF instead of TGFα is likely to be the physiological ligand for EGF receptor in spermatogenesis.

EXPERIMENTAL PROCEDURES

Animals—Mice were held in the Laboratory Animal Unit of the University of Hong Kong. F1 hybrid mice were routinely raised from breeding pairs of C57BL/6N female × CBA/N male. All mice were maintained on a 12-h light and 12-h dark cycle. Autoclaved food (Purina) and UV-irradiated water were available ad libitum. Principles of laboratory animal care and specific national laws relating to the welfare of animals were followed. Research protocols were approved by an institutional ethics committee.

Construction of β-Actin Promoter hEGF Plasmid—The structure of the β-actin promoter hEGF plasmid was shown in Fig. 1A. The hEGF cDNA was regulated by the β-actin promoter to give widespread expression in transgenic animals. Construction of the plasmid for hEGF has been described (13). Briefly, the region encoding the 8 EGF-like repeats was deleted by digestion with EcoRI (position 547, GenBank TM accession number X04571) and
mandibular gland; Kid, kidney; Liv, liver; Tes, testis.

BSu361 (3313). The reading frame is conserved after reigation of the end filled sites as checked by sequencing. The resulting cDNA (1.06 kilobases) was ligated with the 4.3-kilobase β-actin promoter and the 0.3-kilobase SV40 poly(A) in pBluescript KS + (Stratagene). For microinjection, insert DNA was released by digestion with XbaI and SalI, purified using the QIAEX gel extraction kit (Qiagen, Chatsworth, CA), and passed through a spin-X column (Costar).

**Generation of Transgenic Mice—**DNA was introduced at a concentration of 5 ng/μl into F1 and FVB/N embryos by standard microinjection procedures (15, 16). At weaning of the resulting pups, DNA from tail biopsies were screened by polymerase chain reaction with primers 5′-AGTGACCTGAAATGTCCCC and 5′-GTTGACATGCCCCCATCTGCTG, which were specific for human EGF. The temperature profile was denaturation at 94 °C for 3 min, followed by 29 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

**Tissue Preparation and Western Blot—**Mice were sacrificed by cervical dislocation. Blood was collected by cardiac puncture immediately after the animal was killed by cervical dislocation. Blood samples were kept on ice for 15 min, followed by centrifugation at ~6,000 × g. The supernatant was aliquoted and stored at −20 °C until assay. Serum samples were measured using a Coat-A-Count total testosterone kit from Diagnostic Products Corp. Controls were age-matched FVB/N and F1 males. Statistical differences between transgenic and control groups were analyzed using the Mann-Whitney Test.

**RESULTS**

**Construction of DNA Microinjection Fragment—**Initially, we tried using the phosphoglucerase kinase-1 promoter to drive both hEGF expression and the reporter gene βgeo with the use of internal ribosomal entry site. The construct has been described before (13). However, this construct was not giving high level of transgene expression in transgenic mice. Under this condition we changed the strategy and constructed the β-actin promoter injection plasmid (Fig. 1B).

**Generation of Transgenic Mice Expressing hEGF—**Five transgenic mice, named T1–T5, were generated independently. T2, T3, and T4 were FVB males from the same litter. T1 and T5 were F1 females from separate litters. Immunoblot showed that hEGF, with the expected size of 40 kDa, was highly expressed in the membrane fraction of all tissues tested, including submandibular gland, kidney, liver, and testis (Fig. 1C). This demonstrates that hEGF protein was widely and highly expressed under the β-actin promoter.

**Retarded Growth of hEGF Transgenic Mice—**All five transgenic animals were found to be smaller than nontransgenic littermate(s), and the reduction in size appeared proportionate. A significant difference in body weight was already apparent in both sexes since birth (Fig. 2A). At adulthood they attained 70% of the weight of nontransgenic littermates (Fig. 2B).

**Immunohistochemical Staining of Submandibular Gland and Testis of Transgenic Mice—**Immunostaining of hEGF in the submandibular gland and gonad was performed for all transgenic animals. They all showed similar immunostaining. In submandibular gland, the whole organ expressed hEGF (brown), with more prominent staining in granular tubule cells (Fig. 3A). In the testis of T2–T4, immunostaining of testis revealed brown staining in all cell types including germ cells, interstitial cells and Sertoli cells. No staining was seen in nontransgenic littermates (Fig. 3B).
Two out of three transgenic males (T3 and T4) never sired offspring till sacrifice at 9 months. T2, which was sacrificed at 2 months old, had 76 offspring from 10 litters but none of them were transgenic. This observation suggests that he was mosaic and that little or no functional sperm was derived from transgenic spermatogonia. For the sterile males, their testis histology revealed similar features (Fig. 4). The tubules were of smaller diameter, but the tubular lumen was enlarged, which is typical of hypospermatogenesis. The thickness of germ cell layers was uneven; some region had just a layer of spermatogonia. Normally, spermatids at particular stage(s) will be present in all tubular sections, but this is not the case in T3 and T4. For them, very few tubular regions contained spermatids and spermatozoa. These postmeiotic II germ cells, if present, were much less than normal. Only a small number of spermatocytes went past the pachytene stage. No sperm was retrieved from epididymis of T3 and T4 in an attempt to collect capacitated sperms for in vitro fertilization and cryopreservation.

Infertility May Be Related to Low Serum Testosterone Level in Male Transgenic Mice—The mean serum testosterone for transgenic mice (n = 3) was significantly lower than for age-matched controls (n = 6) (16.623 ± 1.815 versus 81.715 ± 17.723 nmol/liter, p = 0.01). However, the sizes of seminal vesicles and prostate glands were normal. T3 and T4 seemed to lack mating behavior. Vaginal plugs were not found in females caged with T3 or T4.

DISCUSSION

We have generated hEGF transgenic mice that appeared to be proportionate dwarfs. They were born with only half of the weight of normal littermates. These findings coincide with previous reports that injection of EGF induced growth retardation in newborn rats (19) and inhibited adipose tissue development (8). Calamandrei and Alleva (20) reported that EGF treatment retards both the rate of body growth and the full appearance of several neurobehavioral signs of maturation. Webber et al. (21) reported that transgenic mice overexpressing TGF-α weighed approximately 10% less than the control. In a recent report, Dealy et al. (22) examined the distribution of TGF-α, EGF, and the chicken EGF receptor (encoded by c-erbB) in embryonic chick limbs. They found that exogenous TGF-α and EGF inhibited chondrogenesis and myogenesis of limb mesenchyme in vitro. They concluded that signaling through the EGF receptor via endogenous TGF-α and EGF may be important for initial limb formation, outgrowth of limb mesoderm, and regulation of limb chondrogenic and myogenic differentiation. More recently, Erwin et al. (23) reported the effect of intestinal overexpression of EGF. A murine EGF precursor cDNA construct was produced, and expression of the transgene was targeted to the small intestine with the use of rat intestinal fatty acid-binding protein promoter. Interestingly, their transgenic animals had improved post-resection adaptation. Besides shortened small intestine, no other abnormal phenotype was observed.

The unexpected finding of the present report is male infertility with EGF overexpression. The physiological role of EGF in reproduction is suggested by various studies on the effects of depleting circulating EGF by sialoadenectomy. Studies showed that after sialoadenectomy, serum testosterone either remained unchanged in adult (5) or increased in peripubertal
mice (6). Both studies reported reduced spermatid count that returned to normal with EGF treatment. In Ref. 5, there was accumulation of pachytene spermatocytes, whereas in Ref. 6, a decrease in preleptotene and pachytene spermatocytes was noted. This suggests a relationship between EGF and spermatogenesis, especially at the stage of meiosis II. That EGF deficiency is linked to oligospermia is further shown in streptozotocin-induced diabetic mice. Both the reduction in sperm count and EGF level could be restored by insulin. These effects were abolished by the concomitant administration of EGF antiserum (24). In this report, we showed that EGF overexpression in testis, on the other hand, again led to reduced spermatogenesis, especially in the production of spermatids and spermatozoa. We therefore suggest that proper dose of EGF is important for spermatogenesis, in particular for progress from MI to MII of meiosis. It is interesting to note that maturation arrest of germ cells causes 10–30% of male infertility in humans (25). The most common is arrest at spermatocyte I, also called meiotic I arrest. Similar to the case in our transgenic males, it is characterized by absence or less than 10% of tubules containing cells beyond the pachytene stage (26).

EGF, TGFα, and amphiregulin are known to bind and activate only the EGF receptor (ErbB1) (27). That EGF is the endogenous ligand for EGF receptor in spermatogenesis is supported by expression of EGF but not TGFα protein in specific germ cell types of the tests. In adult mice, EGF precursor immunostaining was limited to pachytene spermatocytes and round spermatids, whereas mature EGF was found in addition in Sertoli cells (12). The source of mature EGF in these cells remained to be determined. EGF, but not TGFα, was also found in the germ cells in boar. In addition, EGF receptor immunostaining varied according to the course of spermatogenesis, with predominant EGF receptor staining in pachytene spermatocytes before and during meiosis and in post-meiotic germ cells (8). Bartlett et al. (28) have also shown increased testicular EGF concentrations in synchronized rat testes that were closed to the meiotic stages.

This paper provides the first in vivo evidence that EGF overexpression can adversely affect spermatogenesis. This contrasts the previous report on overexpression of TGFα in a line of transgenic mice, which highly expressed human TGFα protein in testis and seminal vesicle. These mice did not have abnormal testis morphology or spermatogenesis (7), suggesting that the two growth factors may have different roles in spermatogenesis. Interestingly, both reduction of circulating EGF by sialoadenectomy and EGF overexpression result in hypospermatogenesis. The expression patterns of EGF, TGFα, and their common receptor in testis together with the physiological studies mentioned above and the overexpression studies in transgenic mice all agreed to the suggestion that the EGF-EGF receptor system is involved in the meiotic process. Furthermore, the EGF receptor is shown to be functional in spermatogonia and spermatozoa (29, 30). Although EGF seems to be the physiological ligand in germ cell development, mice with either single or triple null mutations in EGF, TGFα, and amphiregulin did not suffer from reduced fertility (31). This raises the possibility of functional redundancy with heparin-binding EGF, betacellulin, and epiregulin, which bind ErbB4 as well as EGF receptor (reviewed in Ref. 32).

Besides a primary defect in the germ cells, overexpression of EGF in the somatic lineages of the testis may have a secondary effect on spermatogenesis and fertility in our transgenic animals. A large volume of in vitro data have shown the effects of EGF on the proliferation and function of Sertoli and Leydig...
cells. We suggest that reduced serum testosterone in the transgenic mice was due to either direct effect of EGF on testoster-
one production by Leydig cells (33, 34) or indirect effect of EGF on gonadotrophin production. Nevertheless, we propose that lower testosterone was unlikely to be their underlying cause of infertil-
ity, because the serum testosterone level in all three transgenic males were similar and T2 was fertile. The male accessory glands, which are sensitive to serum testosterone levels (35, 36), still developed to appropriate sizes in our trans-
genic males. As a next step in delineating the functions of EGF in spermatogenesis, overexpression in specific cell types, for example in pachytene spermatocytes, will help to establish the autocrine/paracrine role of EGF in spermatogenesis.

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