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Authors: Fukazawa, Yugo, and Iguchi, Taisen

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Effects of Steroid Hormones and Growth Factors on the Development of the Male Mouse Reproductive Tract *In Vitro*

Yugo Fukazawa†, and Taisen Iguchi*

Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236, Japan

ABSTRACT—In order to determine what growth-promoting factors may be involved in androgen-induced male reproductive tract development, the newborn mouse urogenital sinus region with seminal vesicles attached was cultured for 5 days on collagen gel matrix in a serum-free medium composed of DMEM and Ham's F-12 (1:1) supplemented with BSA, insulin, cholera toxin and transferrin. Testosterone and 5 α-dihydrotestosterone (DHT) stimulated development of seminal vesicle (SV), coagulating gland (CG), prostate (P) and bulbourethral gland (BG). Epidermal growth factor (EGF) stimulated development of CG, P and BG, but inhibited that of SV. Transforming growth factor-α (TGF-α) inhibited SV development, but had stimulatory effects on both CG and P. Addition of anti-EGF antibody significantly inhibited the DHT-induced development of CG and BG, but not of SV and P. These findings suggest that both EGF and TGF-α have organ-specific regulatory actions on male reproductive tract development and that EGF may mediate the action of DHT in the development of CG and BG.

INTRODUCTION

Most of male mouse reproductive organs develop from both urogenital sinus and Wolffian ducts from 12 days of pregnancy through 5 days after birth in mice (Robaire and Hermo, 1988). Epididimys, ductus deferens and seminal vesicle originate from the Wolffian duct, coagulating gland, prostate, urethral gland and preputial gland are derived from the urogenital sinus, and bulbourethral gland is an ectodermal derivative (Jost, 1953; Cunha, 1976; Robaire and Hermo, 1988). Development of all these organs is regulated by androgens from the developing testis (Jost, 1947; Cunha, 1976; Cunha *et al*., 1981).

Stroma induces epithelial differentiation during organogenesis of reproductive tracts (Cunha, 1976; Cunha *et al*., 1996). In male mice, urogenital sinus mesenchyme induces prostate ductal morphogenesis, regulates epithelial proliferation, and specifies expression of prostate secretory proteins (Cunha *et al*., 1987, 1992). Similarly, seminal vesicle mesenchyme induces seminal vesicle epithelial proliferation and differentiation (Higgins *et al*., 1989; Cunha *et al*., 1991). Analysis of chimeric prostates and seminal vesicles composed of wild-type mesenchyme plus epithelium from testicular feminization (Tfm) mouse, lacking all male sex accessory organs because of the absence of functional androgen receptors (Wilson, 1992), has demonstrated that the Tfm epithelium can undergo androgen-dependent ductal morphogenesis, epithelial proliferation, and columnar cytodifferentiation (Cunha and Young, 1992; Donjacour and Cunha, 1993). These results indicate the presence of paracrine factors produced by androgen receptor-positive mesenchyme.

Recently, growth factors and their receptors have been reported to mimic androgen or estrogen action in differentiation of male (Gupta *et al*., 1991, 1993) and female (McLachlan *et al*., 1991; Nelson *et al*., 1991) reproductive tracts; epidermal growth factor (EGF) mimics effects of androgen in the fetal development of male reproductive tracts and of estrogen in the stimulation of female genital tract growth and differentiation.

In the present study, we tried to establish a simple organ culture system, in which several organs develop together, to clarify whether growth factors mediate androgenic effect on male reproductive organs.

MATERIALS AND METHODS

Animals

C57BL/Tw mice were provided with pine shavings for bedding, fresh water, and sterilized rodent block diet (CE-2, CLEA, Tokyo) *ad libitum* and kept at 24 ± 1.0°C under 12-hr artificial illumination (from 0800 to 2000). All procedures were carried out according to the NIH Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by our Institutional Animal Care and Use Committee.

* Corresponding author: Tel. +81-45-787-2216;
FAX. +81-45-787-2370.
E-mail: taisen@yokohama-cu.ac.jp

† Present address: Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan.

Media and supplements

All chemicals except for steroids and culture supplements used in the present study were from Wako Pure Chemical Co. (Osaka). HBSS, DMEM/Ham's F-12; 1:1 (DH, vol:vol), Waymouth's medium (× 10), FBS, penicillin and streptomycin sulfate were obtained from Gibco (Grand Island, NY); insulin, BSA fraction V, human transferrin, cholera toxin (CT), 17β-estradiol (E2), testosterone (T), 5α-dihydrotestosterone (DHT) and cholesterol were from Sigma Chemical (St. Louis, MO); mouse EGF was from Collaborative Research (Waltham, MA), human transforming growth factor-α (TGF-α was from Biomedical Technologies (Stoughton, MA) and anti-mouse EGF antibody was from Upstate Biotechnology (Lake Placid, NY).

Organ culture

The male reproductive tract, from seminal vesicle to bulbourethral gland (BG), was dissected out from newborn mice in HBSS (Fig. 1A), and then BG was cut from the remaining urogenital sinus with seminal vesicles attached. These structures were cultured separately on collagen gel for 5 days in a serum-free (SF) medium composed of DH, insulin (10 μg/ml), transferrin (10 μg/ml), CT (10 ng/ml) and BSA (5 mg/ml) with penicillin (100 unit/ml) and streptomycin (100 μg/ml) or in a serum-containing medium composed of DH and 10% heat-inactivated (30 min, 56°C) FBS with the antibiotics. The components of the SF medium in the above concentrations with EGF (10 ng/ml) were optimal for growth of mouse mammary (Imagawa *et al*., 1982), uterine (Iguchi *et al*., 1987) and vaginal epithelial cells (Iguchi *et al*., 1983) and also support proliferation of human prostate tumor LNCaP cells (Iguchi *et al*., 1990). Collagen solution and gel were prepared by a modified method reported previously (Michalopoulos and Pitot, 1975). Collagen mixture (1 ml) was placed in a 35-mm Falcon culture dish (Becton Dickinson, Lincoln Park, NJ) and allowed to gel for 10 min at room temperature.

In order to investigate the effects of hormones and growth factors on development of male reproductive organs, T (10^{-7} M), DHT

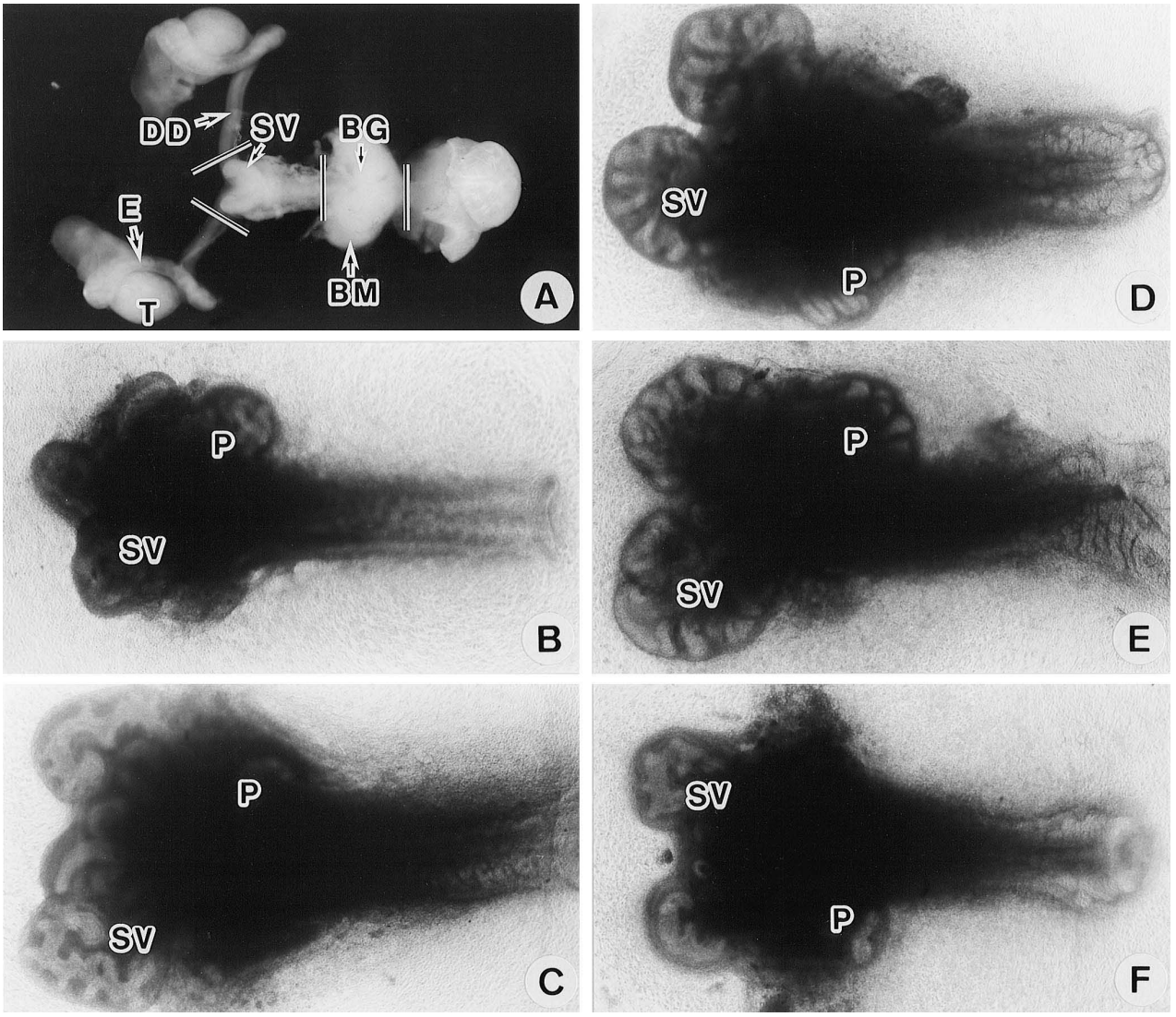

Fig. 1. Effects of sex hormones on morphogenesis of male reproductive organs. Newborn male urogenital sinus with seminal vesicle was cultured for 5 days in serum-free (SF) medium supplemented with (D, E, F) and without (B, C) sex hormones. A, male reproductive tract of newborn mice (dorsal view). Urogenital sinus with seminal vesicles attached and bulbourethral glands were cultured on collagen gel. Cuts are indicated by lines (× 8); B, DH medium; C, SF medium; D, SF medium supplemented with 10^{-7} M testosterone (T); E, SF medium supplemented with 10^{-7} M dihydrotestosterone (DHT); F, SF medium supplemented with 10^{-7} M 17β-estradiol (E_2). T, testis; E, epididymis; DD, ductus deferens; SV, seminal vesicle; P, prostate; BG, bulbourethral gland, BM, bulbocavernosus muscle. (B~F, × 30)

(10^{-7} M), E_2 (10^{-7} M), EGF (1-100 ng/ml), TGF-α (10-100 ng/ml), and anti-mouse EGF antibody (1-20 μg/ml) were added to the culture medium. The medium (1ml) was applied onto collagen gel, and incubated for 30 min at 37°C, then withdrawn so that cultured tissues could face to the atmosphere. The medium was renewed every 2 days by the same method, and 8 to 10 cultured organs in each experiment were harvested on the 5th day. Half of the cultured organs were fixed in 10% buffered formaldehyde solution and examined histologically. The remaining organs were used for DNA determination by the method of Labarca and Paigen (1980; see Iguchi *et al*., 1990) using Hoechst 33258 (Calbiochem, La Jolla, CA).

Measurement of organ volumes as a marker for development

Fixed tissues were embedded in paraffin, serially sectioned at 8 μm and stained with Delafield's hematoxylin and eosin. The area occupied by the epithelium and lumen of each organ was measured by a Color Image Analyzer CIA-102, and the actual volume (μm^3) was calculated as described previously (Iguchi *et al*., 1991).

Eight to 10 tissues of urogenital sinus with seminal vesicle were used for each experimental group. Data were expressed as means ± standard error and analyzed by ANOVA.

RESULTS

Effects of steroid hormones on development of male reproductive organs

Fig. 1A shows the morphology of the male mouse reproductive tract on the day of birth. Testis, epididymis, ductus deferens, seminal vesicles (SV) and BG were recognized, but coagulating glands (CG), prostate (P) and preputial glands were not visually identified. BG was located at dorsal part of the bulbocavernosus muscle.

DH medium maintained these organs for 5 days, but morphogenesis of SV was not observed (Fig. 1B). Supplemented serum-free medium, however, stimulated elongation of SV and budding of P (Fig. 1C). The addition of cholesterol into the SF medium had no additional effect (data not shown), however, the addition of T or DHT to the SF medium induced both morphogenesis of SV and budding of P (Fig. 1D and 1E). Addition of E_2 did not stimulate development of SV and P (Fig. 1F), but extensive outgrowth of fibroblasts occurred, re-

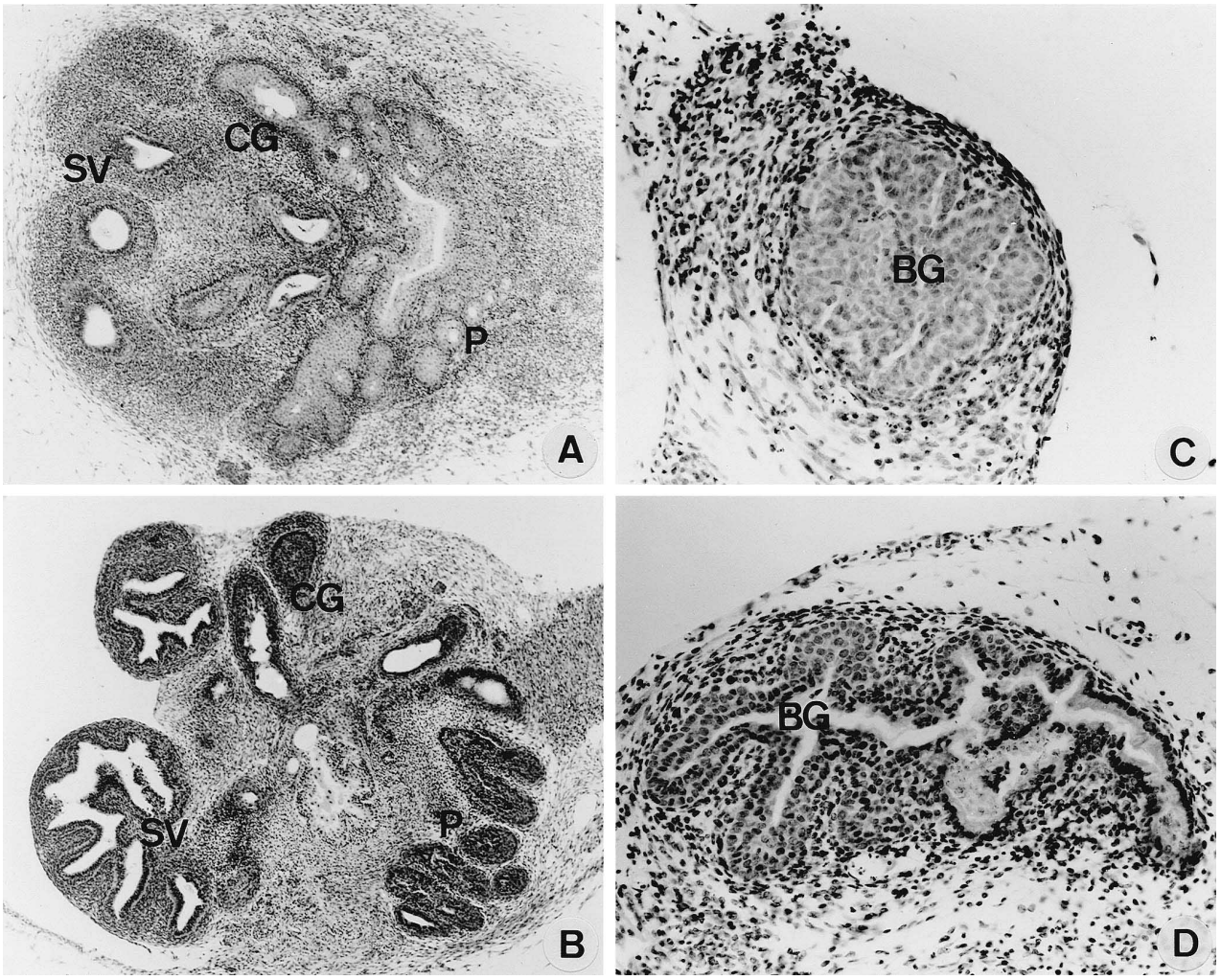

Fig. 2. Histology of urogenital sinus with seminal vesicle cultured in SF medium for 5 days. A and C, cultured in SF medium; B and D, cultured in SF medium supplemented with 10^{-7} M DHT. SV, seminal vesicle; CG, coagulating gland; P, prostate; BG, bulbourethral gland. (A and B, × 70; C and D, × 250)

sulting in a 1.4-fold increase in area compared with that seen with SF or SF containing androgen.

Histologically, the SF medium maintained organs in culture, and addition of DHT to the SF medium stimulated development of SV, CG, P and BG, resulting in expansion of epithelial and/or luminal areas in this culture system (Fig. 2).

Volumes of SV, CG, P and BG were estimated by accumulation of each sectional area surrounded by basement membrane using the image analyzer (Fig. 3). Those of all organs except BG were significantly increased 5 days after culture in SF medium over those before the culture. The addition of T to the SF medium significantly increased volumes of SV, P and BG; DHT also significantly increased volumes of SV, CG, P and BG. However, cholesterol and E_2 had no effect.

The DNA amount in the urogenital sinus with SV attached from 5-day-old mice was about 3 times abundant when compared with that in newborn mice. The DNA amount in the urogenital sinus with SV attached was not increased after 5 days of culture in DH medium; however, SF medium significantly stimulated DNA synthesis. T, DHT and E_2 significantly increased DNA; however, cholesterol did not (Fig. 4).

Effects of growth factors on development of male reproductive organs

Addition of 10 ng/ml EGF or TGF-α to SF medium inhibited morphogenesis of SV and stimulated growth of prostatic buds (Fig. 5 B. C, respectively). Histological analysis showed that volume of SV was reduced by addition of 10 and 100 ng/ml EGF and of 10 and 100 ng/ml TGF-α (Fig. 6). Volume of CG was significantly increased by addition of 100 ng/ml EGF and 100 ng/ml TGF-α. Volume of P was significantly increased by 100 ng/ml EGF and by 10 and 100 ng/ml TGF-α and that of BG was significantly increased by 10 and 100 ng/ml EGF, but not by TGF-α.

Addition of 10 ng/ml EGF into SF medium did not affect DNA amount of cultured tissue (17.6 ± 0.31 μg) when compared with that of the tissues cultured in SF medium only (18.6 ± 0.32 μg); however, these amounts were significantly higher than that before culture (12.4 ± 0.59 μg).

Effects of anti-EGF antibody on DHT-induced development of male reproductive organs

DHT (10^{-7}M) significantly increased the volumes of SV, CG, P and BG by *ca.* 130, 140, 180 and 490%, respectively, over those in DHT-free SF medium (Fig. 7). Addition of anti-EGF antibody to SF medium supplemented with 10^{-7}M DHT significantly inhibited the increase of volumes of CG (10 μg/ml, 20 μg/ml) and BG (20 μg/ml), whereas that of SV (20 μg/ml) was not altered. Volume of P treated with 10 or 20 μg/ml anti-EGF antibody was suppressed to that seen in the DHT-

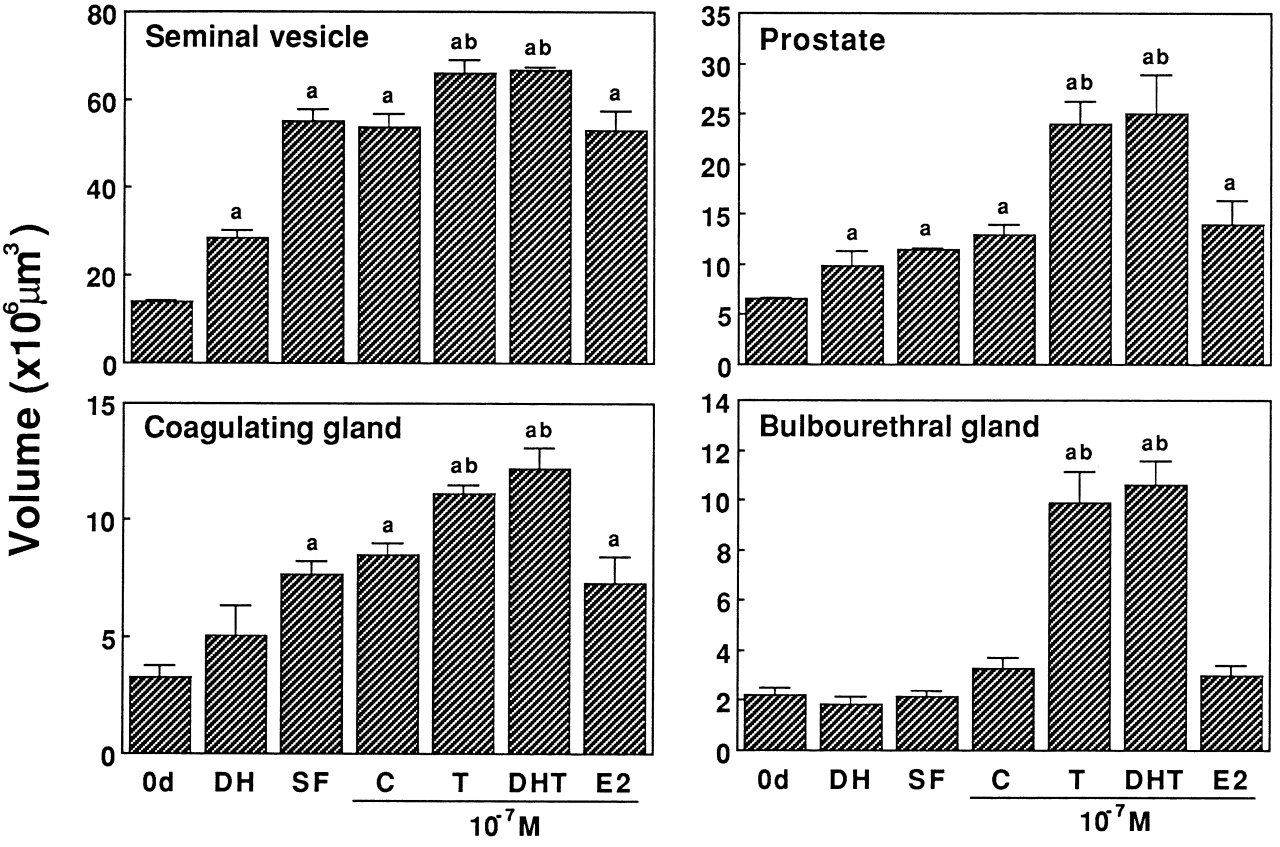

Fig. 3. Effects of steroid hormones on volumes of seminal vesicle, coagulating gland, prostate and bulbourethral gland. DH, Dulbecco's modified Eagle's medium/Ham's F-12 (1:1); SF, serum-free medium; C, cholesterol; T, testosterone; DHT, 5α-dihydrotestosterone; E2, 17β-estradiol. a, $P < 0.05$ vs. 0 day; b, $P < 0.05$ vs. SF (ANOVA)

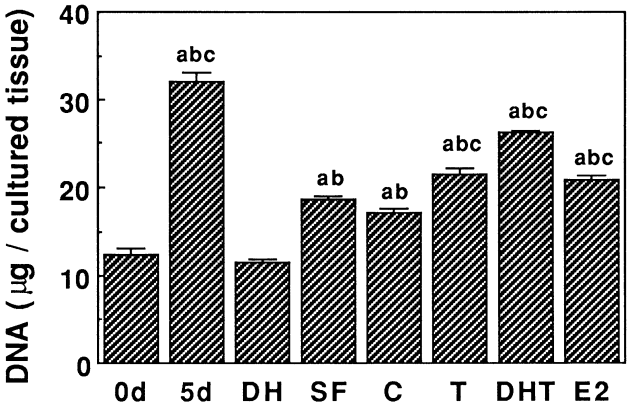

Fig. 4. DNA amount in urogenital sinus with seminal vesicle *in vivo* and *in vitro*, and *in vitro* effects of steroid hormones. 0d, newborn mice; 5d, 5-day-old mice. a, $P < 0.001$ vs. 0d; b, $P < 0.001$ vs. DH; c, $P < 0.001$ vs. C (ANOVA)

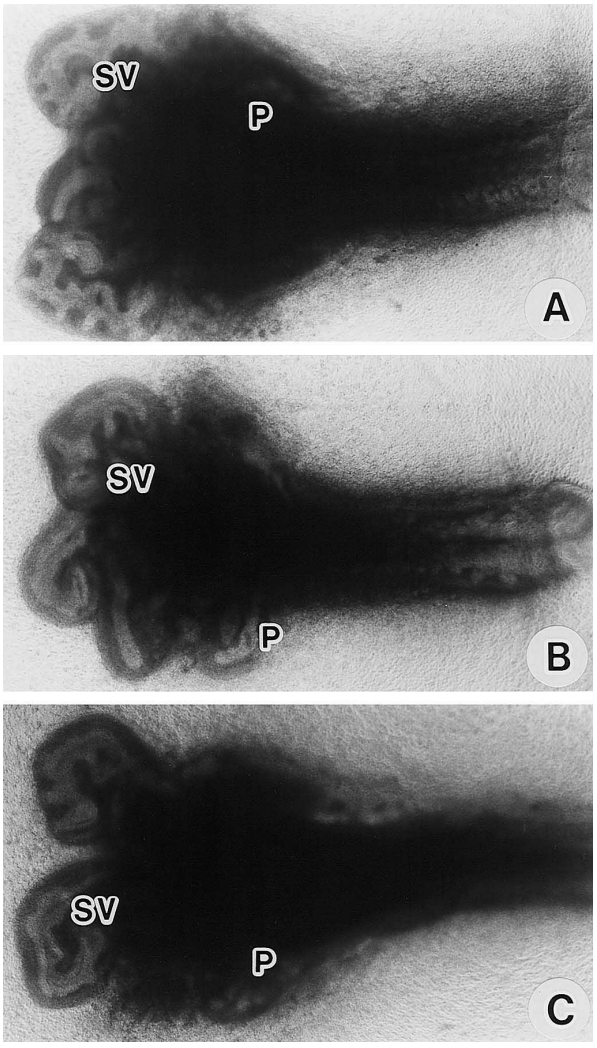

Fig. 5. Effects of EGF and TGF-α on morphogenesis of male reproductive organs. Newborn male urogenital sinus with seminal vesicle were cultured for 5 days in SF medium supplemented with growth factors. A, SF medium; B, 10 ng/ml EGF; C, 10 ng/ml TGF-α; SV, seminal vesicle; P, prostate. (× 30)

free SF medium. Addition of 20 μg/ml normal rabbit IgG to SF medium supplemented with DHT did not inhibit DHT effects (data not shown).

DISCUSSION

Androgen induces development and morphogenesis of the Wolffian duct within the urogenital ridge (Tsuji *et al.*, 1991) and other male reproductive organs (Cunha *et al.*, 1981; Cooke *et al.*, 1987a,b; Shima *et al.*, 1990). In the present study, androgens significantly stimulated the development of seminal vesicle, coagulating gland, prostate and bulbourethral gland. SF medium significantly stimulated DNA synthesis and development of the cultured organs, but not morphogenesis; however, androgens added to the SF medium stimulated morphogenesis as well as DNA synthesis and development. SF medium without insulin did not support the survival (data not shown), suggesting that insulin and/or insulin-like growth factors (IGFs) might be essential for perinatal maintenance of these organs.

EGF replaces sex-hormone action on male (Gupta *et al.*, 1991) and female genital tracts (McLachlan *et al.*, 1991; Nelson *et al.*, 1991) in mice. The developmental expression of EGF, TGF-α, IGF-I and their receptors (Wilcox and Derynck, 1988; Kapur *et al.*, 1992) and stimulation of EGF gene expression by androgen (Gupta and Singh, 1996) has been reported in mouse genital tracts. In the present study, EGF significantly increased volumes of coagulating gland and prostate, but not seminal vesicle, indicating that EGF is involved in the development of organs derived from urogenital sinus. Cultured Wolffian duct from fetal mouse degenerates in the presence of anti-EGF antibody in a dose-dependent manner, and addition of exogenous EGF to the culture nullifies the anti-EGF effect (Kapur *et al.*, 1992), suggesting that growth factor responsiveness may change after differentiation. TGF-α did not stimulate the development of the bulbourethral gland, although TGF-α is thought to be bound to the EGF receptor (Reynolds *et al.*, 1991). This inconsistent result may suggest the presence of a specific TGF-α receptor not reported yet.

Basic fibroblast growth factor (bFGF) stimulates the development of male genital ridge, especially the epididymis, but not of urogenital sinus (Alarid *et al.*, 1991), and keratinocyte growth factor (FGF-7) mimics androgen action on the development of seminal vesicle and prostate (Alarid *et al.*, 1994; Cunha *et al.*, 1996). These findings suggest that growth factors may have an important role as a mesenchymal paracrine mediators, which act specifically on each organ, of androgen-induced development and morphogenesis. In the present study, addition of anti-EGF antibody inhibited the androgen-promoted development of cultured coagulating gland, prostate and bulbourethral gland, but not of seminal vesicle, suggesting that EGF may be one of the androgen mediators which acts specifically on male reproductive organs derived from urogenital sinus. Addition of the antibody, however, did not completely inhibit androgen action on prostate and bulbourethral gland, suggesting that androgen action on each male

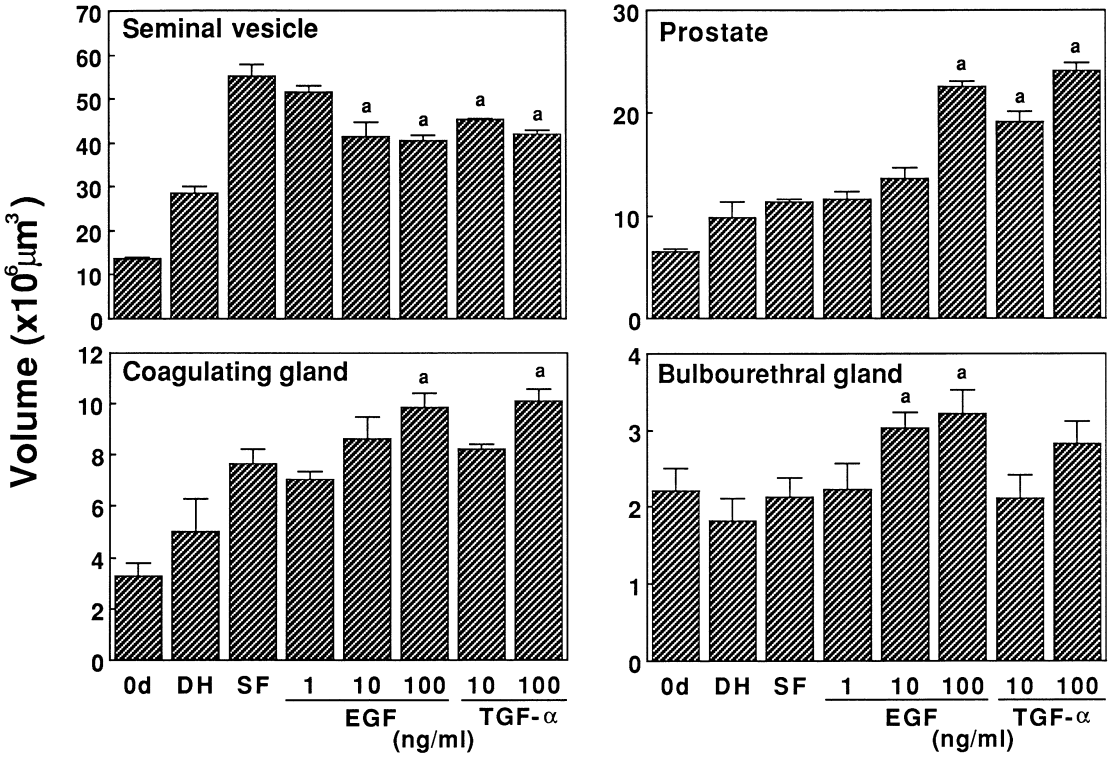

Fig. 6. Effects of EGF and TGF-α on volumes of seminal vesicle, coagulating gland, prostate gland and bulbourethral gland. a, $P < 0.05$ vs. SF medium. (ANOVA)

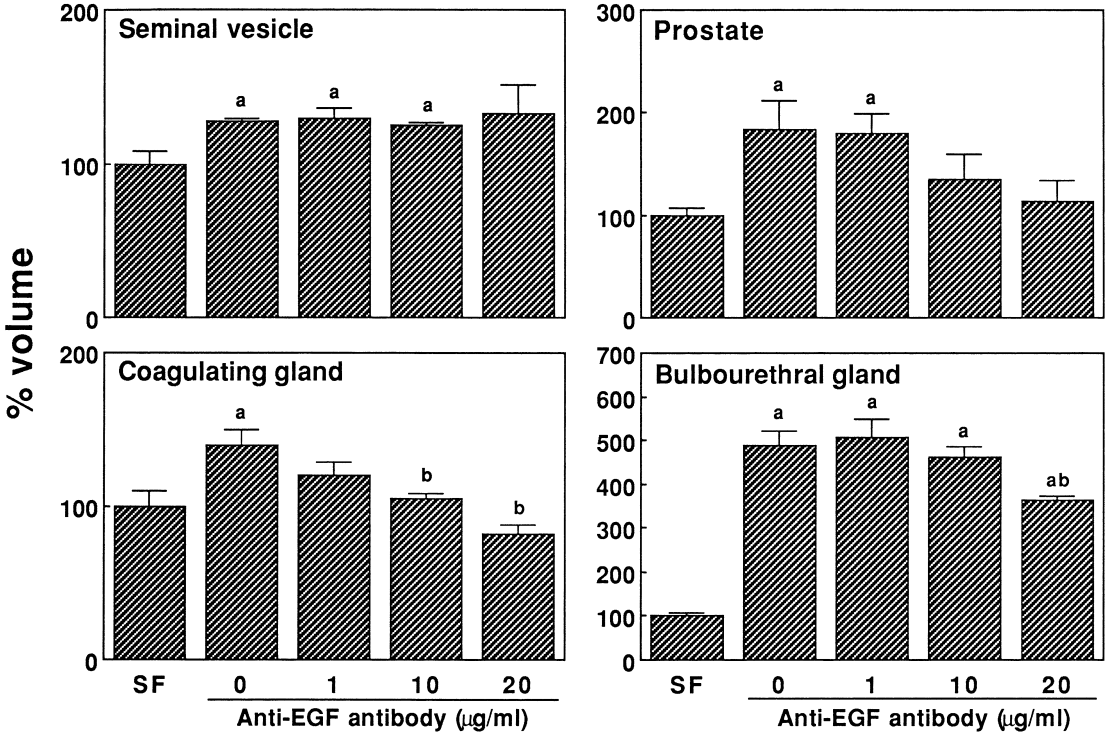

Fig. 7. Effects of anti-EGF antibody on DHT-induced development of seminal vesicle, coagulating gland, prostate and bulbourethral gland. Newborn male urogenital sinus with seminal vesicle was cultured for 5 days in SF medium supplemented with 10^{-7}M DHT and various concentrations of anti-EGF antibody. Data are expressed as % volume of each organ cultured in SF medium. a, $P < 0.05$ vs. SF medium; b, $P < 0.05$ vs 0 μg/ml (without) anti-EGF antibody. (ANOVA)

reproductive organ may be mediated by in combination with other growth factors, such as TGF-α, bFGF and KGF. Thus, a variety of mediators may produce organ specific characteristics.

In seminal vesicle derived from Wolffian duct, anti-EGF antibody did not inhibit the androgen action and EGF or TGF-α inhibited the development and morphogenesis of the seminal vesicle in androgen-free SF medium, indicating that these growth factors do not mediate androgen action on the seminal vesicle. Thus, EGF and TGF-α have organ-specific action in male reproductive tract development. SF medium stimulated the development of seminal vesicle, suggesting that some growth-promoting factors such as KGF may be produced under the serum-free condition. On the other hand, transforming growth factor-β (TGF-β), which is known as a growth inhibitor, particularly for epithelial cells, has been detected in fetal and neonatal mouse prostate and seminal vesicle (Timme *et al*., 1994). TGF-β1 has inhibitory effects on *in vitro* development of seminal vesicle induced by DHT (Tanji *et al*., 1994). Further studies on cooperative effects of several growth factors are needed for understanding the role of growth factors on the development of reproductive organs.

Perinatal estrogen exposure causes hyperplastic and metaplastic changes in the epithelium of the seminal vesicle, epididymis, anterior and ventral prostates in rats and mice (Mori, 1967; Arai, 1968; McLachlan *et al*., 1975; Ohta, 1977; Jones, 1980; Sato *et al*., 1994; for review, McLachlan, 1979; Arai *et at*., 1983; Iguchi, 1992). In the present study, estrogen stimulated DNA synthesis and an extensive outgrowth of stromal cells but not morphogenesis in cultured organs from newborn mice, indicating that stromal cells in male genital organs of perinatally estrogenized mice may promote abnormal development of the genital epithelia.

In conclusion, the development and morphogenesis of several androgen target organs (seminal vesicle, coagulating gland, prostate and bulbourethral gland) occur in the serum-free organ culture system. EGF and TGF-α may be mediators of androgen action on male reproductive organs derived from urogenital sinus, but not on the seminal vesicle derived from Wolffian duct, rather they inhibit the development and morphogenesis of seminal vesicle under the androgen-free condition.

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