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Systemic treatment with epidermal growth factor but not insulin-like growth factor I decreases the involution of the prostate in castrated rats

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Abstract Epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) are strong inducers of proliferation to prostate cells cultured in serum-free medium. Accordingly we wanted to study the growth of the prostate gland in castrated rats after treatment with EGF, IGF-I and testosterone. Castrated Wistar rats were treated with growth factors (EGF 35 μg/rat per day; IGF-I 350 μg/rat per day) or testosterone (2 mg/rat per day) for 3 days either immediately after or 10 days after castration. Prostate tissue was examined by stereological and immunohistochemical techniques and by enzyme-linked immunosorbent assay (ELISA). Treatment with EGF inhibited the involution of the prostate ($P < 0.05$), whereas treatment with IGF-I did not affect the prostate involution as compared to castrated controls. EGF treatment significantly increased the endogenous rat EGF in the ventral prostate, but cellular proliferation was not affected. Testosterone treatment increased the weight of the prostate, by increase of all tissue components of the prostate, and significantly increased cellular proliferation. Systemic administration of EGF but not IGF-I decreased the involution of the rat prostate induced by castration. Compared with testosterone, the effects of EGF treatment on the prostate involution were moderate, and the effects of EGF were not related to cellular proliferation.

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Introduction

Although androgens are essential for the development, growth and homeostasis of the prostate gland, it is currently believed that other hormones and peptide growth factors are involved in the regulation of prostate growth and differentiation. In the prostate a number of growth factor systems have been described, including the insulin-like growth factor (IGF) [21], and the epidermal growth factor (EGF) systems [4, 23]. IGF-I and EGF are known to induce proliferation in prostate primary cell cultures [1, 15, 19] and cell lines [2, 14], and EGF possesses anti-apoptotic effects in primary epithelial cultures [24]. IGF-I has recently been shown to increase the 5α-reductase activity in scrotal fibroblasts in vitro [10], suggesting that IGF-I exerts its effect by regulating the androgen metabolism in the gland. Expression of EGF in the prostate is dependent upon circulating androgens and will decrease following androgen ablation [20], suggesting that the EGF system may function as a mediator of androgen effects in the prostate. Although the effects of the EGF and IGF systems have been studied extensively in cell culture systems of the prostate, the effects and importance of the growth factors in vivo have been investigated sparsely. We have previously shown that systemic administration of IGF-I for 3 days and 7 days in adult rats induces growth of the prostate [26]. To further elaborate the role of IGF-I and EGF in prostate physiology and their dependence upon androgen metabolism in the gland, the present study was undertaken to investigate the effect of systemic treatment with IGF-I and EGF on the prostate after castration in rats.

Materials and methods

Study animals

The study was conducted on 120 male Wistar rats (Møllegaard Avlslab., Eiby, Denmark), 10 weeks of age. The animals were housed two in each cage (temperature 21 C; humidity 55 ± 5%; dark-light cycle, 12-h shift). Animals were fed a standard laboratory diet of Altromin-1324 (Lage, Germany) and water ad libitum. Principles of laboratory animal care were followed, and the study complied with Danish regulations for care and use of laboratory animals (J.nr. 1996-101-22). Animals were housed for 2 weeks prior to the experiments, and body weight was determined once a week.

Study design

The animals were randomly allocated into groups of eight to ten rats for treatment, as shown in Table 1. The study was divided into two parts. In group A to G, rats were castrated and immediately treated for 3 days with growth factor, testosterone or vehicle and then killed. In group H to N, rats were castrated and, after 10 days, treated with growth factor, testosterone or vehicle for 3 days and then killed. Animals were anaesthetized with pentobarbital 30 mg/kg i.p. Human recombinant IGF-I, 350 μg/rat per day (Ciba Geigy, Basle, Switzerland), and human recombinant EGF, 35 μg/rat per day (Upstate Biotechnology, New York, USA) were dissolved in 0.1 M acetic acid (vehicle), and delivered by subcutaneously implanted pumps (Alzet-1003D, Alza corporation, Calif., USA), at an infusion rate of 1 μl/h. Osmotic pumps were implanted subcutaneously. Placebo- and sham-operated rats were given subcutaneously implanted pumps filled with vehicle. Testosterone-17-propionate, 2 mg/rat per day (Riedel de Haën, Seelze, Germany), was dissolved in peanut oil and delivered as subcutaneous injections once a day.

On the day that they were killed, the rats were anaesthetized with pentobarbital 50 mg/kg i.p. Non-fasting blood samples were drawn from the retrobulbar venous plexus and collected through heparinized capillary tubes. The serum samples were kept at −20 °C for later analysis. The ventral and dorsolateral lobes of the prostate and the seminal vesicles were removed within 5 min of anaesthesia and weighed. One lobe from the ventral and the dorsolateral prostate, and one of the seminal vesicles or coagulating glands from each rat were immediately frozen in liquid nitrogen and stored at −80 °C. The other lobes of the prostate and the remaining seminal vesicle or coagulating gland were fixed by immersion in 4% phosphate-buffered formaldehyde.

Tissues

The fixed tissue of the ventral lobes were embedded in paraffin wax at 56 °C. With a random starting point within the lobe, three adjacent sections (each 5 μm thick) were cut. This was repeated throughout the gland at regular intervals to obtain approximately four to six levels in each gland. In tissue from rats treated with EGF or vehicle immediately after castration, the sections were repeated at intervals of 400 μm, and in tissue from rats treated with EGF, IGF-I, testosterone or vehicle 10 days after castration, the sections were repeated at intervals of 100 μm throughout the ventral prostate.

Histological and stereological tissue investigations

Tissue sections from rats treated with EGF or vehicle immediately after castration and from rats treated with EGF, IGF-I, testosterone or vehicle 10 days after castration were deparaffinized and stained with hematoxylin and eosin. Determination of the volume fractions of the different tissue components was performed by a two-dimensional stereological technique, essentially as described previously [9, 26]. The specific density of all tissue components was assumed to be 1.0 g/cm^3. All counting procedures were performed blind. A total number of three to seven tissue sections from each gland were used for quantifying the different tissue components.

Immunohistological staining procedures and quantification of proliferating cells

Cellular proliferation was determined in tissue sections from rats treated with EGF, IGF-I, testosterone or vehicle 10 days after castration. Sections were incubated in a humidified chamber at 60 °C for 30 min. After immersing the sections in xylene, twice for 5 min, and in 99% ethanol, twice for 5 min, endogenous peroxidase activity was quenched by 0.45% H_2O_2 in 99% ethanol, for 30 min, and sections were rinsed in 0.1 M phosphate buffer (PB; 0.25% Triton X-100, pH 7.4) for 5 min. The antigenic epitopes were demasked by immersing the sections in sodium citrate buffer 0.01 M, in a microwave oven at 600–700 W, for 30 min, followed by rinsing the sections in PB for 5 min. Sections incubated with an anti-Ki-67 antibody, NCL-Ki67-MM1 (Novocastra laboratories, Newcastle upon Tyne, England), diluted 1:100 in PB containing 1% bovine albumin (PBA; Sigma, Mo., USA), for 16 h. at 4 °C, followed by rinsing in PB for 10 min. Subsequently the sections were incubated with a biotin-labeled rabbit anti-mouse antibody, E0354 (DAKO, Glostrup, Denmark), diluted 1:400 in PBA for 30 min at room temperature and rinsed in PB for 10 min. Peroxidase conjugated streptavidin (DAKO, Glostrup, Denmark), diluted 1:400 in PBA, was added and incubated for 30 min at room temperature, followed by rinsing in PB for 10 min. Sections were developed by 3-amino-9-ethylcarbazole (ACE) for 15 min, counterstained in Mayers hematoxylin for 1 min, and rinsed thoroughly in water before mounting in wet mounting medium. Cell proliferation was quantified by a two-dimensional stereological technique, using the same magnification and computer software system as described previously [26]. Four counting frames were superposed

Table 1 Experimental design (*C* castration, *SH* sham operation, *P* pump implantation, ✔ animal killed, *EGF* epidermal growth factor, *IGF-I* insulin-like growth factor I)

Group (*n*)	Day 0	Treatment	Day 3	Day 10	Treatment	Day 13
A (n = 10)			✔			
B (n = 9)	SH + P	Sham	✔			
C (n = 7)	C + P	Placebo	✔			
D (n = 8)	C + P	EGF	✔			
E (n = 8)	C + P	EGF + IGF-I	✔			
F (n = 8)	C + P	IGF-I	✔			
G (n = 9)	C	Testosterone	✔			
H (n = 9)	C			✔		
I (n = 10)	C					✔
J (n = 9)	C			P	Placebo	✔
K (n = 8)	C			P	EGF	✔
L (n = 8)	C			P	EGF + IGF-I	✔
M (n = 8)	C			P	IGF-I	✔
N (n = 8)	C				Testosterone	✔

within each field of vision, which were systematically distributed in each tissue section. Approximately 10–20 fields of vision were examined within each section. A total number of three to seven tissue sections from each gland were used for calculating the index of proliferation. Within each counting frame, stained and unstained nuclear profiles were counted. The index for proliferation in the ventral prostate tissue was calculated as follows:

$$\frac{100 \times \Sigma\text{stained nuclear profiles}}{\Sigma\text{stained nuclear profiles} + \Sigma\text{unstained nuclear profiles}}$$

All counting procedures were performed blind.

Determination of EGF in serum and prostate tissue

For determination of human recombinant EGF in rat tissue and serum, an ELISA measuring human EGF was employed. The assay shows negligible cross reactivity to rat EGF [16].

Endogenous rat EGF was also determined by ELISA [12]. The assay shows negligible cross reactivity towards human recombinant EGF or rat TGF. For each of the two peptides, 200 nmol/l was measured as less than 0.03 nmol/l.

Determination of serum IGF-I

Serum IGF-I was determined by IGF-I radioimmunoassay as previously described [6]. This quantifies rat IGF-I and exogenous recombinant human IGF-I.

Statistics

Data are given as mean ± SEM. Statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Bonferroni's test between selected groups. Data showing non-Gaussian distribution were analyzed by the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Two-tailed tests were employed; $P < 0.05$ was considered as the limit of significance. The weight of the prostate and seminal vesicles or coagulating glands of one rat treated with vehicle for 3 days immediately after castration deviated more than +3 SD from the mean of the group, and this case was excluded from statistical analysis.

Results

The animals thrived throughout the study. Body weight determined on the day they were killed did not differ between the rats treated with growth factors or testosterone immediately after castration and the rats treated 10 days after castration.

Weights of the prostate and seminal vesicle or coagulating gland

Rats treated immediately after castration

The mean wet weight of the rat prostate (ventral + dorsolateral lobes), relative to body weight is shown in Fig. 1. There was no difference between the weight of the prostate in the sham-castrated rats and the non-castrated rats (control), indicating that the physiological stress induced by the operation did not affect the wet weight of the prostate. Treatment with EGF resulted in significantly decreased involution of the gland as compared to placebo: 1.42 ± 0.06 mg EGF/g; 1.18 ± 0.08 mg placebo/g ($P < 0.05$). Treatment with IGF-I or EGF + IGF-I did not affect the wet weight of the prostate as compared to placebo. Treatment with testosterone significantly increased the mean wet weight of the prostate as compared to placebo treatment ($P < 0.001$).

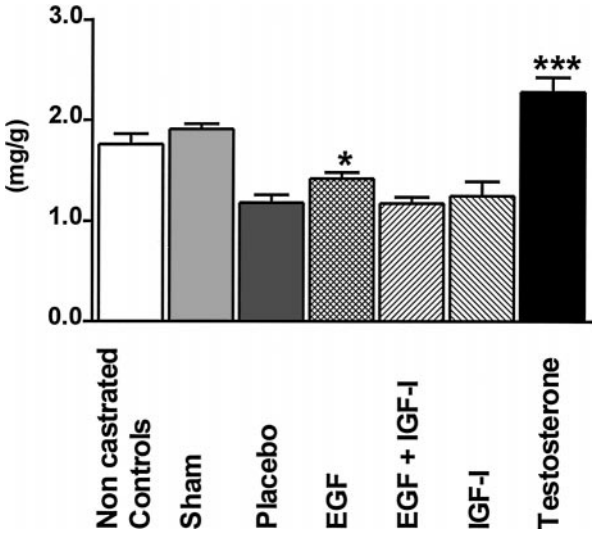

Fig. 1 Wet weight of the prostate (ventral + dorsolateral lobes), relative to body weight (milligrams per gram) in rats, castrated and immediately treated with epidermal growth factor (*EGF*), EGF + insulin-like growth factor I (*IGF-I*), IGF-I, vehicle or testosterone for 3 days, in sham-operated rats and in non-castrated controls. Data are given as mean ± SEM. Statistical analysis by Kruskal-Wallis test followed by Dunn's multiple comparison test. Two-tailed *P*-values were derived: $*P < 0.05$; $***P < 0.001$ versus placebo

Treatment with growth factors did not affect the mean weight of the seminal vesicle and the coagulating gland compared to placebo, whereas treatment with testosterone significantly increased the weight relative to placebo and control ($P < 0.001$; not shown).

Rats treated 10 days after castration

The mean wet weight of the rat prostate (ventral + dorsolateral lobes) relative to body weight is shown in Fig. 2. Rats treated with EGF had a significantly larger prostate than placebo: 0.50 ± 0.03 mg EGF/g, mean ± SEM; 0.38 ± 0.03 mg placebo/g ($P < 0.05$). Treatment with EGF + IGF-I or IGF-I did not affect the weight of the prostate when compared with placebo. Treatment with testosterone more that doubled the weights of the prostate gland as compared to placebo and control ($P < 0.001$).

Treatment with growth factors did not affect the weight of the seminal vesicle and the coagulating gland, but treatment with testosterone significantly increased the weight as compared to placebo and control ($P < 0.001$; not shown).

IGF-I in rat serum

Castration did not affect serum IGF-I as compared to controls, and serum levels were comparable in

sham-operated and non-castrated controls, indicating that serum IGF-I was not affected by operative stress (Tables 2, 3). As expected, treatment with IGF-I increased the serum IGF-I level as compared to placebo ($P < 0.001$). Also the groups receiving EGF and IGF-I showed significantly increased serum IGF-I levels, but less than in rats receiving IGF-I alone ($P < 0.01$). Treatment with EGF decreased the IGF-I level as compared to placebo ($P < 0.05$). This is in agreement with previously published data [8]. Treatment with testosterone did not significantly affect the IGF-I level as compared to placebo or non-castrated controls.

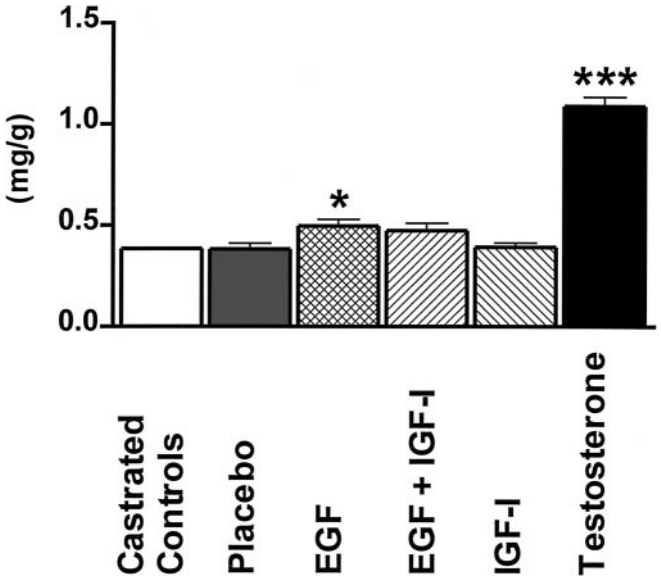

Fig. 2 Wet weight of the prostate (ventral + dorsolateral lobes) relative to body weight (milligrams per gram) in rats, castrated and, after 10 days, treated with EGF, EGF + IGF-I, IGF-I, vehicle or testosterone for 3 days, and weight of the prostate in rats 13 days after castration (castrated controls). Data are given as mean ± SEM. Statistical analysis by Kruskal-Wallis test followed by Dunn's multiple comparison test. Two-tailed *P*-values were derived: $*P < 0.05$; $***P < 0.001$ versus placebo

EGF in rat serum and prostate tissue

Human EGF in rat serum was determined in rats receiving treatment with growth factors or placebo immediately after castration. As expected, human EGF was detectable only in rats treated with EGF (0.13 ± 0.02 pmol/ml), indicating that EGF was released from the osmotic pump and entered the circulation.

Endogenous EGF was measured in prostate tissue from rats treated with EGF and vehicle and from non-castrated controls, as shown in Fig. 3a. The concentration of rat EGF was significantly increased in rats treated with human EGF versus non-castrated controls ($P < 0.01$). Determined as total content of rat EGF per ventral lobe, rats treated with human EGF significantly increased the rat EGF compared with controls ($P < 0.05$; Fig. 3b), indicating that EGF treatment induced de novo synthesis of EGF in the rat ventral prostate.

Stereological investigations

Treatment with EGF for 3 days immediately after castration tended towards an increase in the lumen, although this was not significant (Tables 4, 5). Treatment with EGF starting 10 days after castration resulted in significantly increased stromal volume as compared to placebo ($P < 0.001$). Treatment with

Table 2 Mean ± SEM serum IGF-I in castrated rats (groups A to G) treated immediately with EGF, EGF + IGF-I, IGF-I, vehicle or testosterone. Castrated rats were treated for 3 days with EGF (35 μg/rat per day), IGF-I (350 μg/rat per day). EGF and IGF-I were administered through subcutaneously implanted pumps at an infusion rate of 1 μl/h. Testosterone (2 mg/rat per day) was administered s.c. once a day

	Control (A)	Sham (B)	Placebo (C)	EGF (D)	EGF/IGF-I (E)	IGF-I (F)	Testosterone (G)
Serum IGF-I (ng/ml)	785 ± 42	781 ± 24	827 ± 42	688 ± 28*	1056 ± 41**,***	1293 ± 66**	693 ± 38

* Serum IGF-I significantly decreased versus placebo ($P < 0.05$)
** Serum IGF-I significantly increased versus placebo ($P < 0.001$)
*** Serum IGF-I significantly decreased versus treatment with IGF-I alone ($P < 0.01$)

Table 3 Mean ± SEM serum IGF-I in castrated rats (groups H to N) treated after 10 days with EGF, EGF + IGF-I, IGF-I, vehicle or testosterone. Castrated rats were treated for 3 days with EGF (35 μg/rat per day), IGF-I (350 μg/rat per day). EGF and IGF-I were administered through subcutaneously implanted pumps at an infusion rate of 1 μl/h. Testosterone (2 mg/rat per day) was administered s.c. once a day

	Control-10 (H)	Control-13 (I)	Placebo (J)	EGF (K)	EGF + IGF-I (L)	IGF-I (M)	Testosterone (N)
Serum IGF-I (ng/ml)	824 ± 29	867 ± 22	766 ± 18	554 ± 23[a]	1060 ± 28[b,c]	1451 ± 50[b]	727 ± 34

[a] Serum IGF-I significantly decreased versus placebo ($P < 0.05$)
[b] Serum IGF-I significantly increased versus placebo ($P < 0.001$)
[c] Serum IGF-I significantly decreased versus treatment with IGF-I alone ($P < 0.01$)

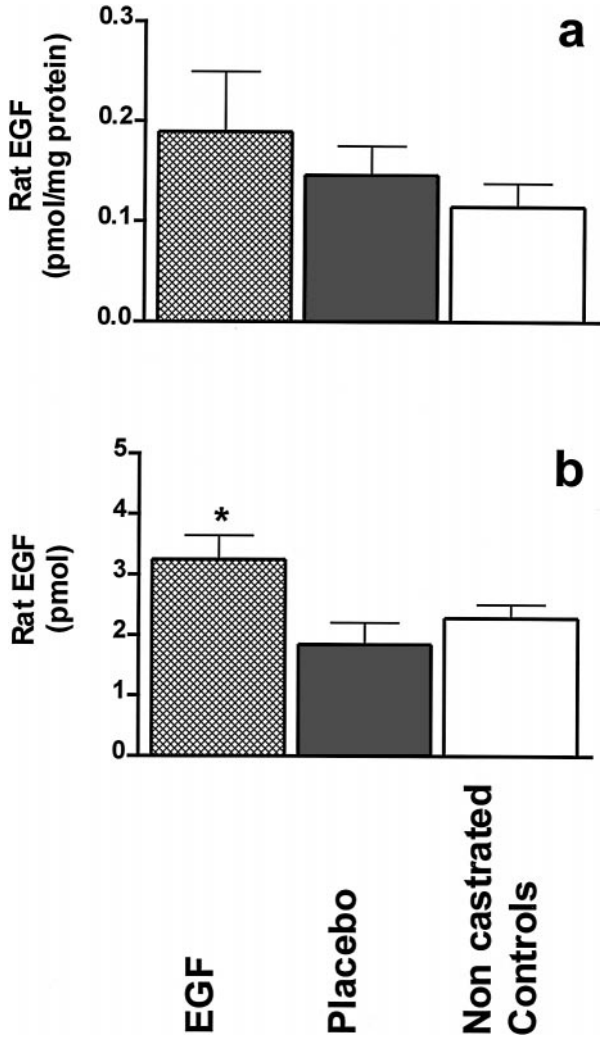

Fig. 3 **a** Endogenous EGF (picomoles per milligram protein) in the ventral prostate of rats treated with EGF or vehicle for 3 days immediately after castration, and in non-castrated controls. **b** Total content of endogenous EGF (picomoles) in the ventral prostate. Data are given as mean ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by Bonferroni's test between selected groups. Two-tailed *P*-values were derived: $*P < 0.05$ versus placebo; $**P < 0.01$ versus non-castrated controls

Table 4 Stereological examination of the ventral prostate in castrated rats treated immediately with growth factors, vehicle or testosterone. Volume of the histological components of the rat ventral prostate in rats treated with EGF (35 μg/rat per day), IGF-I (350 μg/rat per day), testosterone (2 mg/rat per day) or vehicle. Data are mean ± SEM

	Epithelium	Stroma	Lumen
EGF (mm^3 = mg)	66.4 ± 5.5	50.6 ± 6.9	126.3 ± 15.8
Placebo (mm^3 = mg)	67.3 ± 8.1	49.1 ± 4.2	96.5 ± 13.2

IGF-I did not affect the tissue component volumes of the ventral prostate. All component volumes were significantly increased by treatment with testosterone ($P < 0.001$).

Table 5 Stereological examination of the ventral prostate in rats treated 10 days after castration with growth factors, vehicle or testosterone. Volume of the histological components of the rat ventral prostate in rats treated with EGF (35 μg/rat per day), IGF-I (350 μg/rat per day), testosterone (2 mg/rat per day) or vehicle. Data are mean ± SEM

	Epithelium	Stroma	Lumen
EGF (mm^3 = mg)	17.3 ± 1.5	37.1 ± 1.9*	5.7 ± 0.4
IGF-I (mm^3 = mg)	15.0 ± 1.0	26.4 ± 1.8	4.8 ± 0.8
Testosterone (mm^3 = mg)	62.5 ± 4.0*	45.8 ± 3.8*	30.0 ± 5.3*
Placebo (mm^3 = mg)	17.3 ± 1.3	24.0 ± 1.3	4.6 ± 0.9

* Significantly increased as compared to placebo ($P < 0.001$)

Effects on cell proliferation after treatment with EGF, IGF-I and testosterone

Treatment with EGF or IGF-I immediately after castration did not affect the proliferation index as compared to placebo: 0.46 ± 0.14 (EGF); 0.20 ± 0.12 (IGF-I); 0.31 ± 0.12 (placebo); whereas treatment with testosterone significantly increased the index: 7.5 ± 1.12 as compared to placebo ($P < 0.001$).

Discussion

This study was performed to investigate the effects on the prostate, following systemic treatment with EGF and IGF-I as compared to testosterone, in castrated rats. A number of EGF agonists, including EGF [27], TGFα [11], heparin-binding EGF (HB-EGF) [7], and amphiregulin [28] are expressed in the prostate in humans and rats. These ligands all activate the EGF receptor, and systemic administration of EGF mimics the effect of any of these EGF agonists. Administration of IGF-I mimics the endogenous IGF-I, which is synthesized predominantly in the rat liver. Systemic administration of recombinant human EGF [29, 30] and IGF-I [26] has been shown previously to possess growth-promoting effects on the rat urogenital system.

In rats treated with EGF immediately after castration, the weight of the prostate was significantly higher than in rats treated with vehicle, indicating that the weight loss induced by castration was reduced by EGF treatment. The stereological data showed a tendency towards an increase in the luminal tissue. This is in agreement with a previous study showing a minor effect of EGF treatment on organ DNA content in mouse seminal vesicle or coagulating gland, but an increase in the volume of the lumen [13]. Supporting this, we found that EGF treatment doubled the rat EGF content within the ventral lobe, by increasing the synthesis of endogenous EGF. Autoinduction of the EGF system after systemic treatment with EGF has been demonstrated in the rat liver [31]. We have shown previously that rat EGF is synthesized in the luminal epithelial cells and is secreted into the prostate lumen [27].

In rats treated with EGF 10 days after castration, the weight of the prostate gland was significantly higher than placebo. This was seemingly not induced by cell proliferation, since the proliferation index was unaffected by treatment with EGF. In conclusion these results suggest that EGF affects the secretory activity of the rat prostate.

Treatment with IGF-I did not affect the weight of the prostate compared with placebo, and IGF-I did not affect the cell proliferation of the prostate in castrated rats. In cell culture systems, IGF-I acts via stimulation of specific receptors expressed on prostate epithelial cells [1] and/or by regulating the androgen metabolism in the gland [10]. Treatment with a long-acting luteinizing hormone-releasing hormone analogue increases the binding capacity to IGF-I in prostate tissue [5], indicating that IGF-I receptors are present on the cells after androgen withdrawal. In a previous study applying a similar concentration of IGF-I in non-castrated rats, we showed that IGF-I provoked growth and metabolic induction in the prostate after 3 days and 7 days of treatment [26]. The present data indicate that castration abolishes the effect of IGF-I on prostate growth and shows that an intact androgen supply to the prostate is essential for IGF-I to exert its growth-promoting effect. It is accordingly possible that IGF-I exerts the effect on the prostate mainly by stimulating the androgen turnover in the gland, via up-regulation of the 5α-reductase [10] or via direct stimulation of the androgen receptor [3]. A mechanism behind the abolition of IGF-I effects after castration is suggested by data showing increased gene expression for IGFBP-2, -3, -4 and -5 rapidly following castration, reaching an increase of five- to tenfold of control level 72 h after castration [18, 25]. High levels of IGFPB peptide in the prostate gland following castration may complex to free IGF-I or IGF-II and hamper the activation of IGF receptor in the gland. Recently Cohen et al. showed that IGFPB-3 is able to induce apoptosis in a prostate cell line, through an IGF-independent mechanism [22], a mechanism also shown in the MCF7 breast cancer cell line [17]. It is possible that increased IGFBP-3 following castration has a dual effect by complexing free IGFs in the prostate and directly inducing apoptosis in the prostate gland.

In conclusion, systemic treatment with EGF but not IGF-I reduces the degree of involution of the prostate in rats after castration. Compared with treatment with testosterone, the effect of EGF on the prostate involution was modest, and the effect was not mediated by increasing cell proliferation. Administration of EGF increased endogenous EGF in the gland, indicating that EGF affects the secretory activity in the prostate.

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