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Original Article

Keratinocyte growth factor (KGF) can replace testosterone in the ductal branching morphogenesis of the rat ventral prostate

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ABSTRACT Prostatic growth occurs through ductal elongation and branching into the mesenchyme. Ductal branching morphogenesis in the prostate is elicited by androgens via mesenchymal-epithelial interactions mediated by paracrine influences from mesenchyme. The role of keratinocyte growth factor (KGF) was investigated in the developing prostate as KGF has been suggested to be a paracrine acting factor. KGF transcripts were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in neonatal rat ventral prostates (VPs) *in vivo*, in VPs cultured *in vitro*, and in isolated VP mesenchyme. KGF receptor was detected in VP's by RT-PCR and was localized specifically to the epithelium by *in situ* hybridization. KGF was investigated as a potential paracrine mediator during androgen-induced prostatic development by examining neonatal rat VPs cultured for 6 days under serum-free conditions using a basal medium supplemented only with insulin and transferrin. When testosterone (10^{-9} to 10^{-8} M) was added to the basal medium, VPs grew and underwent ductal branching morphogenesis similar to that *in situ*. Neutralization of endogenous KGF with a monoclonal antibody to KGF (anti-KGF) or a soluble KGF receptor peptide inhibited androgen-stimulated VP growth (DNA content) and reduced the number of ductal end buds after 6 days of culture. When KGF (50 or 100 ng/ml) was added to the basal medium in the absence of testosterone, VP growth and ductal branching morphogenesis were stimulated. The number of ductal end buds was about 70% of that obtained with an optimal dose of testosterone (10^{-8} M), and DNA content of VP's cultured with 100 ng/ml KGF was equivalent to that of glands cultured with testosterone. The stimulatory effect of KGF was partially blocked by cyproterone acetate, a steroidal anti-androgen. These data imply that KGF plays an important role as a mesenchymal paracrine mediator of androgen-induced epithelial growth and ductal branching morphogenesis in the rat VP.

KEY WORDS: *keratinocyte growth factor, mesenchymal-epithelial interactions, prostate, testosterone, branching morphogenesis, epithelial growth*

Introduction

Under the influence of androgens the prostate originates from the outgrowth of solid epithelial cords (prostatic buds) from the endodermal urogenital sinus immediately below the developing bladder (Cunha *et al.*, 1987). Prostatic buds emerge in the rat at 19 days of gestation (Price and Ortiz, 1965), and elongate and arborize postnatally into urogenital sinus mesenchyme (UGM) (Hayashi *et al.*, 1991; Timms *et al.*, 1994). Reciprocal mesenchymal-epithelial interactions play critical roles in prostatic development (Cunha *et al.*, 1992b, 1987). UGM induces prostatic ductal morphogenesis, regulates epithelial proliferation and elicits the expression of epithelial androgen receptors and prostate-specific secretory proteins (Cunha *et al.*, 1983, 1980, 1992a; Takeda *et al.*, 1990; Donjacour and Cunha, 1993). In reciprocal fashion the epithelium of the developing prostate induces the surrounding mesenchyme to differentiate into smooth muscle cells (Cunha *et al.*, 1992b).

Through the analysis of chimeric prostates prepared with wild-type mesenchyme and testicular feminization (Tfm) epithelium it

Abbreviations used in this paper: KGF, keratinocyte growth factor; RT-PCR, reverse-transcriptase-polymerase chain reaction; VP, ventral prostate; Tfm, testicular feminization; FGF, fibroblast growth factor; KGF-R, keratinocyte growth factor receptor; RT, reverse transcriptase; dNTPs, deoxynucleotide triphosphates; T, testosterone.

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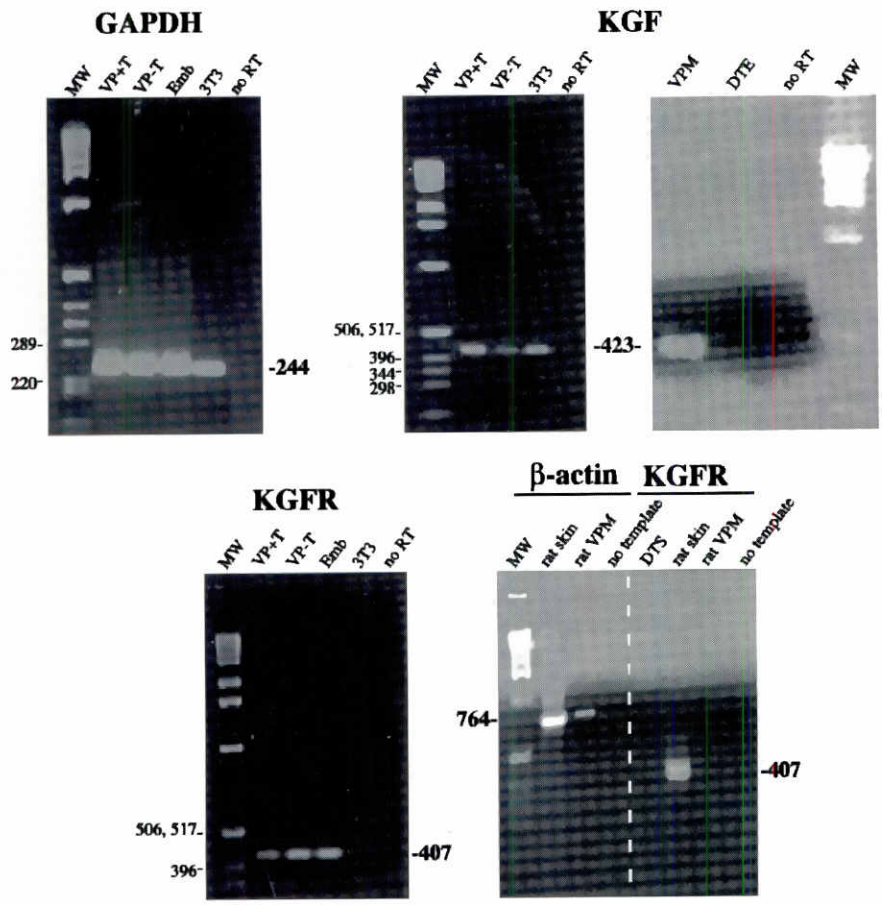

Fig. 1. Detection of transcripts for glyceraldehyde phosphate dehydrogenase (GAPDH), KGF, and KGF-R by RT-PCR *(see Materials and Methods for techniques). MW, molecular weight markers; +T, testosterone at 10^{-8} M; -T, without testosterone, 3T3, 3T3 cells; DTE, Dunning tumor epithelial cells; DTS, Dunning tumor fibroblasts; Emb, 14 day embryonic rat; no RT or no template, deletion of reverse transcriptase reaction product.*

has been concluded that androgenic effects on prostatic epithelial development are mediated via paracrine influences from UGM. Tfm/Y mice are genetic males that are completely insensitive to androgens due to the absence of functional androgen receptors and completely lack all male accessory sexual organs (including prostate) (Ohno, 1979; He *et al.*, 1991; Wilson, 1992). In chimeric prostates composed of wild-type rat UGM plus Tfm mouse epithelium the androgen receptor-negative Tfm epithelium is induced to undergo prostatic ductal morphogenesis, epithelial proliferation and simple columnar cytodifferentiation, all of which are androgen-dependent (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1984; Sugimura *et al.*, 1986a; Donjacour and Cunha, 1993). Thus, in a strict sense certain «androgenic effects» in epithelial cells appear to be elicited by paracrine (probably nonsteroidal) factors produced by androgen receptor-positive mesenchyme and do not require epithelial androgen receptors. Certainly the Tfm/wild-type tissue recombinant studies suggest that mesenchyme is the actual target and mediator of a variety of androgenic effects on developing prostatic epithelium. While the identity of the androgen-regulated paracrine factors of mesenchymal origin remains unknown, growth factors are likely candidates. Growth factors from 6 major families (Thompson, 1990; Gelmann, 1991; Story, 1991) have been described in the adult prostate: insulin-like growth factors, platelet derived growth factor, nerve growth factor, epidermal growth factor/transforming growth factor-alpha, transforming growth factor-betas (TGFß), and heparin-binding growth factors, although the relation of these growth factors to actual prostatic growth and development is far from clear as discussed earlier (Cunha *et al.*, 1992a).

KGF is a member of the fibroblast growth factor (FGF) gene family which contains 9 related genes (Mason, 1994). KGF is about 30-45% homologous at the RNA level to other FGFs (Finch *et al.*, 1989; Yan *et al.*, 1991). The receptor for KGF (KGF-R) is a splice variant of the protein encoded by the *bek* gene (FGFR2) which is expressed in epithelial cells (Bottaro *et al.*, 1990; Miki *et al.*, 1991, 1992). Many FGFs are widely expressed during development. FGFs have been implicated in ventral mesodermal induction in *Xenopus* (Slack, 1994). FGF-3 (also known as int-2) and FGF-4 are involved in spatial patterning during development. Transcripts encoding FGF-3 and FGF-8 exhibit a dynamic and transient expression pattern in the developing central nervous system (Lumsden, 1990; Mason, 1994; Crossley and Martin, 1995). In the developing limb bud FGF-4 is synthesized in the apical epidermal ridge and can substitute for the apical epidermal ridge (Niswender *et al.*, 1993; Vogel and Tickle, 1993). Recently engineered FGF-5 knockout transgenic mice have sustained hair growth and represent a previously described line of mutant mice called angora (Herbert *et al.*, 1994). FGF-7 (KGF) has emerged as a paracrine mediator of mesenchymal-epithelial interactions (Finch *et al.*, 1989; Rubin *et al.*, 1989; Yan *et al.*, 1991) and is expressed during ductal branching morphogenesis in mesenchyme of the developing mammary gland, salivary gland, and lung (Mason *et al.*, 1994; Finch *et al.*, 1995). Recently, KGF has been identified in the rat and human prostate (Yan *et al.*, 1992; Lin *et al.*, 1994; Peehl and Rubin, 1995) and has been shown to play a role in androgen-dependent growth and development of the neonatal mouse seminal vesicle (Alarid *et al.*, 1994). In this study, androgen-dependent prostatic development was investigated to determine the possible role of KGF using serum-free organ culture.

Results

Expression of KGF and KGF-R transcripts

Since organ culture experiments using rat VPs indicated that KGF may be involved in testosterone-induced ductal branching morphogenesis, RT-PCR analysis was performed to assess transcripts for KGF and KGF-R (Fig. 1). Transcripts for glyceraldehyde phosphate dehydrogenase (GAP) or β-actin were detected for all specimens. KGF transcripts were detected in samples of 14 day embryonic rat tissue (not shown), 3T3 cells, VPs cultured with and without testosterone (10^{-8} M) and in mesenchymal cells isolated from neonatal rat VPs. KGF-R transcripts were detected in samples of 14 day rat embryonic tissue, rat skin, VPs cultured with and

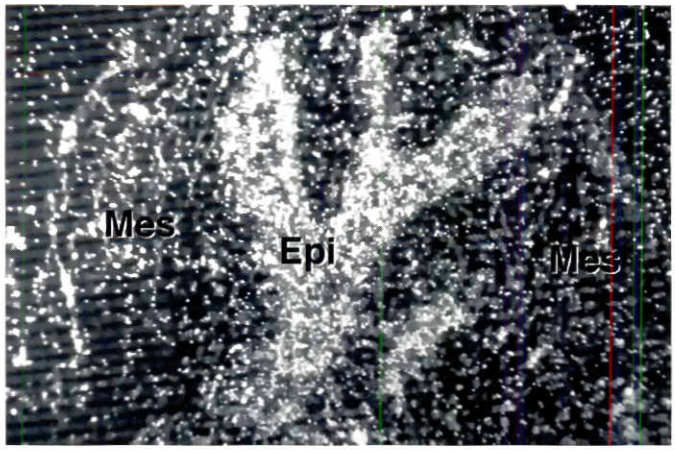

Fig. 2. Detection of transcripts for KGF-R by *in situ* hybridization. *Note labeling of the epithelium using a ^{33}P-labeled anti-sense riboprobe. Labeling of the mesenchyme is considerably lower and based upon hybridization with a sense riboprobe represents background. For controls see (Finch et al., 1995).*

without testosterone (10^{-8} M), but not in 3T3 cells, Dunning tumor fibroblasts or isolated rat VP mesenchyme. By *in situ* hybridization KGF-R transcripts were observed in the epithelial ducts and not in the surrounding mesenchyme (Fig. 2).

Exogenous KGF supports prostatic ductal branching morphogenesis in the absence of testosterone

The newborn rat VP is composed of 3 to 4 main ducts each having 3 to 6 branches surrounded by mesenchyme (Fig. 3a). In the presence of testosterone neonatal rat VPs grew and underwent extensive ductal branching morphogenesis in a dose-dependent manner during 6 days of culture (Fig. 3b-d). Testosterone at 10^{-8} M gave maximal growth and ductal branching morphogenesis, both of which were greatly reduced in VPs cultured without testosterone (Fig. 3e) even though development proceeded for a few days in the absence of exogenous testosterone. This appears to be due to the carry over of endogenous testosterone because rat VPs cultured without testosterone, but with the antiandrogen cyproterone acetate (10^{-7} M) exhibited less development than rat VPs simply cultured without testosterone (Fig. 7a,d). As expected cyproterone acetate (10^{-6} and 10^{-7} M) in combination with testosterone (10^{-9} M) inhibited prostatic growth and ductal branching morphogenesis of rat VPs (Fig. 4).

DNA content of VPs cultured with testosterone increased in a dose-dependent manner. Maximal response was observed at a concentration of 10^{-8} M testosterone (Fig. 5). Because the VPs tended to flatten and thus appeared to enlarge substantially during the culture period, the apparent increase in size of the cultured explants was not associated with a comparable increase in DNA content. Nonetheless, DNA content increased with increasing testosterone concentrations to statistically significant levels at dosages of testosterone $\geq 10^{-8}$ M relative to explants cultured without testosterone. KGF (50 or 100 ng/ml) in the absence of testosterone was able to elicit extensive ductal growth and branching morphogenesis. The pattern of ductal branching morphogenesis induced by KGF was comparable and similar in magnitude to that elicited by testosterone (Fig. 6a-c). The morphogenetic effect of KGF in eliciting coordinated ductal branching in the absence of testosterone was not mimicked by either EGF (2.5, 5, 10, 20, 40 ng/

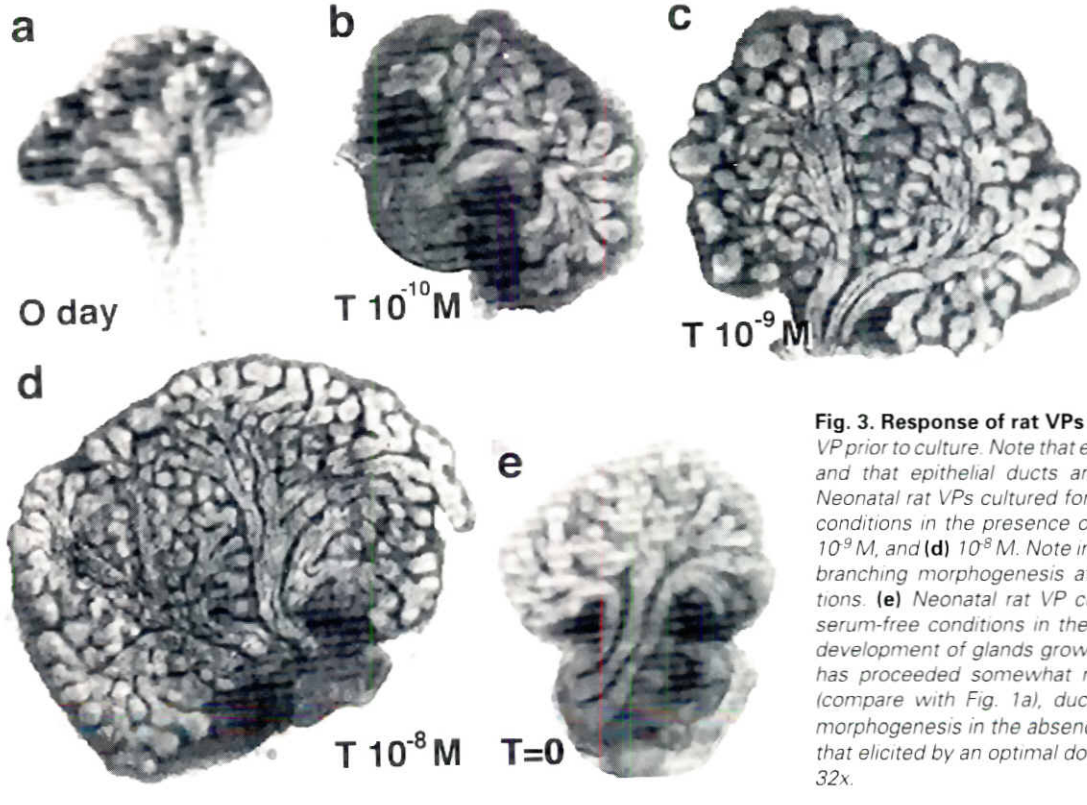

Fig. 3. Response of rat VPs to testosterone *in vitro*. (a) *Rat VP prior to culture. Note that each main duct has 3 to 6 distal tips and that epithelial ducts are surrounded by mesenchyme. Neonatal rat VPs cultured for 6 days in vitro under serum-free conditions in the presence of testosterone at* **(b)** *10^{-10} M,* **(c)** *10^{-9} M, and* **(d)** *10^{-8} M. Note increased ductal growth and ductal branching morphogenesis at higher testosterone concentrations.* **(e)** *Neonatal rat VP cultured for 6 days in vitro under serum-free conditions in the absence of testosterone. While development of glands grown in the absence of testosterone has proceeded somewhat relative to glands before culture (compare with Fig. 1a), ductal growth and ductal branching morphogenesis in the absence of testosterone is far less than that elicited by an optimal dose of testosterone. Magnification 32x.*

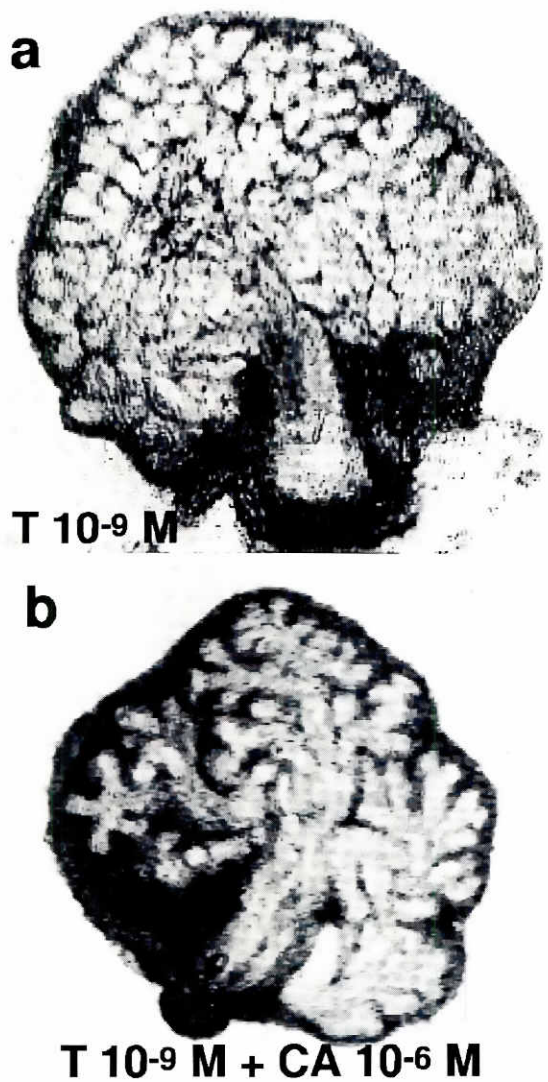

Fig. 4. Neonatal rat VPs cultured for 6 days *in vitro* under serum-free conditions in the presence of (a) testosterone at 10^{-9} M and (b) testosterone at 10^{-9} M plus cyproterone acetate at 10^{-6} M. *Note in* **(a)** *extensive ductal growth and ductal branching morphogenesis with testosterone. Neonatal rat VP cultured for 6 days with testosterone at 10^{-9} M plus cyproterone acetate at 10^{-6} M* **(b)** *exhibited reduced growth and ductal branching morphogenesis. Magnification, 32x.*

ml), TGFα (5, 10, 20, 40 ng/ml), acidic FGF (3 or 30 ng/ml), basic FGF (3 or 30 ng/ml) or HGF (1.8, 2.5, or 25 ng/ml) even though each of these growth factors promoted growth of either the epithelium or mesenchyme to variable extents in VP explant grown for 6 days *in vitro* (not illustrated). With the exception of KGF, none of the above growth factors were capable of eliciting normal ductal branching. An average of 82 ductal tips developed in glands cultured with testosterone 10^{-8} M. The number of ductal tips was increased from 23 (without testosterone) to 60 (100 ng/ml KGF alone) per explant which represents 73% of that elicited by testosterone 10^{-9} M (Fig. 6d). The amount of growth as measured by DNA content of VPs cultured with KGF (50 ng/ml and 100 ng/ml) in the absence of testosterone was comparable to that elicited by testosterone at 10^{-8} M (Fig. 6e). Prostatic development elicited by KGF was inhibited by cyproterone acetate (10^{-7} M) (Fig. 7). Suppression of ductal growth and branching morphogenesis was more profound in explants grown without testosterone plus cyproterone acetate versus explants grown simply without testosterone (Fig. 7).

Histological analysis demonstrated that newborn rat VPs before culture consisted of solid epithelial cords surrounded by an undifferentiated mesenchyme (Fig. 8a). During 6 days of culture in the presence of testosterone 10^{-8} M, most of the solid epithelial cords canalized and in such ducts the epithelium differentiated into tall columnar cells (Fig. 8b). VPs grown without testosterone contained solid epithelial cords surrounded by an undifferentiated mesenchyme, and thus changed little from glands obtained directly from newborn rats (compare Fig. 8a and c). Ductal lumen formation was just beginning in VPs cultured for 6 days in the absence of testosterone but with KGF at either 50 or 100 ng/ml, but was not as advanced as that in cultures treated with testosterone (Fig. 8d).

Neutralization of endogenous KGF with a monoclonal antibody or an inhibitory peptide

To further assess the role of KGF in prostatic development, rat VPs were cultured as explants for six days in the presence of testosterone (10^{-8} M) plus either the 1G4 anti-KGF Mab or peptide 412, a soluble portion of the KGF-R which can bind KGF and thus compete with active receptors for endogenous KGF. Both agents dramatically reduced testosterone-induced ductal growth and branching morphogenesis (Fig. 9) during 6 days of culture. To quantify the effects of Mab 1G4 on cultures of rat VPs, the following parameters were assessed: (a) the number of ductal tips per specimen, (b) epithelial area per specimen, and (c) DNA content per specimen (Fig. 10). When the anti-KGF Mab was added to testosterone containing medium, there was significant reduction in the number of ductal tips, epithelial area and DNA content (Fig. 10).

Discussion

While androgens are known to elicit their developmental effects via high affinity androgen receptors (French *et al.*, 1990), the effects of androgens on prostatic epithelial growth and ductal branching morphogenesis are mediated by paracrine activity of the associated androgen receptor-positive mesenchyme (Cunha *et al.*, 1992a; Cunha, 1994). This conclusion is supported principally by 2 lines of evidence: (a) the ontogeny of androgen receptors and

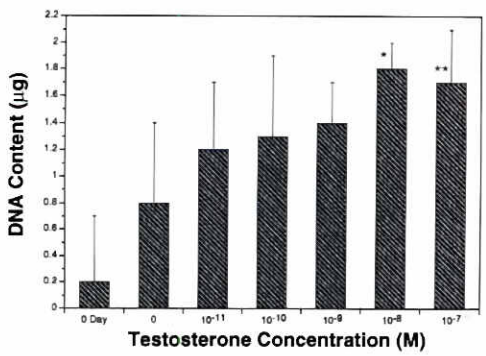

Fig. 5. DNA content of newborn rat VP's cultured for 6 days in serum-free medium at different concentrations of testosterone. *Data are based upon the analysis of 4 to 6 specimens per point. *, P< 0.05; **, P<0.001.*

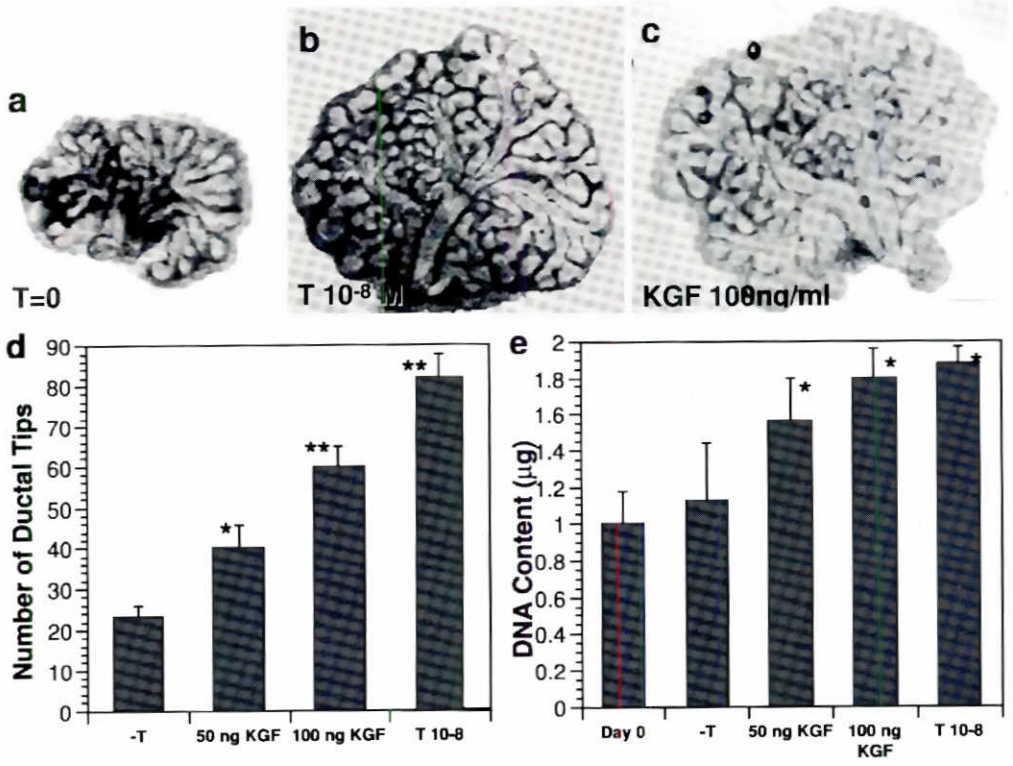

Fig. 6. Representative images of rat VPs *cultured for 6 days in serum-free medium* **(a)** *without testosterone,* **(b)** *with testosterone (10^{-8} M),* **(c)** *without testosterone+KGF 100 ng/ml. Note that ductal growth and branching morphogenesis elicited by KGF is comparable to that elicited by testosterone. Magnification 32x.* **(d)** *Number of ductal tips of VPs cultured for 6 days in serum-free medium with KGF or testosterone at the concentrations indicated.* **(e)** *DNA contents of VPs cultured for 6 days in serum-free medium at different concentrations of KGF or testosterone at the concentrations indicated. Data are based upon the analysis of 4 to 6 specimens per point. *, P< 0.05; **, P< 0.001*

(b) tissue recombination studies in which androgen receptor-negative Tfm (testicular feminization) epithelial cells were grown in association with androgen receptor-positive wild-type urogenital sinus mesenchyme. Ontogenic studies employing steroid autoradiography and immunocytochemistry have demonstrated androgen receptors in urogenital mesenchyme several days before androgen receptors can be detected in the epithelium. In fact, urogenital epithelia undergo "androgenic effects" before epithelial androgen receptors are detectable (Cooke *et al.*, 1991; Husmann *et al.*, 1991; Takeda and Chang, 1991; Prins and Birch, 1995). For example, androgen-induced prostatic buds appear at 19 days of gestation (Price and Ortiz, 1965), while androgen receptors in prostatic buds remain undetectable until shortly after birth (Cooke *et al.*, 1991; Husmann *et al.*, 1991; Takeda and Chang, 1991; Prins and Birch, 1995). Thus, there is a period of several days during which androgen-induced prostatic epithelial development occurs even though epithelial androgen receptors are undetectable. Even more compelling are the so-called Tfm/wild-type tissue recombination studies in which androgen-dependent epithelial growth and development is expressed in androgen receptor-negative Tfm epithelial cells grown in association with androgen receptor-positive mesenchyme (Cunha and Young, 1991; Donjacour and Cunha, 1993). These latter results clearly indicate that the effect of androgens on prostatic development involves paracrine effects of mesenchyme on the epithelium.

The following observations implicate KGF as an important mesenchyme-derived paracrine regulator of androgen-induced ductal growth and branching morphogenesis: (a) KGF transcripts are produced by fetal urogenital sinus mesenchyme (Finch *et al.*, 1995; Thomson and Cunha, unpublished), neonatal prostatic mesenchyme (present study), neonatal mouse seminal vesicle mesenchymal cells, and adult prostatic stroma (Yan *et al.*, 1992; Alarid *et al.*, 1994; Peehl and Rubin, 1995). (b) Neonatal and adult prostatic epithelial cells and neonatal mouse seminal vesicle epithelial cells express the KGF-R (Yan *et al.*, 1992, 1993a; Cunha *et al.*, 1994; Peehl and Rubin, 1995). (c) KGF stimulates growth of adult prostatic epithelial cells (Yan *et al.*, 1992, 1993a; Peehl and Rubin, 1995). (d) KGF (but not EGF, TGFα, acidic FGF, basic FGF or HGF) specifically stimulates coordinated ductal growth and branching morphogenesis of neonatal rat prostates (present study). (e) Neutralization of endogenous KGF with a monoclonal antibody to KGF inhibits androgen-induced ductal growth and ductal branching morphogenesis of neonatal rat prostates (present study); anti-KGF also inhibits androgen-induced growth and development of the neonatal mouse seminal vesicle (Alarid *et al.*, 1994). (f) A soluble peptide representing the KGF-binding domain of the KGF-R inhibits androgen-induced ductal growth and ductal branching morphogenesis of neonatal rat prostates (present study). This latter finding clearly implicates the epithelial KGF-R in the mechanism of action of KGF in androgen-induced VP development. Thus, KGF produced by androgen receptor-positive mesenchymal cells plays an important role in epithelial growth and ductal branching morphogenesis of the rat VP via the KGF-R on the epithelial cell surface. KGF has been also implicated as a paracrine mediator of progesterone action in the monkey uterus (Koji *et al.*, 1994).

KGF may have additional affects on the developing prostate as KGF has been implicated in the expression of epithelial differentiation markers in other models (Marchese *et al.*, 1990; Culig *et al.*, 1994). In the current study, potential affects of exogenous KGF on prostatic epithelial differentiation were assessed histologically. Testosterone stimulated epithelial differentiation as manifested by ductal lumen formation and differentiation of a tall columnar epithelium, neither of which occurred in explants grown without testosterone. A differentiation effect of exogenous KGF on the developing

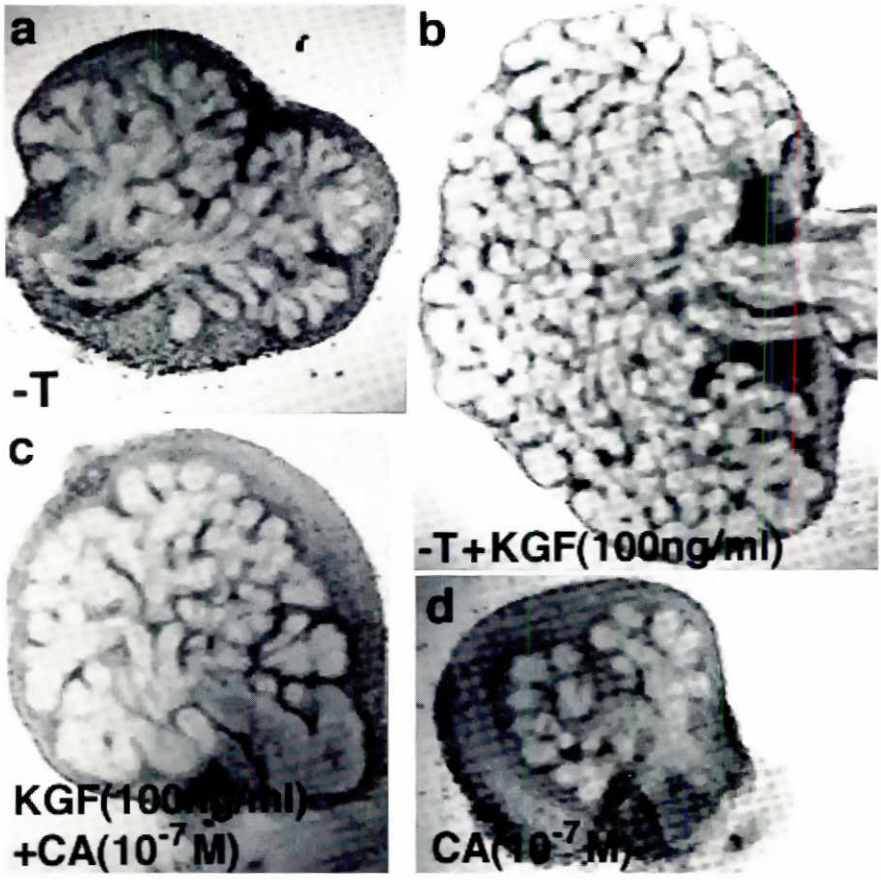

Fig. 7. Representative images of rat VPs *cultured for 6 days in serum-free medium* **(a)** *without testosterone,* **(b)** *without testosterone +KGF 100 ng/ml,* **(c)** *without testosterone+KGF 100 ng/ml+cyproterone acetate at 10^{-7} M, or* **(d)** *without testosterone +cyproterone acetate at 10^{-7} M. Note in* **(b)** *extensive ductal growth and ductal branching morphogenesis elicited by KGF which* **(c)** *is greatly inhibited by cyproterone acetate at 10^{-7} M. Magnification, 32x.*

rat VP was observed as initiation of lumen formation of the solid ducts. While the effect of KGF in the absence of testosterone on epithelial differentiation was considerably less robust than that of testosterone, this may be due to a requirement of direct action of testosterone on the developing prostatic epithelium mediated by intra-epithelial androgen receptors that normally appear shortly after birth. In this regard, recent studies indicate that the initial expression of prostatic secretory proteins early in puberty involves a synergistic interaction between KGF and testosterone (Lopes *et al.*, 1996) which suggests that the KGF signaling pathway and the androgen receptor pathway may intersect at some level. This suggestion is supported in the present study by the observation that the growth and morphogenetic effects of KGF on the developing prostate can be inhibited by a steroidal anti-androgen, cyproterone acetate, and by recent studies on transfected prostatic carcinoma cell lines (Culig *et al.*, 1994). Thus, KGF may influence the androgen-signaling pathway normally required for prostatic development in an androgen-depleted environment.

KGF transduces its signal to the cytoplasm through transmembrane tyrosine kinase receptor which is a variant of FGFR-2 (FGFR-2IIIb or KGF-R) (Johnson and Williams, 1993). Orr-Urtreger and collaborators examined the developmental expression of the KGF-R by *in situ* hybridization (Orr-Urtreger *et al.*, 1993). During organogenesis KGF-R was localized to the surface ectoderm and epithelium of internal organs. This pattern of KGF-R expression has been confirmed and extended by Finch *et al.*, who demonstrated that KGF-R transcripts are expressed in the epithelium of the embryonic prostatic anlagen (urogenital sinus), while KGF transcripts are expressed generally in mesenchyme and specifically in urogenital sinus mesenchyme (Finch *et al.*, 1995). Given the extensive expression of KGF and the KGF-R in the developing embryo, it might be expected that disruption of the KGF gene would have profound effects on the development of structures composed of epithelium and mesenchyme. Surprisingly, a KGF knockout mouse appears to be completely normal except for defects in hair development (Guo *et al.*, 1996). However, the prostates of these animals were not examined. The KGF knockout mouse may represent yet another example in which compensatory pathways have been utilized.

Another potentially interesting splice variant of FGFR-2 that occurs in prostatic epithelial cells involves the deletion of exon 16 from the intracellular domain that contains an autophosphorylation site (tyrosine 789) required for interaction with phospholipase Cγ1 (Yan *et al.*, 1993b). This splice variant may have a reduced capability of interacting with intracellular signal-transducing molecules. The potential importance of dominant negative forms of FGFR-2 is emphasized by the findings of Peters and collaborators who expressed in transgenic mice a membrane-bound truncated form of FGFR-2IIIb using the surfactant promoter. Tissue specific expression of this truncated form of the KGF-R lacking the intracellular domain inhibited ductal branching morphogenesis of embryonic lung buds (Peters *et al.*, 1994). Similarly, the targeting of this truncated form of the KGF-R to skin with the keratin 14 promoter causes a thinning of the epidermis and impaired wound healing (Werner *et al.*, 1993) as well as dramatic impairment of ductal growth in the mammary epithelium (Cunha and Young, unpublished). The fact that in the absence of testosterone exogenous KGF elicited ductal growth and coordinated ductal branching morphogenesis in the rat ventral prostate (present study), but only elicited epithelial growth without ductal branching morphogenesis in the mouse seminal vesicle *in vitro* (Alarid *et al.*, 1994) may mean that different splice variants of the FGFR-2 gene may be utilized in these 2 developing glands.

Ductal branching morphogenesis is a highly coordinated process involving many cellular and molecular events including: epithelial proliferation, changes in cell shape, cell-extracellular matrix interactions (ECM), and remodeling of the basement membrane and ECM. While KGF is an epithelial mitogen both *in vitro* (Aaronson *et al.*, 1990; Marchese *et al.*, 1990) and *in vivo* (Pierce *et al.*, 1994; Ulich *et al.*, 1994a,b; Yi *et al.*, 1994), the present study suggests that KGF may also be an epithelial morphogen responsible for patterning ductal branching. For exocrine glands mesenchyme

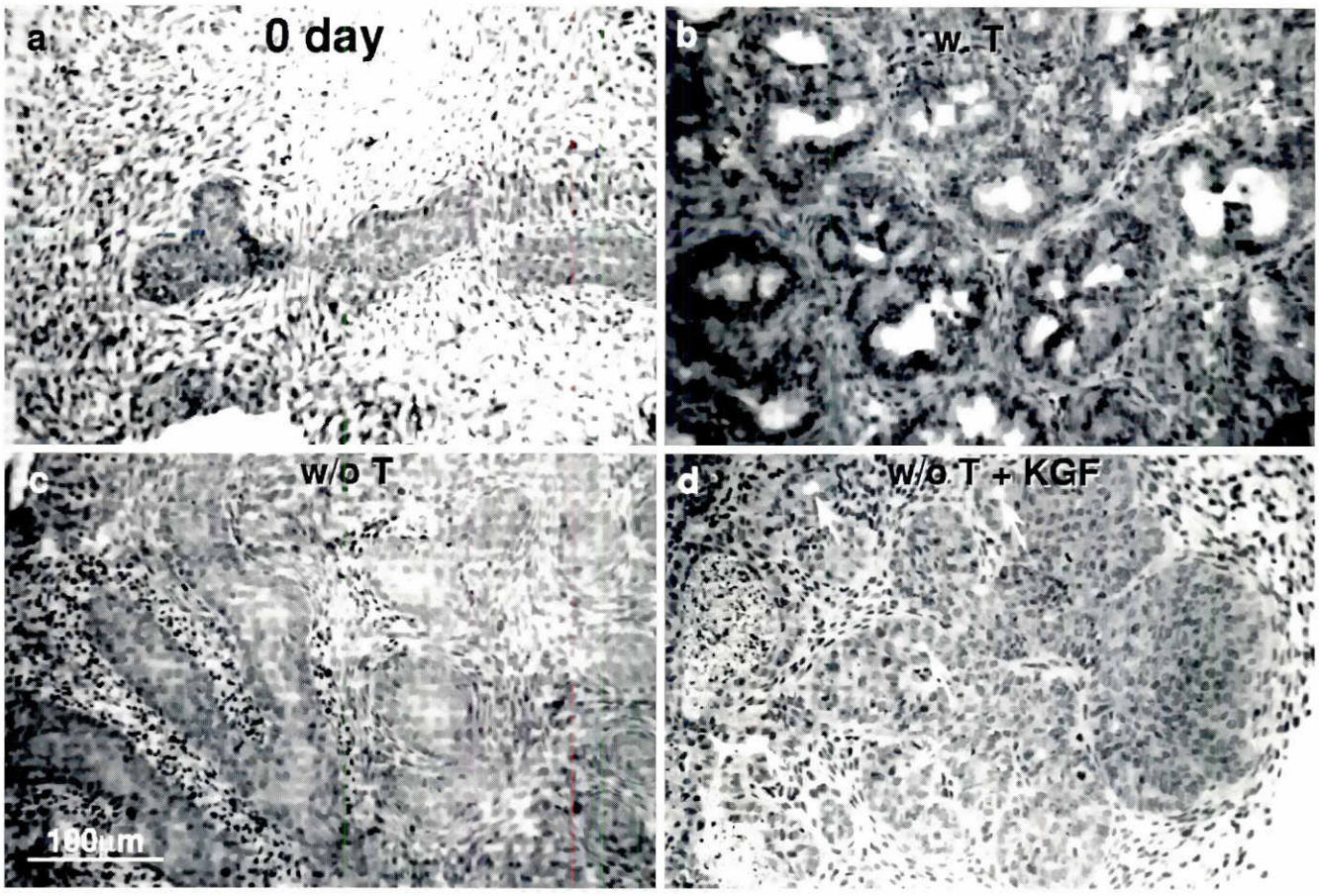

Fig. 8. Histological analysis of VPs cultured with testosterone or KGF for 7 days in a serum-free organ culture system. **(a)** *Newborn rat VP (0 day) before culture. Note solid cords of undifferentiated epithelium. For* **b-d** *all specimens were cultured for 6 days.* **(b)** *Newborn rat VP cultured in basal medium containing testosterone (10^{-9} M) (w. T). Note that lumen formation has occurred with the differentiation of tall columnar epithelial cells.* **(c)** *Newborn rat VP cultured in basal medium without testosterone (w/o T). Note solid cords of undifferentiated epithelium.* **(d)** *Newborn rat ventral prostate cultured in basal medium without testosterone containing KGF (100 ng/ml) (w/o T+KGF). Note that the solid epithelial cords have begun to canalize. Many small lumina (arrows) are present. Bar, 100 μm.*

specifies the pattern of ductal branching (Wessells, 1977). In the case of the prostate the overall developmental process is driven by androgens and results in the development of the various lobar subdivisions of the gland (ventral, dorsal, lateral and anterior lobes) each having a distinctive pattern of ductal branching (Sugimura *et al.*, 1986b; Cunha *et al.*, 1987; Hayashi *et al.*, 1991). It should be emphasized that epithelial growth per se constitutes only part of the overall process of ductal branching morphogenesis. The results reported herein demonstrate that only KGF in the absence of testosterone could elicit ductal growth and branching morphogenesis comparable to that induced by testosterone. Other growth factors (EGF, TGFα, acidic FGF, basic FGF or HGF) stimulated VP growth to variable extents, but did not elicit normal ductal branching. This means that KGF can orchestrate the complex processes (epithelial proliferation, changes in cell shape, cell-ECM interactions, and remodeling of the basement membrane and ECM) which are required to generate specific patterns of ductal branching. A morphogenetic role for KGF is also demonstrated by the inhibition of seminal vesicle morphogenesis by anti-KGF (Alarid *et al.*, 1994) and the targeting of a dominant negative KGF-R to the developing lung as described above (Peters *et al.*, 1994).

Our studies provide the first direct evidence that KGF is a paracrine mediator of androgens in normal prostatic development affecting ductal growth, ductal branching morphogenesis and epithelial differentiation. Previous studies have used prostatic tumor cell lines (Yan *et al.*, 1992, 1993a), cell cultures derived from human prostatic clinical specimens (histologically normal, adenocarcinoma-containing tissues as well as benign prostatic hyperplasia specimens) (Lin *et al.*, 1994; Peehl and Rubin, 1995), or cells derived from growth-quiescent adult rat prostate (Yan *et al.*, 1992, 1993a). Cell culture studies have shown (a) that adult prostatic stromal cells produce KGF, (b) that adult prostatic epithelial cells proliferate in response to KGF *in vitro*, and (c) that adult prostatic epithelial cells have receptors for KGF. Whereas the adult prostate is growth-quiescent and morphogenetically dormant (Tuohimaa, 1980; Sugimura *et al.*, 1986c; Banerjee *et al.*, 1991), neonatal and pubertal prostates are characterized by intense morphogenetic activity and high rates of prostatic epithelial DNA synthesis (Sugimura *et al.*, 1986b,c; Hayashi *et al.*, 1991). Nonetheless, earlier cell culture studies are in agreement with the developmental studies presented herein, although the actual role of KGF in the growth-quiescent adult prostate remains to be determined. In this

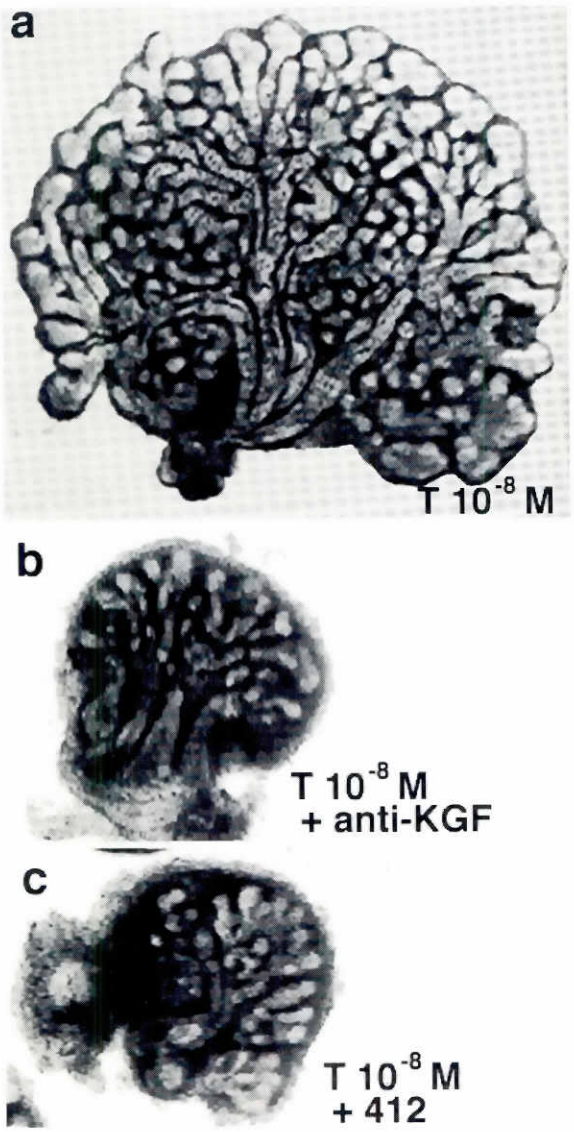

Fig. 9. Rat VPs incubated for 6 days *in* **(a)** *the presence of testosterone (10^{-8} M) plus mouse IgG (60 μg/ml) or* **(b)** *testosterone in combination with anti-KGF Mab (60 μg/ml) or* **(c)** *peptide 412 (60 mg/ml). Note dramatic inhibition of growth and ductal branching morphogenesis elicited by anti-KGF and peptide 412. Magnification 32x.*

regard, the *in vivo* growth-quiescence of adult prostatic epithelial cells is in stark contrast with high rates of epithelial proliferation achieved by adult prostatic epithelial cells *in vitro*. To fully understand the role of KGF in prostatic growth, at the very least the expression and action of KGF must be examined during periods of actual prostatic growth.

A key feature of prostatic growth is that DNA synthesis does not occur homogeneously throughout the gland but is regulated by androgen in a spatially precise manner with DNA synthesis being concentrated at the tips of elongating ducts (Sugimura *et al.*, 1986c; Banerjee *et al.*, 1991; Prins *et al.*, 1992). Enhanced DNA synthesis at the tips of elongating prostatic ducts could be due to focal synthesis of trophic growth factors, focal expression of growth factor receptors, focal release or activation of growth factors, focal differences in proteoglycan distribution and turnover, or focal synthesis of growth inhibitory factors such as TGFβ. Glands growing *in vitro* in the presence of exogenous KGF receive global stimulation that presumably is homogeneous throughout the gland. Surprisingly, global stimulation by exogenous KGF *in vitro* elicits normal ductal patterning which implies a degree of spatial regulation of epithelial DNA synthesis in the face of a homogenous stimulation by exogenous KGF. This paradox suggests the possibility that KGF-R activity may be non-uniform with increased activity at the ductal tips and lower activity proximally. The extraordinary complexity of splice variants possible for FGFR-2 including the expression of dominant negative forms of the receptor, if expressed in a spatially meaningful pattern, may explain this interesting paradox.

Materials and Methods

Animals and organ culture

VPs were dissected from newborn Sprague-Dawley rats which were obtained from Simonsen Laboratories (Hollister, CA). VPs were grown in an organ culture system as previously described with modifications (Shima *et al.*, 1990). Briefly, neonatal rat VPs were placed on Millipore CM filters (Millipore Corporation, Bedford, MA) which were supported on triangular metal grids in 35 mm Petri dishes (Falcon, Cockeysville, MD). Alternatively, the filters were simply floated on the medium. A basal medium of DMEM-Ham's F-12 (1:1 vol/vol) supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), and gentamycin (50 μg/ml) was utilized in all experimental groups. Where indicated, testosterone, cyproterone acetate (a gift from Schering AG, Berlin), KGF, a neutralizing monoclonal antibody (1G4) to KGF or soluble inhibitory KGF receptor peptide 412 were added at concentrations indicated (Bottaro *et al.*, 1993). The medium was changed every three days.

KGF, KGF monoclonal antibody, and peptide 412

Human KGF was generated with a T7 vector bacterial expression system (Ron *et al.*, 1993) and purified by heparin-Sepharose chromatography to ~90% homogeneity as judged by silver-stain analysis of SDS-polyacrylamide gels and immunoblot analysis. The murine monoclonal antibody (Mab) to KGF, designated 1G4 (Bottaro *et al.*, 1993), was purified and concentrated to 1 mg/ml from hybridoma-conditioned cell culture

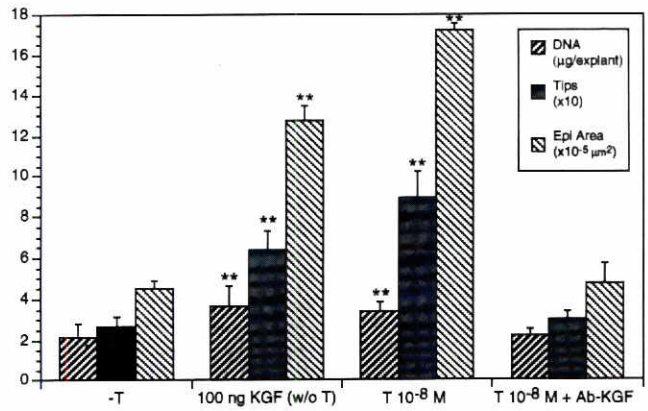

Fig. 10. Effect of testosterone, KGF, and anti-KGF on rat VPs *as assessed by DNA content per rat VP, (μg/explant), the number of ductal tips per rat VP, and epithelial area (μm^2) per rat VP following 6 days of incubation in serum-free medium. Data are based upon the analysis of 4 to 6 specimens per point. **, P< 0.001.*

medium with Gamma Bind resin (Pharmacia; Piscataway, N.J.) as per the manufacturers instructions. Peptide 412 is a synthetic peptide corresponding to amino acid residues 199-223 of the mouse KGF-R sequence (IISGINSSNAEVLALFNVTEMDAGEY). This peptide, which comprises a portion of the KGF-binding domain of the receptor, was shown to block KGF mitogenic activity as a consequence of its ability to bind directly to KGF (Bottaro *et al.*, 1993) Thus, 412 competes with the KGF-R for endogenous KGF (Bottaro *et al.*, 1993).

Measurement of DNA content

Cultured VPs were harvested and stored at -70°C until DNA assays were performed. The samples were thawed in diaminobenzoic acid (Hinegardner, 1971), and incubated at 60-65°C for 1 h. Following incubation, the solution was neutralized with 1N HCl, and DNA content was measured by fluorospectrography. Statistical differences were determined using Student's *t* test for independent samples.

Image analysis

Images of whole-mount photographs of VPs were captured and digitized with a Dage-MTI CCD-72 TV camera interfaced to a Macintosh IIfx computer and processed with Prism View software (Dapple, Sunnyvale, CA) as previously described (Alarid *et al.*, 1994). To quantify epithelial area, the outline of the epithelium was traced manually using Prism View's drawing tools. The outline was selected by Prism View's thresholding tools, converted to a binary image, and epithelial area was computed by Prism View. To assess the number of ductal tips per explant, whole-mount images of VPs were digitized and printed using a 600 dpi Laserwriter printer. Individual ductal tips were counted manually from the prints. Statistical significance was determined using Student's *t* test for independent samples.

Reverse transcriptase-polymerase chain reaction

Tissues dissected from rats or harvested from culture were solubilized in 100 μl of guanidine extraction buffer (4 M guanidine thiocyanate, 0.5 M sodium acetate, 0.005 M EDTA pH 7.5, 1% sarcosyl, 1 M ß-mercaptoethanol). RNA was purified by pelleting 100 μl of the sample through 100 μl of a CsCl cushion (5.7 M CsCl, 0.1 M EDTA, 0.01 M Tris-Cl pH 7.5) by ultracentrifugation. For first strand synthesis 49 μl of the purified RNA solution was incubated with 5x reverse transcriptase (RT) buffer (250 mM Tris pH 8.3, 30 mM $MgCl_2$, 200 mM KCl), the four deoxynucleotide triphosphates (dNTPs, Pharmacia), random hexamer nucleotide primers (2 μg/μl stock, Pharmacia) and reverse transcriptase (NBL) for 90 min at 42°C.

PCR was performed according to Rappolee *et al.* with slight modifications (Rappolee *et al.*, 1989). The reaction was run in tubes containing 10xPCR buffer (500 mM KCl, 100 mM TrisCl pH 8.3, 15 mM $MgCl_2$), dNTPs (1.25 mM each), Amplitaq (2.5 units, Perkin-Elmer) and the product from the RT reaction. For all reactions a Ericomp thermocycler was programmed as follows: 94°C for 1 min; 60°C for 2 min; 72°C for 3 min for 45 cycles. The following primers were used to detect rat KGF mRNA : 5'-AGAATTCCAACTGCCACAGTCATGATTTC and 5'-CAATCTAGAATTCACAGATAGGAGGAGGC (Yan *et al.*, 1991) giving an amplified fragment of 423 base pairs. Oligonucleotide primers for rat KGF-R were as follows: 5'-CACTCGGGGATAAATAGCTCCAAT and 5'-TATCCTCACCAGCGGGGTGTTGGA giving an amplified fragment of 407 base pairs (Miki *et al.*, 1991). Primers for glyceraldehyde phosphate dehydrogenase (GAPDH) were 5'-TGATGACATCAAGAAGGTGGTGAAG and 5'-TCCTTGGAGGCCATGTGGGCCAT (Ercolani *et al.*, 1988) giving an amplified fragment of 244 base pairs. Oligonucleotide primers for β-actin were as described previously (Rappolee *et al.*, 1988). Products of PCR reactions were run on 2% Seachem ME agarose gels (FMC) in 0.5 M TBE buffer containing ethidium bromide. Tissue specificity was investigated for all PCR assays.

In situ hybridization

In situ hybridization was performed on deparaffinized tissue sections using antisense riboprobes essentially as described previously (Finch *et al.*, 1995). ^{33}P labeled riboprobes were applied to tissue sections at a concentration of 0.2 ng/ml/kb length of cloned fragment in hybridization buffer (50% deionized formamide; 0.3 M NaCl; 20 mM Tris HCl, pH 8.0; 5 mM EDTA; 10% Dextran sulfate; 1xDenhardt's buffer; 0.5 mg/ml yeast RNA). Tissue sections were hybridized at 55°C for 18 h and then washed under high stringency conditions (2xSSC, 50% formamide at 65°C). Non-hybridized riboprobe was digested with 20 mg/ml RNase A for 30 min at 37°C. Further washing of the slides under high stringency conditions was followed by dehydration through graded alcohols containing 0.3 M ammonium acetate. Sections were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in H_2O, air dried and exposed at 4°C. After appropriate exposure times, slides were developed photographically in Kodak D-19 developer and counterstained with 0.02% Toluidine Blue.

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