Androgen-Stimulated Human Prostate Epithelial Growth Mediated by Stromal-Derived Fibroblast Growth Factor-10

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Abstract. It has been suggested that prostate homeostasis is regulated indirectly by androgens through stromal-epithelial interactions in part by factors from the stromal cells acting on receptors in epithelial cells. In this report, the role of fibroblast growth factor (FGF)-10 in prostatic epithelial proliferation was investigated. The expression of FGF-10 mRNA was apparent in primary-cultured stromal cells, but not in epithelial cells derived from human tissue from patients with benign prostatic hyperplasia (BPH). The mitogenic activity of human recombinant FGF-10 assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation was demonstrated in isolated epithelial cells, but not in cultured stromal cells. No mitogenic activity of dihydrotestosterone (DHT) for either epithelial or stromal cells could be demonstrated, but quantitative PCR (real-time PCR) with a double-labeled fluorogenic probe demonstrated that expression of FGF-10 in stromal cells was enhanced 5.3-fold at a DHT concentration of 100 pM. Androgen receptor mRNA levels showed no significant change with DHT at concentrations less than 100 pM, but were reduced to 50% of control levels at a DHT concentration of 10 nM. These results suggest that stromal-derived FGF-10 stimulates human prostatic epithelial growth and its mRNA expression is induced by androgens, without an increase in the androgen receptor mRNA. Moreover, FGF-10 may be involved in the development or support of human BPH.

Key words: Fibroblast growth factor, Androgen, Stromal-epithelial interaction

ANDROGENS are essential for development, growth, differentiation and maintenance of the prostate [1–10]. The regulation of these processes requires the activation of gene networks that are responsive and dependent on activation of the androgen-receptor complex [8].

Although several hypotheses have been proposed to explain the basis of androgen-induced cell growth, the exact mechanisms remain to be elucidated [1, 5, 6, 10]. There is little evidence for a direct response of DNA synthesis to androgens in isolated cells [3, 11]. Much evidence indicates that the mitogenic action of androgen on the prostatic epithelium is mediated indirectly by androgen action on stromal cells, although both stromal and epithelial cells have androgen receptors [3, 11].

Ductal branching morphogenesis of the rat prostate epithelium is thought to be elicited by androgen via mesenchyme through directional paracrine action from mesenchyme to epithelium [1]. This “paracrine-theory” suggests that upon exposure to androgen, stromal cells are stimulated to synthesize and release factors that act on epithelial cell receptor [3, 12]. Several polypeptide growth factors have been suggested as candidates. These include members of the fibroblast growth factor family, insulin-like growth factors, epidermal growth factor, hepatocyte growth factors and transforming growth factor alpha [13–18]. Among these, FGF-7, also called keratinocyte growth factor, is a strong candi-
date since it is made by stromal cells, specifically stimulates proliferation of prostatic epithelial cells through a specific receptor, and is increased by androgen in the rat [6]. In human prostate, FGF-7 expression by stromal cells is increased by synthetic androgen R1811 [3]. Expression of FGF-7 was limited to the human prostatic stroma in primary culture, and exhibited a mitogenic effect on cultured prostatic epithelial cells [19]. FGF-7 also exhibited properties of a mesenchyme-derived paracrine mediator of androgen-induced epithelial growth and ductal branching morphogenesis in rat ventral prostate organ cultures [1].

Recently, FGF-10, a sequence homologue of FGF-7, has been identified in rat [20] and human lung [21], and is localized in chromosome 5p12-p13 of the human genome [21]. FGF-10 is expressed at high levels in lung in mesoderm [20] and has a mitogenic effect on lung morphogenesis of the rat [22] and both initial budding and continuous outgrowth of vertebrate limbs in the chick [23]. FGF-10 may also act during wound healing [24]. Recently, expression of FGF-10 has been localized to the stromal cells from both normal prostate and well-differentiated transplantable rat prostate tumors (Lu W et al., submitted for publication). Similar to FGF-7, the expression of FGF-10 was androgen-responsive and this factor exhibited high affinity for the epithelial cell receptor, FGF receptor (FGFR) 2 IIIb. But the pattern of expression of FGF-10 is largely restricted to muscle-like stromal cells relative to FGF-7 which is expressed in both muscle-like and fibroblast-like stromal cells. In contrast to FGF-7, FGF-10 has affinity for FGFR1 IIIb and binds to 4 times more peri-cellular matrix heparan sulfate sites than does FGF-7. These properties suggest that FGF-10 may have both overlapping and unique activities compared to FGF-7.

In human prostate, it is unknown how FGF-10 functions in stromal-epithelial interactions. In this study, the properties of FGF-10 were evaluated with a primary culture model derived from human benign prostatic hyperplasia (BPH) in order to clarify the molecular mechanisms of androgen-induced growth of prostate epithelial cells and stromal interaction. The objectives of the present study were to test the hypothesis that stromal-derived FGF-10 induces epithelial growth and its expression is regulated by androgen.

Materials and Methods

1. Isolation of epithelial and stromal cells from human prostatic tissue

Well-developed nodule of tissues exhibiting BPH, the pathologic diagnosis of which was myoadenomatous hyperplasia, excised from surgical specimens obtained from two patients who underwent subcapsular prostatectomy at the Gunma University Hospital. Primary-cultured stromal cells were obtained by means of an explant technique as reported previously [25]. Briefly, the tissue was cut into pieces less than 1 mm³, and plated on uncoated dishes. The plated fragments were maintained with RITC 80-7 medium (Kyokuto, Tokyo) with 10% fetal bovine serum (FBS; Life Technologies, New York, NY) and 10⁻⁸ M dihydrotestosterone (DHT; Sigma, St.Louis, MO). Within 8 weeks, stromal cells predominated.

Similar to stromal cells, epithelial cells were cultured with RITC 80-7 medium supplemented with 10% FBS. After the appearance of epithelial-like cells around the fragments, the culture medium was changed to serum-free PrEBM media (Clonetics, Walkersville, MD) containing bovine pituitary extract, triiodothyronine, epinephrine, hydrocortisone, transferrin, insulin and retinoic acid (all from Clonetics), and cells were grown at 37°C in a humidified incubator in 5% CO₂. Second-passage cells were used for the following experiments.

Both prostate epithelial and stromal cells were immunocytochemically characterized. The cells were cultured in Chamber Slide (Lab-Tek, Naperville) for 2-3 days. After removal of the culture medium, the cells were immediately fixed in acetone-methanol solution for 5 min. The antibodies to cytokeratin, prostate specific antigen (PSA), desmin and smooth muscle alpha-actin (EPOS antibody; DAKO Japan, Tokyo) were used at a manufacturer’s recommended concentrations for 5 min. The antibodies to FGF-10 functions in stromal-epithelial interactions. In this study, the properties of FGF-10 were evaluated with treatments were visualized after 5-min incubation with 3,3’-diaminobenzidine tetrahydrochloride as chromogen (Dako Japan), and counterstained with hematoxylin for 10 seconds.

The concentration of PSA in the medium was measured with an AIA-PACK PA kit (TOSOH, Yamaguchi) in cultured conditioned medium.
2. **Reverse transcriptase — polymerase chain reaction (RT-PCR)**

Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Isolated epithelial and stromal cells, and BPH tissue were employed for detection of FGF-10 mRNA. Prostate cancer tissue, pathologically diagnosed as poorly-differentiated adenocarcinoma, was obtained by needle-core biopsy. LNCaP cell line was purchased from American Type Culture Collection (Rockville, MD). First-strand cDNA from the RNA template was generated with Ready-To-Go You-Prime-First-Strand Beads (Pharmacia Biotech K. K. Tokyo). A primer pair that recognizes the unique region of human FGF-10 cDNA among FGF family members was used for PCR with AmpliTaq Gold (Perkin Elmer, Foster City, CA). The sequences were 5’-CTTCTCCTCTCCTCCAGCG-3’ for the forward primer and 5’-TTGACGGCAA-CAACTCCG-3’ for the reverse primer. The PCR reaction mixture, with MgCl2 at a concentration of 2.5 mM, was heated at 58°C in the annealing step and amplified for 35 cycles. The DNA sequences of each PCR product were confirmed with a Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

3. **Cell proliferation assay**

The mitogenic assay with recombinant human FGF-7 (Promega, Madison, WI), FGF-2 (Upstate biotechnology, Lake Placid, NY), and human FGF-10 (Sumitomo Pharmaceuticals, Osaka) was performed on prostatic epithelial and stromal cells. Primary cultured prostatic epithelial cells were trypsinized and plated at a density of 3000 cells per well in a 96-well plate (Corning Glass Works, Corning, NY) with PrEBM serum free medium supplemented with insulin (5 mg/ml), dexamethasone (1 mM), bovine serum albumin (BSA) (2.5 mg/ml), and oleic acid (0.1 mg/ml) along with different concentrations of FGF-10, FGF-7 and DHT. After 48 h of incubation, BrdU incorporation was assessed with a kit, cell proliferation ELISA, BrdU (colorimetric) (Boehringer Mannheim K. K., Tokyo) according to the manufacturer's instructions. As well as the epithelial cells, the mitogenic activity of stromal cells was evaluated after a 48-h incubation in serum-free RITC medium with 2.5 mg/ml insulin, 0.1 mg/ml BSA, 4 mg/ml oleic acid and 10 mg/ml transferrin in the presence of various concentrations of FGFs or DHT. Recombinant FGFs were solubilized with 1% BSA plus 0.1% [3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS: Sigma) in PBS to prevent the loss of FGFs due to their adherence to the surface of the wells [26].

4. **Quantitative mRNA analysis of FGF-10 and androgen receptor (AR)**

For quantitative mRNA analysis, isolated stromal cells were incubated in T-25 flasks with serum-free RITC 80-7 supplemented with 2.5 mg/ml insulin, 0.1 mg/ml BSA, 4 mg/ml oleic acid, and 10 mg/ml transferrin for 24 h prior to 10-h exposure with DHT at concentrations of 10^{-12}, 10^{-10}, and 10^{-8} M. The assay medium in the absence of DHT was used as a control.

RNA from stromal cells was transcribed to cDNA as above. Sample cDNAs were amplified in the Model 7700 Sequence Detector (PE Applied Biosystems, Perkin Elmer Japan, Chiba) with the FGF-10 forward primer 5’-GGTGCTGTCTTGGTGTTCTT-CC-3’, FGF-10 reverse primer 5’-CTGGTGACACC-ATGTCTTGAC-3’ and FGF-10 dual-labeled fluorogenic probe (TaqMan probe) 5’(FAM)-TCCCTGT-CACCTGCCAAGGCTT-(TAMRA)-3’ for FGF-10 with the TaqMan PCR Reagent Kit. AR expression was assessed with the AR forward primer 5’-AGGAA-CTCGATCTCTATCTTCG-3’, AR-reverse primer 5’-CTGCCATTATTTCCGGAA-3’ and TaqMan probe 5’(FAM)-CGCTTTCTACCAGCTCACCAGA-GCTCCT-(TAMRA)-3’. Known concentrations of serially diluted FGF-10 cDNA (Sumitomo Pharmaceuticals, Osaka) or AR cDNA generated by PCR were employed as the standard for quantitation of sample cDNAs. To determine the copy number of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA, TaqMan GAPDH Control Reagents (PE Applied Biosystems) were used according to the manufacturer’s instructions. The cDNA copy number of FGF-10 or AR was normalized to the GAPDH result from the same volume of sample.
5. **Statistical analysis**

Data are shown as the mean ± SD. Statistical analysis was carried out by the nonparametric Mann Whitney U-test with StatView J-4.02, Macintosh program [27]. A probability of <0.05 was considered significant.

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**Results**

1. **Isolation and characterization of epithelial and stromal cells**

Both epithelial and stromal cells were isolated from the transition zone [26] of human prostate by the explant technique [25]. Epithelial cells were stained positively with cytokeratin 19 and PSA antibody (Fig. 1a–d), whereas spindle-shaped stromal cells exhibited smooth muscle-like properties which included display of desmin and smooth muscle alpha actin (Fig. 1e–h). These cells showed no cytokeratin antigen on immunocytochemical staining. PSA declined less than 0.2 ng/ml in the conditioned medium of epithelial cells at second passage and 4 days of culture. The epithelial cells failed to divide after the fourth passage.

2. **Stromal cell-specific expression of FGF-10 in human prostate**

Expression of FGF-10 was determined by RT-PCR of 20 ng of total RNA per sample. The results indicated that FGF-10 mRNA was amplified specifically in isolated stromal cells and could not be detected in epithelial cells. Strong mRNA signals were observed.
in samples of BPH tissue but only weak mRNA levels were observed in cancer tissue. FGF-10 mRNA could not be detected in the LNCaP cell line, an androgen-responsive prostatic carcinoma cell line [10] (Fig. 2).

3. Specific mitogenic activity of FGF-10 on prostatic epithelial cells

The mitogenic activity on epithelial cells of recombinant human FGF-10 was compared to FGF-7. FGF-10 enhanced proliferation on cultured prostatic epithelial cells by 27% at a concentration of 1 ng/ml. The level of BrdU incorporation increased to 130% at 10 ng/ml, which was comparable to the mitogenic activity induced by FGF-7 (Fig. 3a). By contrast, DHT depressed DNA synthesis with no positive effect on epithelial growth (Fig. 3a).

Similar to FGF-7, FGF-10 showed no mitogenic effect on stromal cells but FGF-2 had a strong mitogenic action at a concentration of 1 ng/ml. DHT had no effect on the growth of stromal cells (Fig. 3b).

4. Androgen-induced expression of FGF-10 in cultured prostatic stromal cells

Real-time PCR is a novel method for quantitation of cDNA content in the 5’ nuclease assay [28, 29]. Figure 4a shows amplification plots of different reactions, which were performed on 1:10 dilutions of full-length human FGF-10 cDNA. Amplification plots shifted to the right as the input target quantity was reduced. The threshold cycle (Ct), defined as the point at which the amplification plot crosses the PCR baseline [28, 29], decreased logarithmically with the input cDNA copy number. The Ct of each cDNA sample extracted from stromal cells was within the linear phase, so that Ct values can be used as a quantitative measurement (Fig. 4b). The coefficients of correlation between Ct values and the starting target copy number were 0.996, 0.986, and 0.991 when measuring cDNA of FGF-10, GAPDH and AR, respectively.

The real-time PCR demonstrated that FGF-10 mRNA was significantly induced with DHT at concentrations of $10^{-12}$, $10^{-10}$ and $10^{-8}$ M (Fig. 4c).
The level of expression peaked at 5.3-fold with DHT at $10^{-10}$ M, a concentration below the physiologic level ($10^{-9}$ to $10^{-7}$ M) [30].

Androgen receptor mRNA was reduced 50% with DHT at a concentration of $10^{-8}$ M, whereas there were no significant differences at concentrations less than $10^{-10}$ M (Fig. 4d).

**Discussion**

Of the current 18 members of the FGF family, FGF-7 is most clearly implicated in prostate growth and differentiation. When added to co-cultures of rat epithelial and stromal cells by means of...
double-chamber culture, testosterone stimulated epithelial cell growth without any effect on stromal cell growth via FGF-7 [5, 6]. In neonatal rat prostate, FGF-7 has been proposed to mediate induction of branching differentiation by androgen and a mesenchyme-derived paracrine mediator of androgen-induced epithelial growth and ductal branching morphogenesis in the rat prostate [1]. This morphological change was blocked by soluble FGF-7 receptor or anti-FGF-7 monoclonal antibody [1]. FGF-7 promotes human prostate epithelial cell growth in culture [3, 12] and is expressed in the human prostate, and considered to be the major andromedin [6, 32, 33] in human prostate. Recently it was demonstrated FGF-10 has both similar and unique functional properties relative to FGF-7 that suggest the two factors may have both compensatory and unique functions in the context of specific tissues (Lu W et al., submitted for publication).

Isolated prostate stromal and epithelial cells are useful for understanding the interactions between epithelial and stromal cells in the absence of confounding environmental variables in vitro. In this study, cultures of prostate stromal and epithelial cells were established by explant techniques [25]. This simple method does not require tissue digestion with trypsin or collagenase as in previous reports [15, 31]. PSA positivity confirmed that isolated epithelial cells originated in the prostate, whereas the stromal cells were characterized as myofibroblasts. Stromal cells exhibit collagen Type I and III synthesis induced by TGF-R1 [25] as well as physiologic function in a gel contraction assay [34], when stimulated by FBS or endothelin, but not by DHT (manuscript in preparation).

Real-time PCR is a rapid and highly sensitive method developed to determine mRNA levels of FGF-10 and AR. In addition, this method allowed for the analysis of numerous samples within 3 hours (40 cycles). This technique can generate yes-or-no results much faster than Northern blotting, ribonuclease protection assay or several other quantitative RT-PCR technique [35, 36].

The human prostate is a heterogeneous mixture of epithelia, fibromuscular stroma and connective tissue surrounding a glandular component [15, 37]. Benign prostatic hyperplasia originates in the periurethral and transition zones of the prostate [38, 39]. With advancing age and exposure to endogenous androgen, microscopic foci of BPH increase in size [37, 40]. BPH is thought to occur as the result of a disruption of normal epithelial-stromal interactions within the normal endocrine milieu [40], and usually exhibits significant hyperplasia and abnormalities in the stromal compartment in man [37]. The morphologic peculiarities seen in ducts that are tangent to BPH nodule borders suggest that inducers operate to stimulate epithelial activity in BPH [39]. The earliest lesion of BPH in transition zone is a proliferation of glandular epithelium and often with a reduction in a relative amount of stroma [39]. Our results show that FGF-10 mRNA was strongly expressed in both BPH tissue as well as cultured stromal cells, but not in cultured epithelial cells. FGF-10 was also demonstrated to have unique properties as a paracrine factor in the prostatic epithelium. Therefore, overexpression of stromal-derived FGF-10 may contribute to initiate the BPH genesis. In contrast, it has been reported that FGF-7 mRNA expression is undetectable in BPH tissue relative to normal human prostate although it was apparent in cultured stromal cells [8, 19]. A role of FGF-7 cannot be ruled out since this may reflect changes in the cellular composition of BPH tissue and the assay method.

The concept that genesis of BPH nodules lies in alterations at the local tissue level does not rule out the possibility that hormones play a role in pathogenesis [39]. Our data showed that FGF-10 mRNA in stroma was regulated by DHT. It is implied that androgen can indirectly stimulate the epithelial growth via stromal-derived FGF-10 in human prostate. By means of immunohistochemistry, the AR protein was detected in nuclei of the stromal cells (data not shown). In the current study, AR mRNA expression in stromal cells was reduced by DHT. In another in vivo experiment, the administration of androgen to castrated rats decreased AR mRNA in the ventral prostate to the levels in intact controls [7]. Because the present study showed a difference between the patterns of expression of FGF-10 mRNA and AR mRNA, FGF-10 mRNA expression may be indirectly regulated by androgen.

In contrast to BPH, FGF-10 expression was reduced in human carcinoma tissue which may again
reflect changes in cellularity in particular, loss of stroma, or reduced expression of FGF-10 in stroma present in carcinoma. The fact that the human prostate carcinoma cell line, LNCaP failed to respond to FGF-10 (data not shown) may indicate a loss of sensitivity of tumor cells to FGF-10.

As revealed in a recent experiment by two of us (Lu W et al., submitted for publication), FGF-10 as well as FGF-7 exhibited identical mitogenic activities for mouse keratinocytes and affinity for a complex of heparin and the resident epithelial cell receptor isoform, FGFR2IIIb. Nevertheless, FGF-10 exhibits a more specific pattern of expression than FGF-7 among cell types, interaction with 5 times the number of heparan sulfate sites on peri-cellular matrix of epithelial cells than FGF-7 and affinity for FGFR1 IIIb isoform which FGF-7 will not bind to at all. The role of each of these differences in activities of FGF-10 remains to be elucidated in human normal prostate, BPH and carcinoma, but the results suggest that FGF-10 may have unique activities that are subject to modification by androgen in human tissues.

In summary, stromal-derived FGF-10 stimulates human prostatic epithelial growth, and its mRNA expression is induced by androgen, without an increase in the androgen receptor mRNA. Moreover, FGF-10 may be involved in the development or support of human BPH.

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References


