Fibroblast Growth Factor Receptor 2 Limits and Receptor 1 Accelerates Tumorigenicity of Prostate Epithelial Cells

Shuju Feng, Fen Wang, Akio Matsubara, Mikio Kan, and Wallace L. McKeegan

Center for Cancer Biology and Nutrition, Albert B. Alkek Institute of Biosciences and Technology and Department of Biochemistry and Biophysics, Texas A & M University, Houston, Texas 77030-3303

ABSTRACT

Progressive loss of the differentiated phenotype and communication with stroma accompanies the transition of nonmalignant rat prostate epithelial cells to anaplastic, malignant tumors. Here we show that cell surface expression of the fibroblast growth factor receptor 2 (FGFR2) tyrosine kinase is reduced in malignant tumor cell populations (type II) and undetectable at the mRNA level in 30% of cells. This is in addition to the irreversible loss by splice switching of the FGFR2 ectodomain that abrogates response to FGF-7 and homologues from the stroma. One hundred % of type II malignant cells express FGFR1, which is normally expressed in the stroma. Expression of the FGFR1 kinase in premalignant type I tumor epithelial cells by transfection accelerated progression to the malignant phenotype. In contrast to the FGFR2 kinase fused to the ectodomain of FGFR1, the FGFR1 kinase failed initially to support a mitogenic response to FGF-2 in type I tumor cells. However, the FGFR1-transfected cells acquired a mitogenic response after extensive proliferation of the cell population. Resident FGFR2 and ectopic FGFR1 appeared to be partitioned in the type I cells, because neither full-length nor truncated isoforms of FGFR1 affected the mitogenic response of the other. Restoration of the FGFR2IIIB kinase to malignant cells expressing FGFR1 depressed tumor growth rates, restored responsiveness to stromal cells, and restored epithelial cell differentiation. These observations reveal that homologous FGFR1 and FGFR2 kinases play very different roles in cell growth and differentiation and in development and support of the malignant phenotype.

INTRODUCTION

A central problem in management of prostate cancer is understanding the transition of relatively benign, slowly growing, differentiated, androgen-sensitive tumors into rapidly growing, anaplastic, hormone-insensitive malignant tumors. Throughout this study, the former are referred to as pre- or nonmalignant type I tumors and the latter as malignant type II tumors rather than nonmetastatic and metastatic tumors, because metastasis was not studied in detail in this report. Differentiated androgen-responsive rat tumors in the Dunning R3327 series, which can be maintained as s.c. implants in syngeneic males, undergo the transition when hosts are subjected to androgen ablation (1). During the process, distinct changes occur in expression of members of the FGF4 family of polypeptides and FGFR kinases (2–5). This includes a switch from exclusive expression of the IIIB splice variant of the FGFR2 gene in the type I tumor epithelial cells to exclusive expression of the IIIC isoform in type II tumors (5). FGFR2IIIB in epithelial cells is the receptor for the androgen-regulated paracrine signal carried by FGF-7 (keratinocyte growth factor) and its homologues from the stromal compartment (5, 6). FGF-7 binds only FGFR2IIIB, whereas FGFR2IIIC recognizes other FGF polypeptides (5–7). In addition to the switch from exclusive FGFR2IIIB to FGFR2IIIC expression, abnormal (ectopic) expression of the FGFR1 gene, which is normally exclusively found in the stromal compartment, occurs in the type II tumor epithelial cells along with elevated expression of FGF-1, FGF-2, FGF-3, and FGF-5 (2, 5). FGF-1 expression is very low, and FGF-2, FGF-3, and FGF-5 are absent in normal prostate and nonmalignant type I tumor epithelial cells. When implanted in rat hosts in the absence of stromal cells, cloned epithelial cells from the type I tumors progress to type II tumors. The resultant type II tumors exhibit the identical phenotype of type II tumors that evolve from the parent type I tumor after androgen ablation (5). However, when type I tumor epithelial cells are implanted together with type I tumor stromal cells, the progression to type II tumors is prevented, and slowly growing, differentiated, nonmalignant tumors with a well-defined stromal and epithelial compartment are the result (5). The cloned type I tumor epithelial cells, the cellular and molecular properties of which can be dissected and manipulated in vitro, are thought to represent the epithelial cells in the parent Dunning R3327PAP tumor, which evolve into type II tumors after androgen ablation. We have proposed that the loss of the directional paracrine signal carried by FGF-7 from stromal cells to FGFR2IIIB in the epithelial cells of type I tumors may allow subsequent events to unfold that drive progression of the type II tumor phenotype (5). One of those events by correlation appears to be the gain of a new autocrine loop between ectopically expressed FGFR1 and FGF-2, which supports the malignancy that is observed in malignant tumor cells (5). Differences in mitogenic signaling and proteins phosphorylated have been reported between FGFR3 and FGFR4 and FGFR1 and FGFR2, but none have been reported between FGFR1 and FGFR2 (8–11). In this study, we compare the effect of FGFR1 and FGFR2 isoforms on development and reversal of the type II tumor phenotype when transplanted into nonmalignant type I or malignant type II tumor epithelial cells, respectively. The results show that, despite the high sequence and structural homology, the FGFR2 and FGFR1 kinases play very different roles in epithelial cells in the progression to malignancy and maintenance of the malignant cell phenotype. This suggests that the two FGFRs couple to different signal transduction pathways that affect mitogenesis and phenotype in different cell types.

MATERIALS AND METHODS

Cell Culture and Mitogenic Assays. Primary cultures and stock cultures of cloned cell lines from rat prostate, the Dunning R3327PAP and R3327AT3 tumors, and tumors reconstituted from cell lines were prepared and maintained by described methods (5, 12). Mitogenic activity was assayed by DNA synthesis in cells at low cell density and in serum-free medium. Cells (2 × 10⁴) were inoculated into each well of 24-well culture plates in culture medium containing 5% fetal bovine serum for 12 h, and the medium was replaced with new medium containing 0.5% fetal bovine serum for 24 h and then with serum-free medium for 72 h. The cells were washed twice with PBS followed by addition of 500 µl of medium containing 2 µg/ml heparin and 4 ng/ml of the recombinant FGF indicated in the text. After incubation at 37°C for 16 h, 5 µl of [methyl-3H]thymidine (50 µCi/ml, DuPont NEN Research Products, Boston, MA) were added. After 4 h, cells were washed with PBS, fixed in 1
FGFR1 and FGFR2 in Prostate Tumors

Respectively. In FGFR2: open double line. exon IIlb; solid double line. exon Ilic. @. acidic box sequence domain; U. FGFRI kinases; D. FGFR2 kinases; 1. point of truncation of the epithelial (DTE) cells derived from the Dunning R3327PAP tumor and type II XhoI and XbaI sites.

Intracellular domains are shown on the left and right of the transmembrane (TM) looplike modules I, II, and III with putative disulfides of the ectodomain and the various domains that were the means of triplicates.

Recombinant FGFR cDNAs and Transfection of Prostate Tumor Cells. Full-length recombinant constructs are summarized schematically in Fig. 1. Human FGFR1α1, FGFR1β1, FGFR1α2, FGFR1β2, and FGFR2βIIlb1 were prepared as described (4, 13, 14). Full-length rat FGFR2βIIlc1 was generated by ligation of cDNA coding for the extracellular domain of the rat FGFR2βIIlc (GenBank accession no. L19111) and the type I intracellular domain of FGFR2 (L19107) at the EcoRI site. FGFR2βIIlbTr (residues 1–418) was generated in the PCR with human sense primer pB1 (13) and antisense primer pB2 (GGCTGAAATCCGGTCAGCAACGCGGTG-GCT) with rat prostate cDNA template. cDNAs were cloned into pBluescript SK vector for restriction enzyme and sequence analysis and then cloned into mammalian expression vector pcDNAI/neo (Invitrogen Co., San Diego, CA, 92121) at the HindIII and XbaI sites.

The chimeric cDNA FGFR1β2R2 was constructed by ligation of the extracellular region of human FGFR1β to the intracellular part of rat FGFR2 at a Kpnl site with a link generated by PCR with sense primer R21 (CGAATGAA-GACCGGTACCAAGAAGCCA) and antisense primer R22 (AGGGGTGTCGCCCAGCTAGCTCATC) and rat FGFR2β1 cDNA template. The PCR product was then cloned into SK vector, sequenced, and excised with KpnI and BamHI for subsequent construction of the chimeric cDNA (Fig. 1). The chimeric FGFR1β/β2R2 cDNA was then generated by ligation of the extracellular region of rat FGFR2βIIlc to the intracellular part of human FGFR1 at the Kpnl site with a link generated by PCR using sense primer R23 (GTCCGT-GACGCCGCCGGGTGTAAC) and antisense primer R24 (TGCGTCTTCTGTAACCCTCTTCTC) and rat FGFR2βIIlc1 template. The PCR fragment was cloned into SK vector for sequence verification and excised with EcoRV and KpnI for the construction of the chimeric cDNA (Fig. 1). Chimeric cDNAs described above were cloned into the pcDNAI/neo expression vector between Xhol and XbaI sites.

Transfected cell lines were prepared from cloned lines of type I tumor epithelial (DTE) cells derived from the Dunning R3327PAP tumor and type II tumor (AT3) cells into 96-well plates. Expression of the FGFR1 and FGFR2 mRNAs as well as specifically the FGFR2βIIb and FGFR2βIIlc isoforms were analyzed in 50 clonal cultures from each of the DTE and AT3 populations using PCR. Total RNA was extracted from each well, and a first-strand cDNA template was generated by reverse transcription. A 1–μl sample containing 10 ng of cDNA was used as a template in a 100-μl reaction mixture at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles. PCR products were run on 2% agarose gels in 0.5× Tris-borate EDTA buffer and visualized with ethidium bromide. Primers yielding the band given in (bp) as follows: FGFR1 (307 bp), R1C5 (5′-TCCCATACGAATCTCGTGTGAGG3′); and R1C3 (5′-GGACCTCAAAAGCGCCTGACTGACGCC-3′); FGFR2βIIb (380 bp), R2P5 (5′-AACCGGAGAGTGAATTAAACGAC-3′); and R2PB (5′-GAGGCC-TATTTATCCCGAGT3′); FGFR2βIIlc (506 bp), R2P5 (given above) and R2PC3 (5′-TGGCGAGAAGCTGCAACCATGC-3′); and FGFR2 (273 bp), R2P5 (given above) and R223 (5′-GGACCTCATATAAACCCTGGC-3′).

Tumor Cell Implantation and Histochemical Analysis. Prior to use in implantation experiments, clonal cultures were derived from wild-type and transfected cell populations by limiting dilution and selected for expression of the desired type and level of FGFR by screening in radioreceptor assays. At least five 100–150-g male Copenhagen rats were implanted s.c. in both flanks with 1×10^5 DTE cells, 1×10^5 AT3 cells, or 3×10^5 stromal-derived DTS cells (5), as indicated in the text. Animals were examined continuously for appearance and progress of tumors over a 6-month period for implants using type I tumor epithelial (DTE) cells and 1 month for type II tumor (AT3) cells. For histochemical analysis, fresh tumor tissues were fixed in 10% polyformaldehyde in PBS and embedded in paraffin. Tissues were sectioned and stained with H&E.

Analysis of Cytokeratins. A portion (100 mg) of freshly excised tumor tissue was homogenized in 0.5 ml of 1% Triton X-100 and protease inhibitors (17). Cell monolayers were extracted directly with the same solution. After clarification by centrifugation at 16,000 × g, the detergent-soluble fraction was mixed with an equal volume of 2× concentrated SDS-PAGE sample buffer [0.0625 M Tris-HCl (pH 6.8), 0.2% SDS, 0.05 M mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue] and boiled for 5 min. The samples were separated by 7.5% SDS-PAGE and electroblotted onto nitrocellulose paper. Duplicate blots were stained with Monoclonal Anti-Pan Cytokeratin (a mixture of mouse ascites fluid containing C-11, PCK-26, CY-90, Ks-l, 30, and M20, and A53-B/A2 clones from Sigma Biosciences). Mouse antibodies on the immunoblots were visualized with a secondary antibody conjugated to alkaline phosphatase.

RESULTS

Expression and Activity of FGFR2 and FGFR1 Differ in Premalignant and Malignant Cells. Normal rat ventral prostate cells and the premalignant type I tumor R3327PAP and derived epithelial cells (NPE and DTE, respectively; Refs. 3 and 12) from both tissues express exclusively the IIlb splice variant of the FGFR2 gene (Ref. 5; Fig. 2 and 3). In normal or premalignant epithelial cells, the
FGFR2IIIb ectodomain recognizes FGF-1 and stromal cell-derived FGF-7, but not FGF-2. A clonal analysis of the expression of FGFR2 and FGFR1 mRNA in the premalignant DTE cell populations indicated that all cells express the FGFR2IIIb mRNA (Fig. 3A). Separate binding and cell growth assays indicated that FGFR2IIIb was translated at sufficient levels to bind radiolabeled FGF-1 and FGF-7 and to support a growth response (data not shown). FGFR1 transcripts were not detected in any of the 50 clonal cultures of DTE cells (Fig. 3A). Type II AT3 tumors and cells that evolve in vivo from parent type I tumors after castration of hosts (1, 5) exhibit a total loss of expression of FGFR2IIIb with a less significant change in expression of total FGFR2 mRNA (5). Type II tumors (E tumors) that emerge from cloned DTE cells in the absence of stroma exhibit the same change (5). However, the level of cell surface FGFR2 that bound FGF-1 in E-C and AT3-C cells derived from the E and AT3 tumors was much more reduced (Fig. 2A) than was predicted by analysis of total FGFR2 mRNA (5). A clonal analysis of expression of FGFR2 and FGFR1 mRNA in the AT3 cell population revealed that about 70% of cells expressed the FGFR2 gene (Fig. 3B). The other 30% were completely devoid of mRNA coding for FGFR2 isoforms. Of the 50 clonal cultures analyzed, none exhibited an FGFR2IIIb. In contrast, all 50 cultures derived from single AT3 cells expressed the FGFR1 mRNA (Fig. 3B). These results suggested that a reduction in total cell surface FGFR2 occurs in the malignant cell population. Some cells exhibit a complete loss of expression of the FGFR2 gene in addition to the loss of expression of FGFR2IIIb by splice switching.

Expression of the FGFR1 gene correlates closely with the malignant phenotype of type II tumors that emerge either from parent type I tumors after castration or cloned type I tumor epithelial cells (5). Therefore, we examined the effect of transfection of the FGFR1 tyrosine kinase into premalignant DTE cells on the mitogenic response to FGF-1, FGF-2, and FGF-7 in vitro and their progression to malignancy in vivo. Untransfected DTE cells display about 12,000 FGF-1 receptor sites per cell with an apparent Kd of about 40 nM and no sites for FGF-2 (2). Radioreceptor assay and covalent affinity labeling of receptors (Fig. 2C) were used to identify transfected cells expressing about the same number of new FGF-2 binding sites. FGF-2 bound to the transfected full-length FGFR1a1 (not shown) or FGFR1B1 kinases on the surface of cells (Fig. 2A). However, similarly the untransfected cells, FGF-2 did not elicit an increase in DNA synthesis (Fig. 4). In addition, expression of neither the full-length kinase isoforms, the kinase-defective FGFR1a2 (not shown), nor the FGFR1B2 altered the mitogenic response to FGF-1 or FGF-7 (Fig. 4).

We examined the response to FGF-2 of DTE cells transfected with cDNAs coding for the native FGFR2IIIc1 kinase. In addition, chimeric constructions comprising the ectodomain of FGFR1B fused to the intracellular domain of FGFR2 and the FGFR2IIIc1 ectodomain fused to the FGFR1 intracellular domain were examined (Fig. 1). Transfected DTE-R1B/R2 and DTE-R2BIIIc1 cells bound FGF-2 (Fig. 2C) and responded to FGF-1, FGF-2, and FGF-7 (Fig. 4). Cells transfected with the chimeric construction coding for the FGFR2IIIc1 ectodomain and the intracellular domain of FGFR1 bound FGF-2 (Fig. 2C) but failed to respond to it (Fig. 4). This showed that the FGFR1 intracellular kinase domain is incapable of eliciting a mitogenic response in the DTE cells and strongly supports the thesis that the mitogenic response to FGF-1 and FGF-7 in wild-type DTE cells is mediated by the FGFR2IIIb1 kinase. The lack of effect of overexpression of FGFR1 constructs with or without a kinase domain on the mitogenic activity elicited by FGF-1 or FGF-7 in the DTE cells (Fig. 4) indicated that neither the ectodomain nor the kinase domain of transfected FGFR1 interacts with the resident FGFR2IIIb1. However, it was observed that the mitogenic response to FGF-1 and FGF-7...
A

DTE

FGFR2

FGFR2IIib

FGFR2IIic

FGFR1

B

AT3

FGFR2

FGFR2IIib

FGFR2IIic

FGFR1

Fig. 3. Clonal analysis of the expression of FGFR2 and FGFR1 mRNA in premalignant and malignant prostate tumor cells. A, DTE cells from Dunning R3327PAP tumors. B, AT3 cells from Dunning R3327AT3 tumors. Cells were isolated and purified, and clonal cultures were derived from single cells by limiting dilution. Expression of the indicated FGFR isoforms was assessed by PCR from 50 clonal cultures, of which 6 representatives (Lanes 1–6) are displayed. N, negative control in which template cDNA was omitted; P, positive control containing the indicated FGFR cDNA; M, markers.

The Response to FGF-2 in Nonmalignant Epithelial Cells Expressing Recombinant FGFR1 Is an Acquired Property. We determined that DTE cells can acquire responsiveness to the FGFR1 kinase prior to the loss of FGFR2IIib and acquisition of the phenotype exhibited by malignant E or AT3 tumor cells. The DTE cells transfected with FGFR1β1 [DTE-R1β1(P1)] that initially showed no response to FGF-2 were subcultured serially 20 times at 1:5 dilutions of the cell population over a period of about 100 days (about 100 population doublings). The mitogenic response of the DTE-R1β1(P20) cells to FGF-1, FGF-2, and FGF-7 was then compared to that of the parent DTE cells, DTE cells that were transfected with the control vector (DTE-CV), and DTE cells that were transfected with kinase-defective FGFR1β2 [DTE-R1β2(P20)]. In contrast to the parent untransfected DTE and DTE cells expressing FGFR1β1 just after emergence from selection medium [DTE-R1β1(P1)], the DTE-R1β1(P20) cells acquired a response to FGF-2 without a change in the response to FGF-1 or FGF-7 (Fig. 4). No change in mitogenic response to the three FGF polypeptides was observed in DTE cells expressing kinase-defective FGFR1β2 that were cultured in parallel for the same period. These results show that the mitogenic response to the FGFR1 kinase in premalignant DTE cells is an acquired property that can occur prior to loss of FGFR2IIib and consequently prior to the loss of response to stromal-derived FGF-7.

Acceleration of the Malignant Phenotype by Expression of FGFR1. We tested whether the presence of the FGFR1β1 kinase accelerated the rate of progression to the malignant type II tumors in the absence of stroma (5). After 6 months, DTE cells transfected with the FGFR1β1 cDNA gave rise to tumors (DTE-R1β1) that were on average nearly 10 times the wet weight of those that arose from untransfected DTE cells in the same time period (Fig. 5; Table 1). DTE cells transfected with the control vector (DTE-CV) or the kinase-defective FGFR1β2 construct yielded tumors that were similar in size to those from untransfected DTE cells.

Restoration of Expression of the FGFR2 Gene in Malignant Type II Tumor Cells Reduces Growth Rate of Derived Tumors. Malignant Dunning R3327AT3 (AT3-C) cells were transfected with the FGFR2βIIIb1 cDNA (Fig. 2A). Untransfected AT3 cells display about 19,000 FGF-1 and FGF-2 binding sites per cell with an apparent $K_d$ of about 50 μM (2). By radioreceptor assay, transfected cells were selected that acquired one to two times the same number of FGFR2 binding sites of similar affinity (Fig. 2A). Separate experiments confirmed that the transfected cells acquired the ability to bind FGF-7 proportional to the number of transfected receptors (not shown). As described previously (1, 5), wild-type AT3 cells (Fig. 6) or their E tumor cell counterparts (not shown) give rise to rapidly growing tumors, which result in multiple organ metastasis and kill the hosts in about 2 months (5). Implantation with stromal cells had no effect on the growth and malignancy of tumors derived from AT3 or E tumor cells (5). In contrast, the size of tumors resulting from AT3 cells expressing FGFR2βIIIb1 (AT3-R2βIIIb1 tumors) was on average
Fig. 4. Mitogenic response to FGF-1, FGF-2, and FGF-7 of premalignant epithelial cells expressing different combinations of the FGFR1 and FGFR2 extracellular and intracellular domains. DNA synthesis was measured in DTE cells transfected with the indicated FGFR construction after stimulation with the indicated FGF. Cloned DTE cells transfected with FGFR1β1 or FGFR1β2 just after selection in geneticin (P1) were frozen in liquid nitrogen or carried through 20 subcultures at a 1:5 dilution of the cell population (P20). P1 cultures were then prepared from frozen stocks, and the mitogenic response of each culture to FGF-1, FGF-2, and FGF-7 was compared. A representative experiment of three reproductions is presented. Data are the means of triplicates (bars, SE).

37% of the size of those resulting from untransfected AT3 cells or cells transfected with the control vector (AT3-CV; Fig. 6 and Table 1). Coinoculation of the transfected AT3 cells with stromal (DTS) cells derived from the differentiated type I R3327PAP tumor (5) dramatically depressed the growth rate of the resultant tumors (AT3-R2βIIIb1 + DTS) to 15% of the size of tumors formed from control cells (Fig. 6). Surprisingly, AT3 cells transfected with a kinase-defective FGF2βIIIb, which was truncated at the beginning of the intracellular juxtamembrane sequence, also reduced the size of the resultant tumors (AT3-R2βIIIbTr; Fig. 6). Transfection of AT3 cells with FGFR1β1 at three to four times the number of endogenous FGFR1 sites (Fig. 6) or a chimera of the FGFR2βIIIc ectodomain and the FGFR1 intracellular domain (not shown) had no effect on size of derived tumors.

Restoration of Expression of the FGFR2 Gene in Malignant Type II Tumor Cells Induces Morphological Differentiation and Response to Stroma. The nonmalignant type I parent tumors (Dunning R3327PAP) of the isolated cells and derived tumors exhibit predominantly glandlike structures surrounded by stromal cells (Fig. 7, DT) and rare foci of squamous differentiation (5). In contrast, the malignant type II tumors that evolve from them consist of a relatively homogeneous, undifferentiated, and anaplastic epithelial cell sheet with no distinguishable stromal cell compartment (Ref. 5; Fig. 7, AT3). In the absence of type I tumor stromal cells, cloned DTE cells give rise to tumors that at early times after implantation comprised predominantly the glandlike structures characteristic of their R3327PAP parents (Fig. 7, DTE-T, left). Extensive glandular differentiation occurs surrounding blood vessels in the tumor. Stromal cells, presumably recruited from the host, because no stromal cells were coinoculated, surround the ductlike structures. After 4—6 months, sarcomatous areas of undifferentiated cells appear among and adjacent to the glandlike structures (Fig. 7, DTE-T, right). The former dominates eventually after 10—12 months as the distinct luminal structures deteriorate progressively and are eventually lost (5). Within two transfers by implantation of a 1-cm³ trocar of intact tumor tissue or implantation of isolated epithelial cells from the tumors, the DTE tumors exhibited the morphological homogeneity of the AT3 tumors. We have previously designated these completely undifferentiated DTE cell-derived tumors "E tumors" to distinguish them from the early-stage DTE tumors (5). Although rare foci of organized cells can still be observed, the DTE-R1β1 tumors derived from DTE cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tumor weight (g)</th>
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<tbody>
<tr>
<td>DTE</td>
<td>5.5 ± 2.1</td>
</tr>
<tr>
<td>DTE-CV</td>
<td>4.7 ± 1.8</td>
</tr>
<tr>
<td>DTE-R1β2</td>
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<tr>
<td>DTE-R1β1</td>
<td>51.0 ± 8.9</td>
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<tr>
<td>AT3</td>
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</tr>
<tr>
<td>AT3-CV</td>
<td>38.5 ± 5.2</td>
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<tr>
<td>AT3-R2βIIIb1</td>
<td>14.3 ± 6.1</td>
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<tr>
<td>AT3-R1β1</td>
<td>35.1 ± 4.8</td>
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<tr>
<td>AT3-R2βIIIc/R1</td>
<td>37.3 ± 3.8</td>
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* Data are means ± SE. Five animals were implanted with the indicated cells.
FGFR1 AND FGFR2 IN PROSTATE TUMORS

transfected with FGFR1 exhibited predominantly the anaplastic phenotype of the AT3 tumors within 6 months in a single host without additional passage (Fig. 7).

AT3 cells transfected with FGFR1β1 or FGFR2β/R1 (not shown) exhibited the histological properties of tumors derived from untransfected AT3 cells (Fig. 7) or those transfected with the control vector (not shown). However, the more slowly growing tumors derived from AT3 cells transfected with FGFR2βIIIb1 (AT3-R2βIIIb1) exhibited properties much different from those of the parent AT3 tumors. Generally, cells were more densely packed and exhibited more cell-to-cell contacts throughout the tumor than wild-type AT3 tumors (Fig. 7, AT3-R2βIIIb1, left). Foci of organized epithelial cells near or actually surrounding blood vessels in the tumor imperfectly resembled the glandlike structures in the DT and DTE tumors (Fig. 7). The anaplastic sheets of cells characteristic of parent AT3 tumors outside the foci of organized cells were infiltrated extensively with lymphocytes, and areas of overt necrosis were apparent. Although tumors resulting from AT3 cells transfected with kinase-defective FGFR2βIIIb (AT3-R2βIIIbTr) were smaller, they exhibited no comparable changes in cellular organization. Rare foci of lymphocyte infiltration and necrosis were observed, but more than 90% of the more slowly growing AT3-R2βIIIbTr tumors exhibited the morphological properties of wild-type AT3 tumors (Fig. 7). In marked contrast, the still-smaller tumors (AT3-R2βIIIb1 + DTS) resulting from coimplantation of the AT3 cells expressing FGFR2βIIIb1 and stromal (DTS) cells from the parent type I tumor exhibited areas of cellular organization characterized by intense squamous differentiation. The foci of epithelial cells were surrounded by stroma. More than 50% of the tumor comprised these S-E cell islands dispersed among the anaplastic sheets of the common AT3 cellular phenotype. Squamous differentiation comprised more than 90% of S-E tumors formed from mixtures of pre-malignant DTE and the DTS cells (5). Independent of the presence of DTS cells, no squamous differentiation could be observed in the more slowly growing tumors that formed from AT3 cells that were transfected with kinase-defective FGFR2βIIIbTr (not shown). These results suggest that the presence of the FGFR2 kinase restores the response to stroma in malignant cells and is involved in or mediates the squamous differentiation induced by the presence of the coinoculated stromal cells.

Expression of the FGFR2 Kinase Restores Expression of Cytokeratins in Malignant Type II Tumors. Both DT tumors (and DTE cells derived from them) and S-E tumors derived from mixtures of DTE and DTS cells expressed a mixture of cytokeratins, whereas total cytokeratins were reduced to undetectable levels in the malignant AT3 and E tumors (reference 5 and Fig. 8). Expression of cytokeratins was reduced in the tumors (Fig. 8, DTE-7) derived from cloned DTE cells and the DTE-R1β1 tumors proportional to their degree of progression toward the type II
phenotype (Fig. 8). Transfection and expression of the FGFR1βIIIb1 gene into the AT3 cells restored the expression of cytokeratin in derived tumors to detectable levels. Tumors from AT3 cells transfected with chimeric FGFR2βIIIc/R1 (not shown) or FGFR1β1 showed no change. When mixed with stromal cells, the transfected AT3 cells expressing FGFR2βIIIb1 exhibited a still higher level of cytokeratins near that of S-E tumors derived from the premalignant epithelial (DTE) cell precursors when they were mixed with the same DTS stromal cells (Ref. 5; Fig. 8). Coincident with the lack of changes in cellular organization, the more slowly growing tumors derived from AT3 cells transfected with kinase-defective FGFR2βIIIbTr showed no increase in cytokeratin expression. Analyses with individual monoclonal antibodies against keratins 1, 5, 6, 10, 13, and 14 confirmed that AT3 and AT3-R1β1 tumors are negative for all. The DT, DTE, DTE-R1β1, S-E, AT3-R2βIIIb, and AT3-R2βIIIb+DTS tumors exhibited keratins 5 and 14. Keratin 1 was not detectable in any of the samples, whereas small amounts of keratins 6, 10, and 13 in the S-E tumors and keratin 13 in the DTE-R1β1 tumors could be detected. These results show that restoration of the FGFR2 kinase to AT3 cells restored the expression of cytokeratin in the malignant cells concurrent with the morphological changes described earlier.

Fig. 7. Histological analysis of prostate tumors. Tissue samples from the parent Dunning R3327PAP tumor (DT) and three indicated tumors resulting from cells transfected with the indicated cDNAs were fixed in 4% polyformaldehyde in PBS overnight, embedded in paraffin, sectioned, and stained with H&E. Magnification, ×200.
expression of cytokeratin. Extracts of freshly excised tissue from the indicated tumors were analyzed by 8% SDS-PAGE and immunoblot with a mixture of monoclonal antibodies against cytokeratin. Primary antibodies were visualized with a second antibody conjugated to alkaline phosphatase. Except for the S-E tumor (SE), samples were taken from the tumors shown in Figs. 5–7. S-E tumors are slowly growing, well-differentiated tumors that result from a mixture of DTE and DTS cells (5).

**DISCUSSION**

Activation of FGFR1 in Premalignant Epithelial Cells Accelerates Progression to the Malignant Phenotype. Previously, we reported that the phenotypic switch from exclusive expression of the IIIb splice variant of the ectodomain of FGFR2 and appearance of the normally stromal cell-associated FGFR1 gene in the epithelial cells are hallmarks of progression to malignancy in the Dunning R3327PAP rat tumor model (5). Here, we show that, in addition, the expression of all FGFR2 gene products in isolated cell populations derived from malignant Dunning R3327AT3 tumors (type II) is reduced and undetectable in 30% of individual cells. Separate experiments not shown here revealed no difference between growth rates and phenotypic properties of tumors derived from clonal cultures of type II tumor cells expressing detectable levels of FGFR2 mRNA or none at all. All type II tumor cells expressed FGFR1. To date, we have detected no type II tumor cells, despite extensive attempts and clonal analysis that expressed only the FGFR2 mRNA. The presence of FGFR1 in 100% of the malignant tumor cells, regardless of whether FGFR2 is expressed, suggests that it is FGFR1 that supports the malignant phenotype. Transfection of FGFR1 into premalignant type I epithelial cells confirmed that ectopic expression of FGFR1 while FGFR2IIIb is still present dramatically accelerated the rate at which the premalignant type I epithelial cells form type II tumors in the absence of stromal cells. Experiments are in progress to determine whether activation of FGFR1 contributes to the loss of responsiveness to stroma-derived FGF-7 by impact on the splice switch from FGFR2IIIb to IIIc, the global loss of the FGFR2 gene, or the signal elicited by the FGFR2 kinase.

Mitogenic Responsiveness to the FGFR1 Kinase Is an Acquired Property in Premalignant Type I Tumor Epithelial Cells. Surprisingly, transfection of the full-length FGFR1 kinase into premalignant type I epithelial cells revealed that the cells supported the binding of FGF-2 to the FGFR1 ectodomain, but were initially incapable of a mitogenic response to FGF-2. The mitogenic response to FGF-2 of cells transfected with a chimeric FGFR comprising the FGFR1 ectodomain and the FGFR2 intracellular domain showed that the failure was not due to the FGFR1 ectodomain, but to the inactivity of the FGFR1 kinase in the premalignant epithelial cells. Despite the initial lack of mitogenic activity of the FGFR1 kinase in the type I tumor epithelial cells, they acquired a mitogenic response to FGF-2 after several cycles of proliferation of the cell population with no change in the response elicited by FGFR-1 and FGFR-7. The acquisition of the FGF-2 response was a consequence of the presence of the FGFR1 kinase, because cells transfected with kinase-defective FGFR1 or the chimeric FGFR1/FGFR2 containing the FGFR2 kinase exhibited no change in response pattern from untransfected cells. The fact that FGFR-1, FGF-2, and FGFR-7 promote similar levels of DNA synthesis suggests that the cells transfected with FGFR1 continue to express active resident FGFR2IIIb1. In notable contrast, the transfection of chimeric FGFR2IIIb1IC/R1, which binds FGF-1 and FGF-2 but not FGFR-7, appeared to depress the response to all three factors.

The results of this series suggest that FGFR isoforms containing the FGFR1 ectodomain and the resident FGFR2IIIb isoforms in the premalignant type I tumor epithelial cell function independently. The FGFR1 and FGFR2 ectodomains appear to be partitioned within the premalignant epithelial cells such that formation of heterodimers or oligomers between them does not affect activity of one on the other.

Transfection of the FGFR2 Kinase into Malignant Type II Tumor Cells Restores Lost Properties Exhibited by Their Nonmalignant Precursors. Accompanying the switch from exclusive expression of FGFR2IIIb to IIIc, the reduction in expression of FGF-2 in general, and the activation of FGFR1 in epithelial cells is the loss of both ductal and squamous differentiation and loss of responsiveness to stromal cells. Transfection of the FGFR2IIIb1IC gene into highly malignant type II tumor cells restores some of these properties. The derived tumors not only exhibit a slower growth rate, but they also exhibit changes in cellular organization and cell-to-cell interactions, the absence of which is a hallmark of the anaplastic type II tumors. In addition, the FGFR2IIIb1IC-transfected cells respond to coinnoculated type I tumor stromal cells by display of areas of intense keratinization similar to those observed in tumors derived from premalignant type I tumor epithelial cells and stromal cells (S-E tumors; Ref. 5). None of these properties were observed in type II tumor cells transfected with the FGFR1 kinase. These results indicate that the extremely malignant Dunning R3327AT3 tumor cells, which have been selected extensively for malignant and metastatic properties, retain growth-limiting and differentiation-promoting pathways that are responsive to the FGFR2 kinase. The loss of FGFR2 activity, either by lack of activating ligands or by reduction in expression, may be contributory or necessary for progression to and maintenance of the malignant phenotype.

The Relative Importance of FGFR2 and FGFR1 to Prostate Tumor Progression. The results of this study support our previous proposal that FGFR2 plays a self-limiting role in the maintenance of epithelial cell homeostasis directed by the stromal compartment and other environmental factors. In contrast, the stepwise acquisition of the mesenchymal cell property of expression and subsequent activation of FGFR1 may be the positive driving force for progression to and support of the malignant phenotype in the epithelial cell (5). Both changes comprise a loss and acquisition that could cooperate to promote malignancy. It is of interest whether a change in one FGFR causes a change in the other or whether the order of reduction and activation of the FGFR2 and FGFR1, respectively, is important to the rate of malignant progression. Our results suggest that FGFR1 functions independently in premalignant epithelial cells and can accelerate progression to malignancy while coexistent with the resident FGFR2IIIb1IC. However, restoration of FGFR2IIIb1IC to the malignant type II tumor cells expressing FGFR1 also appears to override resident FGFR1 in support of the phenotype. Conceivably, the balance between activity of progression-limiting FGFR2 and the progression-promoting FGFR1 determines the rate of progression to malignancy.
Potential Mechanisms Underlying the Different Effects of FGFR1 and FGFR2. Of the four FGFR tyrosine kinases, FGFR1β1 and FGFR2β1 isoforms exhibit the highest similarity. Overall amino acid sequence homology is 72%, 57% in the ectodomains through the beginning of the kinase domains, and 84% in the remainder of the intracellular domain, which includes the kinase and a COOH-terminal domain (20, 21). FGFR2 exhibits tyrosine autophosphorylation sites homologous to those that have been demonstrated in FGFR1 (20, 21), and no unique sites are apparent in FGFR2. Phospholipase Cγ1 is to date the only substrate that exhibits a direct stable association with the intracellular domain through mutual interaction with a defined structural module (22–24). The interaction is not required for either fibroblast growth factor (FGF)-stimulated mitogenic activity (24–26) or neuronal differentiation in glial cells (27). Stimulation of cells expressing either FGFR1 or FGFR2 with FGF-1 results in phosphorylation of multiple intracellular proteins, many membrane-associated, within 15–30 s. Such substrates do not interact with FGFR with sufficient affinity in cell extracts for detection and identification by coprecipitation (9, 10, 11, 23). Among such proteins are 80K-H and SNT-like proteins (28–30), which may link activity of the FGFR kinase to GRB2-SOS complexes and, thereby, the mitogen-activated protein kinase or other signaling pathways that involve low molecular weight GTP-binding proteins. SNT-like proteins also interact with p13α,b1, which is involved in inactivation of the cell cycle catalytic p34cdk2 at anaphase so that mitosis can be completed (31, 32). FGFR3 activates the transcription factor Stat1 and expression of the cell cycle inhibitor p21 (33). Rapid phosphorylation of SNT by FGF and other differentiation promoters was first observed to be associated with differentiation rather than mitogenesis in neuronal cells. From the results, it was proposed that phosphorylation and activation of SNT might indirectly promote differentiation as a consequence of inhibition of cell cycle progression through p13α,b1 and p34cdk2 (32). Subsequent analyses indicated that SNT-like proteins are also rapidly phosphorylated in a wide variety of cell types during the mitogenic response to FGF (28). Thus, it remains to be established how the same FGFR can promote mitogenesis, growth limitations, and differentiated phenotypes, as well as how the four homologous kinases cause cell type-dependent differences in the three end points.

The FGFR2IIIbTr ectodomain without an intact kinase and intracellular domain slows the growth of malignant type II tumors to an extent nearly an equal that of FGFRIIIb1. However, the appearance of phenotypic parameters other than reduction in growth rate, e.g., morphological organization, cytotetator expression, and response to stromal cells, was dependent on the presence of the FGFR2 kinase. The restoration of the differentiated properties of parent type I tumors in the anaplastic tumor appears to be not simply due to a reduction in tumor cell proliferation rate. The dampening effect of the kinase-defective FGFR2IIIbTr on tumor growth rate may reflect dimerization between the transgenic and endogenous FGFR1 by a dominant-negative mechanism. However, the resident FGFR2 and transfected FGFR1 appear to be partitioned in the premalignant type I tumor epithelial cells and do not interact. Conceivably, the malignant cells have lost the ability to partition the two FGFRs. Recently, we have shown that in the absence of restraints imposed by concentration, heparan sulfate, or other cofactors, the four FGFRs interact promiscuously through a highly conserved interaction interface in the extracellular domain that connects immunoglobulin loops II and III (14, 34). The more dramatic impact of the restoration of the FGFR2IIIb1 kinase to the growth rate of the type II tumors can reflect dual effects. One effect is the recoupling of the FGFR2 kinase to signal transduction pathways in the malignant cells that limits tumor cell growth and/or promotes differentiation. The other is the suppression of tumor growth rate by heterodimerization with the resident FGFR1 that is driving proliferation and malignancy. It is noteworthy that the FGFR2 ectodomain fused to the FGFR1 intracellular kinase, which presumably homodimerizes with the resident FGFR2 via the ectodomain, dampens FGF-stimulated mitogenesis in nonmalignant epithelial cells. These observations lead to the conclusion that trans-activation events that occur between FGFR intracellular domains within dimers or oligomers (20–23, 35) is homotypic.

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