Increased Expression of Fibroblast Growth Factor 6 in Human Prostatic Intraepithelial Neoplasia and Prostate Cancer

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ABSTRACT

Fibroblast growth factors (FGFs) are known to play an important role in the growth of normal prostatic epithelial cells. In addition to their effects on proliferation, FGFs can promote cell motility, increase tumor angiogenesis, and inhibit apoptosis. FGFs also have a variety of other biological activities that can play an important role in tumorigenesis. We have shown previously that FGF2, FGF7, and FGF9 are present in high concentrations in normal human prostate (9–11). They are produced by prostatic stromal cells and can act as paracrine growth factors for prostatic epithelial cells and, except for FGF7, as autocrine growth factors for stromal cells. Thus, FGFs are important growth factors for maintenance of the normal prostate in vivo.

Evidence for the importance of FGFs in prostate cancer comes from studies of the Dunning rat model system. Yan et al. (12) have shown that as these transplantable tumors progress from a mixed stromal-epithelial phenotype to a stromal independent phenotype, expression of FGFs not originally present in the tumors (such as FGF3 and FGF5) occurs, and there are changes in the isoforms of FGFs expressed, consistent with autocrine stimulation of growth. We therefore sought to determine whether a similar autocrine production of FGFs occurs in human prostate cancers.

We have found that approximately half of human prostate cancers contain increased amounts of FGF6, and, in the majority of such cases, FGF6 is expressed in the cancer cells. In PIN, the precursor lesion for prostate cancer, the prostatic basal cells express a markedly increased level of FGF6. The biological importance of this overexpression is shown by our finding that FGF6 is a potent growth factor for normal and neoplastic prostatic epithelial cells in culture. Thus, FGF6 may play an important role as both a paracrine and an autocrine growth factor in the initiation and progression of human prostate cancer.

MATERIALS AND METHODS

Tissue Acquisition and Analysis. Prostate cancer tissues and samples of the uninvolved peripheral and transitional zone of the prostate were taken from radical prostatectomies performed for treatment of clinical stage T1cN0 or T2N0 prostate cancer. Tissues were received fresh, and portions were snap-frozen in liquid nitrogen or used to establish primary cell cultures (see below). Additional cancer tissues were obtained from three transurethral resections of prostate performed to treat obstruction in advanced prostate cancer and from a pelvic lymph node metastasis. The frozen tissues were then analyzed by frozen section to confirm the presence or absence of carcinoma or high-grade PIN, and, if present, the percentage of the tissue involved in these processes. The carcinoma tissues contained 30–80% cancer, and tissues with PIN contained 30–50% PIN. To obtain nine tissues with such extensive high-grade PIN, it was necessary to select tissues from over 800 frozen sections performed previously on prostate tissues harvested from radical prostatectomies and, in some cases, to microdissect the tissue to enrich for PIN. All normal peripheral and transition zone tissues were free of cancer and PIN. Additional frozen sections were prepared for immunohistochemistry in some cases, and the remaining tissue was used to prepare cell extracts or RNA.

RNA Extraction, RT-PCR, and Northern Blotting. RNAs were extracted from tissues and cultured cells using the guanidine isothiocyanate procedure, and reverse transcription was performed as described previously using 2 μg of total RNA (9). The cDNA product, corresponding to 0.1 μg of total RNA, was used for PCR amplification as described previously. Primers for FGF6 were CACGAGGAGACCCTTACA (forward primer, exon 1) and
TCCCTTGGTACAAAGTCGA (reverse primer, exon 3). Positive control for FGF6 was skeletal muscle mRNA from Clontech Laboratories, Inc. (Palo Alto, CA). FGF-4 primers were CTTGACCTCGACAGCATGTA (forward primer) and GCACATCTACGAGTTATAGCGG (reverse primer). For each set of PCR reactions, a control in which water was added rather than cDNA was included, and this reaction was uniformly negative. FGF3 and FGF4 primers, Southern blotting of PCR products, Northern blotting, and specific probes for FGF3, FGF4, and FGF6 were as described previously (9). The FGF4–probe was a 600-bp BamHI fragment from the 3′ end of the human FGF4–plasmid SV25E (13). This probe consists primarily of a 3′ untranslated sequence to eliminate cross-hybridization with other FGF4 transcripts.

Cell Proliferation Assays. Primary epithelial cell cultures were established using prostatic tissue samples from areas in the peripheral zone of radical prostatectomy specimens that were free from carcinoma. Primary epithelial cells were plated on collagen-coated 35-mm dishes at 5 × 10⁴ cells/dish in complete epithelial growth medium that includes bovine pituitary extract (a source of FGFs), EGF, insulin, dexamethasone, cholera toxin, and BSA, as described previously (9). We have previously shown such cultures to be of prostatic epithelial origin by immunohistochemistry (9). The next day, medium was changed to epithelial growth medium without bovine pituitary extract or EGF. Cells were kept in this incomplete medium as controls or were supplemented with 10 ng/ml recombinant FGF6 (R&D Systems, Minneapolis, MN). Cells were then trypsinized and counted using a Coulter counter at 2-day intervals.

Prostate cancer cell lines (DU145, PC3, and LNCaP) were maintained in RPMI 1640 with 10% FCS. Cells were plated at 5 × 10⁴ cells/35-mm dish, and the next day, cells were refed with 0.2% FCS with or without 10 ng/ml FGF6. Cells were counted at 2 or 4 days after growth factor addition. PNT1A, an immortalized normal prostatic epithelial cell line (14), was maintained in RPMI 1640 containing 5% FCS. Cells were plated at 2.5 × 10⁴ cells/35-mm dish and refed the next day with RPMI 1640 supplemented with 1% ITS (insulin, transferrin, and selenium; Sigma, St. Louis, MO) with or without 10 ng/ml FGF6. Cells were then counted on days 3 and 5.

Preparation of Cell Extracts. Prostatic tissue samples were weighed, pulverized in liquid nitrogen, and then homogenized by three strokes (each for 10 s) on ice in a lysis buffer, as described previously (10), using 0.5 ml lysis buffer/200 mg tissue. The homogenate was then incubated for 30 min on ice, and insoluble material was removed by centrifugation for 1 min in a micro-centrifuge at 4°C.

Immunohistochemistry. Frozen tissue sections were fixed in acetone for 10 min, postfixed in methanol for an additional minute, and stored at −80°C. Immunohistochemical localization of FGF6 was carried out by the avidin-biotin complex method as described previously (10). All sections were treated with AutoBlocker (R&D Systems) to inhibit endogenous peroxidase and avidin/biotin (Vector Laboratories, Burlingame, CA) to block endogenous biotin. The sections were incubated with rabbit polyclonal anti-FGF6 antibody (200 ng/ml; R&D Systems) at a concentration of 400 ng/ml for 2 h at room temperature. After washing as described above, biotinylated polyclonal anti-FGF6 antibody (BAF 238; R&D Systems) was added at a concentration of 400 ng/ml for 2 h at room temperature. After washing as described above, detection was carried out by adding 100 μl/well of a 1:4000 dilution of streptavidin/peroxidase (Sigma, St. Louis, MO) and incubating for 20 min at room temperature. Wells were washed and incubated with a substrate consisting of a 1:1 solution of H₂O₂ and tetramethylbenzidine (Sigma) at a concentration of 0.1 mg/ml. Stop solution (H₂SO₄) was added with 30 min, and absorbance at 450 nm was determined using an ELISA plate reader. The sensitivity of this ELISA was found to be less than 1 pg/ml. No cross-reactivity with FGF2, FGF7, and FGF9 was detected when these growth factors were added at 10 ng/well.

RESULTS

RT-PCR Analysis of FGF6 Expression in Prostate Cancers. Our initial goal was to screen prostate cancer RNAs for expression of FGF RNAs that were not expressed by normal prostate. To maximize the sensitivity and specificity of this screening, we analyzed the prostate cancer RNAs by RT-PCR using primers for FGFs 1–9 followed by Southern blotting of the PCR products and hybridization with a probe corresponding to the specific product of RT-PCR reaction. Using this extremely sensitive technique, FGFs 1, 2, 5, 7, 8, and 9 gave detectable bands in normal tissues (data not shown) and thus were not useful for this qualitative study to determine whether new FGFs were expressed in the prostate cancer tissues. However, no expression of FGFs 3, 4, or 6 was detected by this technique in RNA from the normal peripheral zone. To determine whether these FGFs are expressed in human prostate cancers, we analyzed a total of 26 clinically localized prostate cancer RNAs from tissues containing 30–80% carcinoma on frozen section by RT-PCR followed by Southern blotting of the PCR products. Expression of FGF6 mRNA was detectable in variable amounts in a subset of prostate cancers (Fig. 1), and no FGF6 was detected in the control normal prostate tissue. No expression of FGF3 or FGF4 was seen (data not shown). Overall, 14 of 26 prostate cancers had detectable FGF6 mRNA by this RT-PCR analysis.

ELISA of FGF6 Concentration. To determine whether the expression detected by RT-PCR corresponded to increased expression of biologically significant quantities of FGF6, we analyzed tissue extracts from a total of 24 prostate cancers for FGF6 content by ELISA. These samples included 20 clinically localized cancers, a pelvic lymph node metastasis, and 3 locally advanced, androgen-independent cancers. All tissue extracts contained 40–90% carcinoma by frozen section analysis. As controls, we also analyzed eight tissue extracts from the normal peripheral zone and four extracts from the normal transition zone. In addition, nine extracts from prostate tissue with high-grade PIN, which is widely believed to be the precursor lesion for invasive prostate carcinoma, were analyzed. As shown in Fig. 2 the normal control tissues did not express detectable quantities of FGF6, whereas 15 of 24 prostate cancers contained between 0.5 and 2.6 ng FGF6/g tissue. No correlation between FGF6 content and clinical behavior was observed. As determined by the Gleason score or clinical or pathological stage, was apparent, but the number of cases is small, and more extensive analysis will need to be performed to definitively

**Fig. 1.** Expression of FGF6 mRNA in human prostate cancer. RNAs from normal prostatic peripheral zone (Lane N) or clinically localized prostate cancers (Lanes 1–12) were isolated and reverse transcribed, and cDNAs were analyzed by PCR with FGF6-specific primers, followed by transfer of PCR products to nylon membranes and hybridization with a radioiodinated FGF6 probe. The washed blot was exposed to X-ray film for approximately 30 min. The FGF6 product was 207 bp.
determine whether FGF6 expression is correlated with clinical or pathological parameters. We also detected between 0.4 and 0.9 ng FGF6/g tissue in four of nine high-grade PIN lesions. Thus, we found expression of FGF6 protein in approximately one-half of PIN lesions and invasive prostate carcinomas, which is similar to the percentage of prostate cancers expressing FGF6 mRNA by RT-PCR analysis.

**Immunohistochemical Analysis of FGF6 Expression.** To determine the cellular localization of FGF6 in the prostatic tissues, we examined normal peripheral zone, PIN, and cancers by immunohistochemistry with affinity-purified rabbit polyclonal anti-FGF6 antibodies. As shown in Fig. 3, in the normal peripheral zone, weak staining of the basal cell layer was detectable (Fig. 3A). In one sample, we also found staining of skeletal muscle fibers (data not shown). It is well known that occasional skeletal muscle fibers can be found in normal prostate, and FGF6 has previously been shown to be expressed by skeletal muscle at other sites (15); therefore, the positive staining observed is consistent with prior observations. In the high-grade PIN lesions, we found a marked increase in the staining of the basal cells in the dysplastic acini (Fig. 3B and C). This staining was distinctly stronger than that seen in adjacent, normal acini in the same section. Preincubation of the anti-FGF6 antibody with excess recombinant FGF6 completely abolished immunostaining (Fig. 3D). A total of 10 carcinomas with detectable FGF6 by ELISA were examined by immunohistochemistry. In eight of these carcinomas, staining of the cytoplasm of the cancer cells was identified. This staining varied from

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**Fig. 2.** FGF6 content of normal and neoplastic prostate tissues. Tissue extracts were prepared as described in “Materials and Methods” from normal peripheral and transition zone tissues (NL), prostate tissues with high-grade PIN, or prostate cancer tissues (CA), and the FGF6 content was determined by ELISA. For the cancer tissues, open circles are the values for a metastasis to a pelvic lymph node (the highest value) and three transurethral resections in men with locally advanced, androgen-independent prostate cancer, and closed circles are the values for clinically localized cancers.

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**Fig. 3.** Immunohistochemistry of prostate, PIN, and prostate cancer with anti-FGF6 antibody. Frozen sections of prostatic tissues were analyzed by immunohistochemistry using anti-FGF6 antibody as described in “Materials and Methods.” A, normal prostate showing weak staining of basal epithelial cells (×400). B, low-power view of high-grade PIN showing intense staining of basal epithelial cells (×40). C, high-power view of a PIN lesion (×400). D, high-power view of a PIN lesion stained with antibody preincubated with excess recombinant FGF6 (negative control; ×400). E, prostate carcinoma showing strong staining of the cytoplasm of the cancer cells (×400). F, prostate cancer with staining of the spindle-shaped fibroblastic cells within the cancer tissue (×200).
light but diffuse (1+) to intense and diffuse (3+). The single case with 1+ staining was derived from a metastatic cancer to a pelvic lymph node that was >90% cancer on frozen section and contained 2.6 ng FGF6/g tissue by ELISA. In six of eight cases, there was 2+ staining for FGF6, characterized by variable weak to intense staining of the tumor cytoplasm. Finally, a single case of 3+ staining with uniform intense staining of the prostate cancer epithelial cells was found (Fig. 3E). In two prostate cancers, there was no staining of the cancer epithelial cells, but staining of stromal cells with fibroblastic morphology was seen (Fig. 3F).

**Mitogenic Activity of FGF6 on Prostatic Cells.** To determine the role of FGF6 in the proliferation of normal and neoplastic prostatic epithelial cells, we assessed the ability of exogenous recombinant FGF6 to induce proliferation in primary cultures of prostatic cells and in normal and neoplastic prostatic epithelial cells lines. As seen in Fig. 4A, FGF6 was mitogenic for both primary cultures of prostatic epithelial and stromal cells. The differences seen are highly statistically significant \((P < 0.001, t \text{ test})\) at 4 and 6 days of treatment for both epithelial and stromal cells. Similar results were seen with PNT1A cells, a cell line derived from normal prostatic epithelial cells immortalized by SV40 large T antigen (Fig. 4B). In addition, a marked proliferative response to exogenous FGF6 was also seen in the prostate cancer cell lines DU145 and LNCaP, which was again highly statistically significant by day 4 of treatment \((P < 0.001, t \text{ test})\). The PC3 prostate cancer cell line had only a slight (but statistically significant) proliferative response to FGF6. Thus, with the exception of PC3 cells, the normal and transformed prostatic epithelial cells tested showed a marked proliferative response to exogenous FGF6.

**Expression of FGFR-4 in Primary Prostatic Cells and Prostate Cancer Cell Lines.** It has been shown by Ornitz et al. (2) that the FGFR with the most potent mitogenic response to FGF6 is FGFR-4. We therefore examined the expression of FGFR-4 in the normal peripheral zone of human prostate and in DU145, PC3, and LNCaP prostate cancer cell lines by RT-PCR. The breast cancer cell line MDA-MB231, which has been shown to express FGFR-4 (16, 17), was included as a positive control. As can be seen in Fig. 5A, all four prostate samples and the prostate and breast cancer cell lines express FGFR-4. A control in which genomic DNA was added results in a larger 1.2-kb product, confirming that the primers span an intron and the product seen in the RT-PCR reactions is not due to genomic DNA contamination. To determine the site of FGFR-4 expression in normal prostate, RNAs from the three prostate cancer cell lines and the immortalized normal prostatic epithelial cell line PNT1A were analyzed by Northern blotting using 1 mg of polyadenylated RNA from the three prostate cancer cell lines and the immortalized normal prostatic epithelial cell line PNT1A reveals a low but detectable expression of FGFR-4 in all four cell lines, with PC3 cells having the lowest FGFR-4 expression (Fig. 6).
FGF6 overexpression observed is shown by the ability of exogenous FGF6 to promote proliferation of normal, immortalized, and fully transformed prostatic epithelial cells. In addition, by promoting the growth of stromal cells, FGF6 may enhance the production of paracrine growth factors by tumor stroma (20), which can further promote tumor progression.

FGF6 is normally expressed in the skeletal muscle and may play an important role as a growth factor in that tissue. Studies by Penault-Llorca et al. (21) have shown expression of FGF6 in 3 of 10 breast cancer cell lines and in 15% of primary breast cancer tissues. No FGF6 expression was detected in normal breast tissue. These same authors detected overexpression of FGFR-4 in 32% of primary breast cancers. Expression of FGFR-4 has been shown by others to be present in the majority of breast cancer cell lines (16, 17). Given that FGFR-4 is the most potent receptor for FGF6 (2), a potential autocrine loop involving FGF6 and FGFR-4 may be established in a subset of breast cancers. We have also found expression of FGFR-4 in all three prostate cancer cell lines and in primary prostate epithelial cultures. It should be noted that this expression is relatively low, and we did not detect it previously in the normal prostate (9); however, using improved PCR primers allowed us to detect this expression. Chandler et al. (16) have also reported expression of FGFR-4 in DU145 cells using a RNase protection assay, which is consistent with our results, but they found barely detectable expression of FGFR-4 in PC3 cells, whereas we found FGFR-4 expression that was lower than but similar to that seen in DU145 cells. Whether this difference reflects the use of different probes and methods for RNA analysis or differences in the PC3 cells between different laboratories is not clear. It is of interest to note that both prostate (22–24) and breast cancers (25) have been reported to have increased expression of FGF8, a potent activator of FGFR-4. Thus, in both breast and prostate cancer, there is evidence that autocrine stimulation of FGFR-4 by FGF6 and FGF8 may occur. It is known that the signaling properties of FGFR-4 differ from those of the other FGFRs (26–29) in that FGFR-4 only weakly stimulates mitogen-activated protein kinase activation (27) and is associated with the induction of the other FGFRs (26–29) have shown that FGFR-4 is the only FGFR that can promote membrane ruffling when tranfected into COS-7 cells. Such membrane ruffling is associated with changes in the actin cytoskeleton related to increased motility. Thus, FGFR-4 activation by autocrine stimulation may be more important in altering motility or other properties when compared to similar stimulation of other FGFRs and may therefore play a unique role in tumor progression.

In addition to FGFR-4, other FGFR isoforms that can respond to FGF6 expression was detected in normal breast tissue. These same authors detected overexpression of FGFR-4 in 32% of primary breast cancers. Expression of FGFR-4 has been shown by others to be present in the majority of breast cancer cell lines (16, 17). Given that FGFR-4 is the most potent receptor for FGF6 (2), a potential autocrine loop involving FGF6 and FGFR-4 may be established in a subset of breast cancers. We have also found expression of FGFR-4 in all three prostate cancer cell lines and in primary prostate epithelial cultures. It should be noted that this expression is relatively low, and we did not detect it previously in the normal prostate (9); however, using improved PCR primers allowed us to detect this expression. Chandler et al. (16) have also reported expression of FGFR-4 in DU145 cells using a RNase protection assay, which is consistent with our results, but they found barely detectable expression of FGFR-4 in PC3 cells, whereas we found FGFR-4 expression that was lower than but similar to that seen in DU145 cells. Whether this difference reflects the use of different probes and methods for RNA analysis or differences in the PC3 cells between different laboratories is not clear. It is of interest to note that both prostate (22–24) and breast cancers (25) have been reported to have increased expression of FGF8, a potent activator of FGFR-4. Thus, in both breast and prostate cancer, there is evidence that autocrine stimulation of FGFR-4 by FGF6 and FGF8 may occur. It is known that the signaling properties of FGFR-4 differ from those of the other FGFRs (26–29) in that FGFR-4 only weakly stimulates mitogen-activated protein kinase activation (27) and is associated with the induction of the other FGFRs (26–29) have shown that FGFR-4 is the only FGFR that can promote membrane ruffling when tranfected into COS-7 cells. Such membrane ruffling is associated with changes in the actin cytoskeleton related to increased motility. Thus, FGFR-4 activation by autocrine stimulation may be more important in altering motility or other properties when compared to similar stimulation of other FGFRs and may therefore play a unique role in tumor progression.

In addition to FGFR-4, other FGFR isoforms that can respond to the autocrine production of FGF6 or by inducing adjacent stromal cells to produce it and, in fact, produce it in larger amounts than in normal tissues. The potential biological importance of the autocrine production of FGF6 or by inducing adjacent stromal cells to produce it and, in fact, produce it in larger amounts than in normal tissues. The potential biological importance of the autocrine production of FGF6 or by inducing adjacent stromal cells to produce it and, in fact, produce it in larger amounts than in normal tissues. The potential biological importance of the autocrine production of FGF6 or by inducing adjacent stromal cells to produce it and, in fact, produce it in larger amounts than in normal tissues. The potential biological importance of the
FGF6, such as FGF-R1 IIIc and FGF-R2 IIIc (2) are also expressed in prostate cancer cells. We have shown that expression of FGF-R1 is increased, particularly in poorly differentiated prostate cancers (20), and we have recently found that FGF-R1 is present only as the IIIc isoform in prostate cancers.4 FGF-R1 IIIc could markedly enhance response to FGF6 in those cases where it is expressed. Similarly, there is evidence that the DU145 prostate cancer cell line and some prostate cancer xenografts express the FGF-R2 IIIc isoform (30) that can bind FGF6. However, FGF-R1 IIIc and FGF-R2 IIIc are not expressed in cultured primary prostatic epithelial cells (9); therefore, in normal prostatic epithelial cells, most of the mitogenic effect of FGF6 must be through activation of FGF-R4. Additional studies are needed to define the expression of the various FGF isoforms that bind FGF6 in prostate cancer cells in vivo and the relative importance of each of these receptors in the biological response of prostate cancer cells to FGF6.

In summary, we have found that FGF6 is expressed by normal prostatic basal cells in extremely small amounts, and expression in basal cells is markedly increased in PIN lesions. Approximately half of prostate cancers express FGF6 in the cancer cell themselves or in the tumor stroma. The acquisition of FGF6 expression by the prostate cancers implies but does not prove that it may play a role in cancer cell proliferation or perhaps in other aspects of tumor progression. Other FGFs, such as FGF2 (20) and FGF8 (22–24), have also been shown to be increased in prostate cancer, and a number of other growth factors, including EGF and transforming growth factor α (31), have also been implicated in prostate cancer. The relative importance of each of these growth factors in prostate cancer progression has yet to be elucidated. Further work will be needed to define the role of the increased expression of FGF6 in human prostate cancer.

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