Long Pentraxin-3 Inhibits FGF8b-Dependent Angiogenesis and Growth of Steroid Hormone–Regulated Tumors

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Abstract

Fibroblast growth factor-8b (FGF8b) exerts nonredundant autocrine/paracrine functions in steroid hormone–regulated tumors. Previous observations had shown that the soluble pattern recognition receptor long pentraxin-3 (PTX3) is a natural selective antagonist for a restricted number of FGF family members, inhibiting FGF2 but not FGF1 and FGF4 activity. Here, we assessed the capacity of PTX3 to antagonize FGF8b and to inhibit the vascularization and growth of steroid hormone–regulated tumors. Surface plasmon resonance analysis shows that PTX3 binds FGF8b with high affinity (Kd = 30–90 nmol/L). As a consequence, PTX3 prevents the binding of FGF8b to its receptors, inhibits FGF8b-driven ERK1/2 activation, cell proliferation, and chemotaxis in endothelial cells, and suppresses FGF8b-induced neovascularization in vivo. Also, PTX3 inhibits dihydrotestosterone (DHT)- and FGF8b-driven proliferation of androgen-regulated Shionogi 115 (S115) mouse breast tumor cells. Furthermore, DHT-treated, PTX3 overexpressing hPTX3_S115 cell transfectants show a reduced proliferation rate in vitro and a limited angiogenic activity in the chick embryo chorioallantoic membrane and murine s.c. Matrigel plug assays. Accordingly, hPTX3_S115 cells show a dramatic decrease of their tumorigenic activity when grafted in immunodeficient male mice. These results identify PTX3 as a novel FGF8b antagonist endowed with antiangiogenic and antineoplastic activity with possible implications for the therapy of hormonal tumors. Mol Cancer Ther; 10(9); 1600–10. ©2011 AACR.

Introduction

Fibroblast growth factor-8 (FGF8), originally cloned from an androgen-dependent mouse mammary carcinoma cell line, belongs to the angiogenic FGF family (1). Experimental and clinical data point to a nonredundant autocrine/paracrine role of FGF8 in the growth of epithelial/stromal cells in steroid hormone–regulated tumors (2, 3), the FGF8 gene containing a functional androgen-response element responsible for its transcriptional activation by steroid–receptor signaling (4). Various alternatively spliced FGF8 isoforms are generated in mice and humans. Among them, the FGF8b isoform is endowed with the strongest tumorigenic and angiogenic potential and is highly expressed in breast, prostate, and ovarian cancers (2, 5), thus representing a possible druggable target for multidrug or multimodality treatment of hormonal tumors (2, 4).

The soluble pattern recognition receptor long pentraxin-3 (PTX3) is a member of the pentraxin family produced locally in response to inflammatory signals (6). PTX3 may serve as a mechanism of amplification of inflammation and innate immunity with nonredundant functions in various physiopathologic conditions including angiogenesis and cancer (7). PTX3 shares the C-terminal pentraxin domain with short pentraxins and possesses a unique N-terminal domain (6). The biological activity of PTX3 is related to its ability to interact with different ligands via its N-terminal or C-terminal domain as a consequence of the modular structure of the protein (6, 7). When assessed for the capacity to interact with a variety of extracellular signaling polypeptides, PTX3 was found to bind FGF2 via its N-terminal extension (8, 9), thus inhibiting FGF2-dependent endothelial cell proliferation in vitro and angiogenesis in vivo (7–10). Under the same experimental conditions, PTX3 did not bind to a wide panel of cytokines, chemokines, and growth factors representative of different classes of soluble polypeptide mediators (8). Accordingly, PTX3 did not affect endothelial cell proliferation triggered by various mitogens, including serum, diacylglycerol, epidermal growth factor, phorbol ester, or VEGF-A (8). Furthermore, PTX3 did not show any interaction with FGF1 and FGF4 (8), thus
indicating that its antagonist activity is selective for a restricted number of FGF family members.

Here, we show for the first time the capacity of PTX3 to bind FGF8b with high affinity, thus inhibiting its angiogenic activity in vitro and in vivo. Also, PTX3 suppresses the angiogenic and tumorigenic potential of the prototypic androgen-regulated Shionogi 115 (S115) mouse mammary carcinoma cells in which testosterone drives a FGF8b-dependent autocrine/paracrine loop of stimulation (11). Thus, PTX3 represents a novel FGF8b antagonist with possible implications for the therapy of hormonal tumors.

Materials and Methods

Chemicals

Purified human recombinant FGF2 and PTX3 (12, 13) were from Tecnogen, human recombinant FGF8b from M. Jalkanen (Biotie, Turku, Finland), recombinant N-terminal fragment PTX3-(1–178; Nterm-PTX3), C-terminal fragment PTX3-(179–381; Cterm-PTX3) and anti-PTX3 rabbit monoclonal antibody from B. Bottazzi (Humanitas Clinical Institute, Rozzano, Italy), sFGFR1(IIIc)/Fc and Noggin/Fc chimeras from RELIATech GmbH, dihydrotestosterone (DHT) from Sigma-Aldrich. K5 and O-sulfated K5 polysaccharide (K5-OS) were from Glycores 2000.

Surface plasmon resonance analysis

A BIAcore X apparatus (BIAcore Inc.) was used to set up the following experimental models: (i) PTX3 (2.2 μmol/L in 10 mmol/L sodium acetate, pH 2.4) was allowed to react with a Fc1 flow cell of a CM4 sensor chip that was previously activated with a mixture of 0.2 mol/L N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 mol/L N-hydroxysuccinimide (35 μL, flow rate 10 μL/minute). These experimental conditions allowed the immobilization of approximately 0.053 pmol/mm² of PTX3. After ligand immobilization, matrix neutralization was done with 1.0 mol/L ethanolamine (pH 8.5; 35 μL, flow rate 10 μL/minute). Activated/deac- tivated Fc2 flow cell was used as a reference cell and for blank subtraction. (ii) Size-defined heparin (13.6 kDa) was activated Fc2 flow cell was used as a reference cell and for blank subtraction. (iii) PTX3 as analyzed for its capacity to inhibit the binding of free FGF8b to immobilized FGFR1. To this purpose, sFGFR1(IIIc)/Fc chimera (100 μg/mL in 10 mmol/L sodium acetate, pH 4.0) was allowed to react with a flow cell of a CMS sensor chip that was previously activated with a mixture of 0.2 mol/L N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 mol/L N-hydroxysuccinimide (35 μL, flow rate 10 μL/minute). These experimental conditions allowed the immobilization of approximately 0.065 pmol/mm² of FGFR1. Similar results were obtained for immobilization of Noggin/Fc chimera (100 μg/mL in 10 mmol/L sodium acetate, pH 4.8), here used as a negative control and for blank subtraction. After ligand immobilization, matrix neutralization was done with 1.0 mol/L ethanolamine (pH 8.5; 35 μL, and flow rate 10 μL/minute). Different doses of FGF8b were injected in HBS-EP buffer (0.01 mol/L HEPES pH 7.4 plus 0.005% surfactant P20, 0.15 mol/L NaCl, and 3 mmol/L EDTA). Injection lasted for 4 minutes (40 μL, flow rate 10 μL/minute) to allow the association of FGF8b with the immobilized ligand and was followed by 2 minutes of dissociation. For kinetic analysis, association and dissociation constants (kₐ and kₒff respectively) were obtained in all experiments by fitting simultaneously all the raw sensorgrams with the 1:1 drifting baseline-bind- ing model by using the nonlinear-curve-fitting software package BLAevaluation 3.2 (Biacore). Equilibrium affinity constants (i.e., expressed as dissociation constant, K_d) were either derived from the kinetic parameters (K_d = kₒff/kₐ) or measured from Scatchard plots obtained under steady-state conditions.

In parallel competition experiments, FGF8b was injected for 4 minutes on PTX3, biotinylated heparin, or sFGFR1(IIIc)/Fc chimera surfaces in the absence or presence of recombinant full-length PTX3, related Nterm- PTX3, or Cterm-PTX3 fragments, or vehicle at the doses indicated in the figure legends. The response (in resonance units) was recorded at the end of injection and binding data were plotted as percentage of maximal bound analyte.

Cell cultures

Fetal bovine aortic endothelial GM7373 cells (8) were obtained in 1989 from NIGMS Human Genetic Mutant Cell Repository (Institute for Medical Research), and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS). Wild-type Chinese hamster ovary (CHO)-K1 cells and the related HSPG-deficient A745 CHO cell mutants (15), kindly provided in 1995 by J.D. Esko (University of California San Diego, La Jolla, CA), were grown in Ham’s F-12 medium supplemented with 10% FCS. FGFR1-transfected A745 CHO fgl-1A cells, bearing about 30,000 FGFR1(IIIc) molecules per cell, were generated in our laboratory by transfection with the IIIc variant of murine FGFR1 cDNA (16). Murine dermal microvascular endothelial cells (SIEC; ref. 17) were obtained in 2007 from Dr. A. Vecchi (Humanitas Clinical Institute, Rozzano, Italy) and grown on gelatin-coated dishes in DMEM supplemented with 1 mmol/L glutamine, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, and 20% FCS in the presence of 10 ng/mL FGF2 and 10 μg/mL heparin. S115 mouse mammary carcinoma cells (18) were kindly provided in 2001 by M. Jalkanen (Biotie, Turku, Finland) and maintained in DMEM supplemented with 5% heat inactivated FCS, 1 mmol/L sodium pyruvate, 1 mmol/L glutamine, and 10 mmol/L DHT. Human prostate cancer LNCaP cells were obtained from Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS.
All cell lines were kept at low passage, returning to original frozen stocks every 3 to 6 months, and tested regularly for *Mycoplasma*. The lines have not been reauthenticated since receipt.

**ERK1/2 phosphorylation analysis**

Serum-starved GM7373 cells were incubated for 15 minutes in medium containing 0.4% FCS plus FGF8b (0.20 nmol/L) or 10% FCS in the absence or the presence of recombinant PTX3 (660 nmol/L). Then, cell lysates (50 μg protein per sample) were resuspended in reducing SDS-PAGE sample buffer, boiled, run on reducing SDS-12% PAGE, and immunoblotted with anti-P-ERK1/2 antibody (Santa Cruz Biotechnology). Equal loading of the lanes was confirmed by immunoblotting with anti-ERK2 antibody (Santa Cruz Biotechnology).

**Cell proliferation assays**

GM7373 cell proliferation assay was done as described (19). Briefly, subconfluent cultures of GM7373 cells were incubated for 24 hours in medium containing 0.4% FCS plus FGF8b (0.20 nmol/L) in the absence or the presence of recombinant PTX3.

S115 and LNCaP cell proliferation assay was done as described (20). Tumor cells were seeded in 48-well plates at 25,000 cells/cm² in medium containing 4% hormone-deprived dextran-coated charcoal-stripped FCS (21) in the absence of DHT, followed by a 24-hour serum starvation in a 1:1 mixture of serum-free Ham’s F-12 and DMEM (22). Cells were then treated for 48 hours with different stimuli, as specified in the figure legends. For all proliferation assays, cells were trypsinized and counted in a Burker chamber at low passage, returning to original frozen stocks every 3 to 6 months, and tested regularly for *Mycoplasma*. The lines have not been reauthenticated since receipt.

**Chemotaxis assay**

Endothelial cell migration assay was done by using a 48-well microchemotaxis chamber (Neuroprobe). Polyvinylpyrrolidone-free polycarbonate filters (Nucleopore; Corning Glass) with a pore size of 8 μm were boiled for 1 hour in water containing 5 mg/L gelatin. Chemotactic stimuli (either FGF8b or FCS with or without PTX3) were placed in the lower chamber in DMEM plus 0.1% FCS. SIECs (55,000 cells per well) were suspended in the same medium and added to the upper chamber. After 4 hours at 37°C, cells that migrated to the lower side of the filter were stained with Diff-Quick (Dif-Quick; Baxter Diagnostics) and 5 random fields were counted for each experimental condition at ×250 magnification.

**FGF8b-mediated cell–cell adhesion assay**

This assay was done as described previously (23) with minor modifications. Briefly, wild-type CHO-K1 cells were seeded in 24-well plates at 150,000 cells/cm². After 24 hours, cell monolayers were washed with PBS and incubated with 3% glutaraldehyde in PBS for 2 hours at 4°C. Fixation was stopped with 0.1 mol/L glycine and cells were washed extensively with PBS. Then, A745 CHO flg-1A cells (50,000 cells/cm²) were added to CHO-K1 monolayers in serum-free medium plus 10 mmol/L EDTA with or without FGF8b (0.40 nmol/L) in the absence or presence of increasing concentrations of PTX3. After 2 hours of incubation at 37°C, unattached cells were removed by washing twice with PBS, and A745 CHO flg-1A cells bound to the CHO-K1 monolayer were counted under an inverted microscope at ×125 magnification. Adherent A745 CHO flg-1A cells have a rounded morphology and can be easily distinguished from the confluent CHO-K1 monolayer lying underneath on a different plane of focus. Data were expressed as the mean of the cell counts of 3 microscopic fields chosen at random. All experiments were conducted in duplicate and repeated twice with similar results.

**Human PTX3 cDNA transfection in S115 cells**

The full-length human PTX3 cDNA (GenBank accession no. X63613) was cloned in the eukaryotic expression pBABE/Puro vector (24), thus generating the pBABE-hPTX3/Puro expression vector (8). S115 cells (6 × 10⁵ cells per 6-mm plates) were transfected with Lipofectamine (Gibco BRL) containing 6 μg of pBABE-hPTX3/Puro (hPTX3_S115 cells) or of the empty vector (mock_S115 cells) according to manufacturer’s instructions. After 48 hours, puromycin (2.5 μg/mL; Sigma) was added to cell cultures. Stable transfected S115 cell populations were tested for human PTX3 expression by semiquantitative reverse transcriptase PCR (RT-PCR) and Western blotting of the cell supernatants.

**Analysis of mRNA expression in transfected S115 cells**

Total RNA was extracted by using TRIzol Reagent according to manufacturer’s instructions (Invitrogen) and 2.0 μg of total RNA were retrotranscribed with MMLV reverse transcriptase (Invitrogen) by using random hexamers in a final 20 μL volume. Then, 1/10th of the reaction was analyzed by semiquantitative RT-PCR by using the following primers:

- **Human PTX3**: 5'-GTGCTCTCTGGTCTCTGAGTG (forward) and 5'-GAGCTCTCCATGTGGCTGCTG (reverse);
- **murine PTX3**: 5'-GACGCCACCGACGACGTCC (forward) and 5'-CTCTGAACTCCAGGTGCCAC (reverse);
- **murine FGF2**: 5'-TCAAAACTAACAACCCCAAAGCAGAA (forward) and 5'-GTAACACACTTTGAAGGCCAGCA (reverse);
- **murine VEGF-A**: 5'-TGGATGTCATATACGCACGACGTG (forward) and 5'-TGTTTTTGACGGAACATTTACGCA (reverse);
- **murine tubulin**: 5'-TCAACTGGTCCCTCTCTGCTGACT (forward) and 5'-GGGTTTGGGATTTTGGGCTGATT (reverse).

**Analysis of PTX3 expression in transfected S115 cells**

Total RNA was extracted by using TRIzol Reagent according to manufacturer’s instructions (Invitrogen) and 2.0 μg of total RNA were retrotranscribed with MMLV reverse transcriptase (Invitrogen) by using random hexamers in a final 20 μL volume. Then, 1/10th of the reaction was analyzed by semiquantitative RT-PCR by using the following primers:

- **Human PTX3**: 5'-GTGCTCTCTGGTCTCTGAGTG (forward) and 5'-GAGCTCTCCATGTGGCTGCTG (reverse);
- **murine PTX3**: 5'-GACGCCACCGACGACGTCC (forward) and 5'-CTCTGAACTCCAGGTGCCAC (reverse);
- **murine FGF2**: 5'-TCAAAACTAACAACCCCAAAGCAGAA (forward) and 5'-GTAACACACTTTGAAGGCCAGCA (reverse);
- **murine VEGF-A**: 5'-TGGATGTCATATACGCACGACGTG (forward) and 5'-TGTTTTTGACGGAACATTTACGCA (reverse);
- **murine tubulin**: 5'-TCAACTGGTCCCTCTCTGCTGACT (forward) and 5'-GGGTTTGGGATTTTGGGCTGATT (reverse).
**Conditioned medium preparation and Western blot analysis**

To assess the levels of PTX3 protein released by S115 transfectants, cell cultures were grown under serum-free conditions for 2 days. Conditioned media were collected and clarified by centrifugation. Western blot analysis was done on 15 μg of total protein by using rabbit polyclonal anti-PTX3 antibodies.

**Chick embryo chorioallantoic membrane assay**

Algin bead cores (5 μL containing vehicle or 11.5 pmoles of FGF8b with or without PTX3 (46 pmoles) or K5-OS (3.0 μg) were prepared as described (25) and placed on top of the chick embryo chorioallantoic membrane (CAM) of fertilized White Leghorn chicken eggs at day 11 of incubation (6–8 eggs per experimental group). In a second set of experiments, mock_S115 cells or hPTX3_S115 cells were delivered onto the CAM (20,000 cells per embryo) after a 24 hour-incubation in the absence or in the presence of 10 nmol/L DHT (26). After 72 hours, blood vessels converging toward the implant were counted by 2 observers in a double-blind fashion under a stereomicroscope (STEMI-SR, ×2/0.12; Zeiss) at ×5 magnification.

**In vivo Matrigel plug angiogenesis assay**

Sixteen-week-old C57BL6/N male mice (Charles River) were injected s.c. with 400 μL of Matrigel (Trevigen) containing 3 × 10⁶ pPTX3_S115 cells or mock_S115 cells as control (4 animals per group, 2 plugs per animal). After 7 days, mice were sacrificed, Matrigel plugs were collected, and divided into 2 parts. One-half was embedded in OCT medium (Olympus) at 200 magnification. The other half was processed for total RNA extraction and quantitative RT-PCR analysis.

**Tumorigenesis studies**

Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December 1987). Nineteen-week-old severe combined immunodeficient (SCID) male mice (Charles River) were injected s.c. with 5 × 10⁶ hPTX3_S115 or mock_S115 cells in 350 μL total volume of PBS or Matrigel into both dorsolateral flanks, and tumor growth was comparable in both flanks. The tumors were measured in 2 dimensions and tumor volume was calculated according to the formula \( V = (D \times d^2)/2 \), in which \( D \) and \( d \) are the major and minor perpendicular tumor diameters, respectively (27, 28). At the end of the experiment, the animals were sacrificed in a CO₂ chamber and tumors were quickly removed, weighed, and divided into 2 parts. One-half was embedded in Tissue Tec OCT (Sigma), snap frozen by immersion in liquid nitrogen–cooled isopentane and analyzed by immunofluorescence microscopy. The other half was processed for total RNA extraction and quantitative RT-PCR analysis.

**Immunofluorescence analysis of Matrigel plugs and tumors**

For immunofluorescence analysis, 8-μm OCT-embedded frozen sections were fixed in cooled acetone (10 minutes), air dried, and washed 3 times with TBS. After blocking with 10% goat serum in TBS, sections were stained with an anti-PTX3 rabbit polyclonal IgG antibody (1/2,000 dilution, 1 hour at room temperature), followed by incubation with Alexa Fluor 488 anti-rabbit IgG antibody (1/500 dilution, 45 minutes at room temperature; Invitrogen). To evaluate microvessel density, sections were incubated with a rat IgG2a antimouse CD31 monoclonal antibody (1/200 dilution, 1 hour at room temperature; BD Pharmingen), followed by incubation with Alexa Fluor 594 anti-rat IgG antibody (1/500 dilution, 45 minutes at room temperature; Invitrogen). For mitotic index analysis, sections were stained with a anti-mouse Ki-67 antigen (clone TEC-3) rat monoclonal antibody (1/200 dilution, overnight at 4°C; Dako), followed by incubation with Alexa Fluor 594 anti-rat IgG antibody (Invitrogen). Nuclei were counterstained with 4,6-di amidino-2-phenylindole (DAPI; Sigma). The percentage of Ki-67⁺ nuclei was evaluated in 2 to 3 sections per tumor. Images were taken by using a Zeiss fluorescence microscope (Zeiss Inc.) equipped with an Olympus N547 digital camera (Olympus) at ×200 magnification.

**RNA extraction and quantitative RT-PCR analysis of Matrigel plugs and tumors**

Total RNA was extracted from Matrigel plugs and tumors and contaminating DNA was digested by using DNase, following indications reported in RNeasy Micro Handbook (Qiagen). Two microgram of total RNA was retrotranscribed with MMLV reverse transcriptase (Invitrogen) by using random hexamers in a final 20 μL volume. Quantitative PCR (qPCR) was done with a Biorad iCycler iQ Real-Time PCR Detection System by using a SYBR Green Supermix (Biorad) according to manufacturer’s instructions. Gene expression levels were analyzed by using tubulin as a reference gene. The qPCR specific primers (final concentration 400 nmol/L) were as follows:

**Human PTX3**, 5′-GGTCTCGACTGGTGCGCGGAGAA (forward) and 5′-TCGTCCGTGGCTTCGAGCAG (reverse);

**Marine CD31**, 5′-CGGTATTGATGATTTCTTGG (forward) and 5′-AAAGGAGAAGACACTTCACTT (reverse);

**Marine FGF8b**, 5′-GTTCACCTTGCTGTCTCGCT (forward) and 5′-CGGGTGTAGTTGGCTTGAATT (reverse);

**Marine VEGF-A**, 5′-ACCTCCACATGCCGAAGT (forward) and 5′-TCAATCGGACCGCCAGTAG (reverse);

**Marine tubulin**, 5′-CCGGACAGTCTGGCAACCCGATCG (forward) and 5′-TGCCCAAGAAGACCTTGAGC-GAACGG (reverse).
Results

PTX3 is an FGF8b antagonist

To characterize FGF8b/PTX3 interaction, increasing concentrations of human FGF8b were injected over a human PTX3-coated BIAcore sensor chip (Fig. 1A, top panel). Analysis of kinetic parameters ($k_{on}$ and $k_{off}$) shows that FGF8b/PTX3 interaction occurs with high affinity ($K_d = 30$ nmol/L). Similar results ($K_d = 90$ nmol/L) were obtained by Scatchard plot analysis of steady-state SPR data (Fig. 1A, bottom panel). As observed for FGF2/PTX3 interaction, the recombinant N-terminal fragment PTX3-(1–178), but not the C-terminal fragment PTX3-(179–381), prevents the binding of FGF8b to immobilized PTX3 (Fig. 1B), thus implicating the N-terminal PTX3 extension in FGF8b interaction.

FGFs exert their biological activity by binding to different members of the tyrosine kinase FGF receptor (FGFR) family (29). Among them, FGF8b binds FGFR4 and FGFR1-3(IIIc) isoforms (30). FGF interaction with signaling FGFRs is mediated by cell-surface heparan sulphate proteoglycans (HSPG), leading to the formation of productive ternary complexes on target cells (31). Accordingly, FGF8b leads to the formation of HSPG/FGF8b/FGFR ternary complexes in a FGF-dependent cell–cell adhesion assay (23) in which FGF8b mediates the adhesion of FGFR1(III)c-overexpressing CHO cells to a HSPG-bearing CHO cell monolayer (Supplementary Fig. S1). Similar to the FGF8b antagonist K5-OS (20), PTX3 inhibits FGF8b-mediated cell–cell adhesion in this assay (Fig. 2A). Also, PTX3 prevents the binding of FGF8b to the extracellular domain of FGFR1(IIIc) immobilized to a CHO cell monolayer, whereas control unmodified K5 polysaccharide (black arrowhead) is ineffective (Fig. 2B and C). Thus, PTX3 impairs the formation of HSPG/FGF/FGFR ternary complexes by inhibiting FGF8b/FGFR interaction with no significant effects on glycosaminoglycan binding. Similar results were obtained when FGF2 was used as a FGFR1 ligand (8).

In keeping with its FGF8b antagonist activity, PTX3 inhibits ERK1/2 phosphorylation and cell proliferation.
induced by FGF8b in bovine endothelial GM7373 cells with no effect on serum stimulation (Fig. 3A and B). Also, PTX3 blocks FGF8b-dependent chemotactic migration of murine microvascular SIECs (Fig. 3C). In both endothelial cell types, PTX3 impairs FGF8b activity with a potency (ED_{50} \approx 220 \text{ nmol/L PTX3}) similar to that required to prevent in vitro FGFR/FGF8b interaction (see Fig. 2C). Moreover, PTX3 significantly inhibits the angiogenic activity of FGF8b when coadministered in a molar excess with the growth factor via an alginate pellet implanted on the top of the chick embryo CAM (Fig. 3D).

**PTX3 inhibits FGF8b-dependent tumor growth and vascularization**

Androgen-regulated S115 cells are a prototypic example of steroid hormone–dependent tumors in which testosterone enhances their tumorigenic activity by inducing FGF8b upregulation, thus activating an autocrine loop of stimulation (see refs. 11, 32; Supplementary Fig. S2). S115 cells do not express PTX3 and FGF2 mRNA as assessed by RT-PCR (Supplementary Fig. S3A). Like the FGF8b antagonist K5-OS (20), here used as a positive control (Supplementary Fig. S2B), PTX3 protein specifically inhibits both DHT- and FGF8b-driven cell proliferation when administered to serum-starved S115 cells, with no effect on serum-induced cell proliferation (Fig. 4A). Again, PTX3 potency was congruent with its effectiveness in preventing in vitro FGFR/FGF8b interaction. As observed for S115 cells, PTX3 inhibits the mitogenic activity exerted by DHT or FGF8b also on androgen-regulated FGF8b-producing LNCaP human prostate tumor cells (2), confirming its ability to suppress the FGF8b-dependent growth of hormone-dependent tumor cells (Supplementary Fig. S4).

On this basis, S115 cells were transfected with an expression vector harboring the full-length human PTX3 cDNA or with the empty vector and stable hPTX3_S115 and mock_S115 cell populations were obtained. PTX3 transfectants release significant amounts of PTX3 (approximately 120 ng/10^6 hPTX3_S115 cells per 48 hours as assessed by semiquantitative immunoblotting; Fig. 4B, inset). As observed for exogenously added PTX3 protein, endogenous PTX3 overexpression inhibits the mitogenic response triggered by DHT or FGF8b in serum-starved hPTX3_S115 cells when compared with mock_S115 cells, with no effect on basal cell proliferation (Fig. 4B).

An increased angiogenic response may contribute to the accelerated growth of FGF8b-producing hormonal tumors (2, 11). To assess the effect of PTX3 overexpression on the angiogenic potential of S115 cells, mock- and hPTX3_S115 cells were grafted onto the CAM after a 24-hour incubation in the absence or in the presence of 10 nmol/L DHT. As shown in Figure 4C, DHT induces an increase of the angiogenic activity of mock-S115 cells that was abolished when cells were grafted in the presence of the FGF8b inhibitor K5-OS. Even though DHT induces a similar upregulation of the expression of FGF8b and VEGF-A (33) in mock- and hPTX3_S115 cells (Supplementary Figure 3).
Each point is the mean after 48 hours. Data, expressed as fold increase in respect to vehicle, are 0.04 nmol/L FGF8b (black bars), or 10 nmol/L DHT (gray bars) and counted starved mock_S115 or hPTX3_S115 cells were incubated with vehicle, Dotted line, angiogenic response in vehicle-treated alginate beads. Inset, immunoblotting of the conditioned media (15 or 5% heat-inactivated FCS (g)) of serum-starved S115 cells were counted 24 hours in the absence (open bars) or in the presence (black bars) of antibody. C, mock_S115 and hPTX3_S115 cells were incubated for 7 days, mRNA levels were measured by quantitative RT-PCR analysis of the harvested plugs. Data, normalized for tubulin expression, are the SEM of 6 plugs per group (*, P < 0.01, Student’s t test).

Fig. S3A), the angiogenic response triggered by DHT-treated hPTX3_S115 cells was significantly reduced when compared with DHT-treated mock_S115 cells and fully abolished by K5-OS. Together, these data suggest that PTX3 overexpression impairs the FGF8b-dependent angiogenic response triggered by DHT in S115 cells.

To confirm these observations, Matrigel plugs containing mock- or hPTX3_S115 cells were implanted s.c. in 16-week-old male mice. After 7 days, despite the similar levels of FGF8b and VEGF-A mRNAs in mock versus PTX3-overexpressing plugs, quantitative real-time RT-PCR analysis revealed a significant reduction of the expression of the endothelial marker CD31 in hPTX3_S115 plugs when compared with mock-S115 plugs (Fig. 4D). Double immunostaining confirmed the paucity of CD31+ neovascularization in the areas of PTX3 production in hPTX3_S115 plugs (Supplementary Fig. S3B). These results confirm the capacity of PTX3 overexpression to inhibit the angiogenic potential of S115 cells.

To assess the effect of PTX3 on the tumorigenic activity of androgen-dependent tumor cells, mock- and hPTX3_S115 transfectants were injected s.c. in SCID male mice. Even though mock- and hPTX3_S115 lesions express similar levels of FGF8b and VEGF-A transcripts (Fig. 5A) and undetectable levels of FGF2 (data not shown), the rate of growth of PTX3-overexpressing tumors was strongly reduced when compared with controls (Fig. 5B and C). Accordingly, hPTX3_S115 tumors showed a reduced Ki-67+ proliferative index (Fig. 5C) and a limited CD31+ vascularity when compared with mock grafts (Fig. 5D). A similar inhibition in tumor growth and vascularity was observed also when PTX3-overexpressing S115 cells were grafted s.c. in SCID male mice within a Matrigel plug, an experimental condition that enhances the tumorigenic potential of mock-S115 cells (Supplementary Fig. S5).

Discussion

FGF8b deeply affects epithelial/stromal compartments of steroid hormone-regulated tumors by exerting a potent autocrine activity on cancer cells and a paracrine proangiogenic function that may contribute to tumor progression (2–5). Indeed, the expression of FGF8 and its FGFRs occurs in a significant proportion of human breast, prostate, and ovarian cancers, FGF8b being detectable in approximately 80% of advanced prostate tumors and bone metastasis samples (reviewed in refs. 2, 5). Also, FGF8b has been implicated in the inappropriate activation of growth factor signaling cascade that overcome steroid hormone sensitivity of these tumors, resulting in the progression toward a refractory neoplasm insensitive to hormone deprivation therapy (4, 34, 35). On this basis, the FGF/FGFR system has been hypothesized as a target for the treatment of steroid hormone–regulated tumors (2, 35–38), also in a possible synergistic combination with γ-irradiation or antineoplastic drug therapies (39). Here, we show that PTX3 represents a novel FGF8b antagonist endowed with antiangiogenic and antineoplastic activity. Indeed, PTX3 binds FGF8b with high affinity, thus preventing FGF8b binding to signaling FGFRs. Accordingly, PTX3 inhibits the angiogenic activity exerted in vitro and...
PTX3 overexpression inhibits S115 tumor growth. A–C, SCID male mice were injected s.c. with 5 × 10⁶ mock_S115 (○) or hPTX3_S115 (●) cells in 350 μL of PBS. Tumor volume was measured with callipers and animals were sacrificed after 53 days. Harvested tumors were analyzed for the expression of human PTX3 and of murine VEGF-A and FGF8b by quantitative RT-PCR (A) and data, normalized for tubulin expression, are the mean ± SEM of 4 lesions per group (*, P < 0.05, Student’s t test). PTX3 overexpression causes a significant inhibition of tumor growth (B) as well as of tumor weight and Ki-67 proliferation index at sacrifice (C). Mean ± SEM of 5 to 6 tumors (**, P < 0.01; *, P < 0.05; Student’s t test). Inset in B, macroscopic appearance of harvested mock_S115 and hPTX3_S115 tumors. D, double immunostaining of representative mock_S115 and hPTX3_S115 tumors with anti-CD31 (red) and anti-PTX3 (green) antibodies shows the decreased vascularity of PTX3-overexpressing lesions. Nuclei are counterstained with DAPI. Original magnification, ×200.

in vivo by FGF8b protein and by FGF8b-producing tumor cells. Also, PTX3 inhibits the proliferation of androgen-regulated murine mammary S115 and human prostate LNCaP tumor cells driven by FGF8b as well as by DHT. As a result of its ability to inhibit both paracrine and autocrine functions of FGF8b, PTX3 exerts a dramatic inhibitory effect on the tumorigenic activity of PTX3-overexpressing S115 tumor grafts in immunodeficient male mice. Interestingly, in keeping with a possible antitumor activity of PTX3 in hormonal cancer, preliminary data mining on the cancer microarray database Oncomine 4.0 (Oncomine DB; ref. 40) reveals that PTX3 is significantly downregulated in various breast and prostate cancer datasets when compared with normal tissues.

It must be pointed out that recombinant PTX3 protein interacts with and inhibits the biological activity of FGF8b at doses comparable with those measured in the blood of patients affected by inflammatory diseases in which PTX3 concentrations rise from less than 2 ng/mL up to 200 to 800 ng/mL (6). Moreover, taking into account that PTX3 is produced locally, concentrations of PTX3 in the tissue at sites of inflammation may be far higher than in blood. Administration of high doses of recombinant PTX3 protein in vivo (up to 50 μg/day per mouse) have been used for the treatment of Aspergillus fumigatus infection.
From our in vitro observations (see above), it can be extrapolated that PTX3-overexpressing S115 grafts produced less than 0.5 μg of PTX3 per day, indicating that the continuous, local production of low doses of PTX3 by transfected tumor cells results in a highly efficacious inhibitory effect when compared with the administration of a bolus of recombinant protein.

Previous observations had shown the capacity of PTX3 to bind FGF2 and to inhibit its biological activity (7–10). Here, we show that PTX3 binds FGF8b with a similar affinity. In contrast, PTX3 does not show any interaction with FGFI and FGFR (8), indicating that its antagonist activity is restricted to defined FGFR families. Thus, our observations identify FGF8b as a novel PTX3 ligand of the FGF family. It must be pointed out that S115 cells do not express FGF2 in vitro and in vivo, thus supporting the hypothesis that the inhibitory effect exerted by PTX3 on S115 tumors is related to its FGF8b antagonist activity. However, when compared with neutralizing anti-FGF8b antibodies (35), the ability to antagonize both FGF2 and FGF8b activities makes PTX3 a putative multitarget agent for those lesions characterized by the coexpression of the 2 angiogenic growth factors. Further studies are required to define the molecular bases of the selective interaction of PTX3 with distinct members of the FGF family.

PTX3 interacts with different ligands via its N-terminal or C-terminal domain (6, 7). An integrated approach that used recombinant N-terminal and C-terminal PTX3 fragments, monoclonal antibodies, and surface plasmon resonance analysis identified the FGF2-binding domain in the PTX3 N-terminus (9). As observed for FGF2 (8, 9), a free recombinant N-terminal PTX3 fragment prevents the binding of FGF8b to immobilized PTX3 (see Fig. 1B), thus implicating the N-terminal PTX3 extension also in this interaction. Recently, N-terminal PTX3-derived low molecular weight FGF antagonists have been identified in our laboratory (10, 43), including the acetylated (Ac) synthetic pentapeptide Ac-ARPCA-NH2 (in single letter code), corresponding to the amino acid sequence 100 to 104 in PTX3 N-terminus (42). Ac-ARPCA-NH2 binds FGF2 via hydrophobic interactions that may mimic the interaction of the growth factor with hydrophobic FGF2-binding domain(s) in FGFRs (42). Similar to FGF2, hydrophobic interactions are implicated in FGF8b binding to FGFRs (30), thus suggesting that this mode of interaction may also occur between Ac-ARPCA-NH2 and FGF8b. Indeed, Ac-ARPCA-NH2 inhibits the mitogenic activity exerted by FGF8b on endothelial cells (42) and S115 tumor cells (Leali D, unpublished observations). Experiments are in progress to translate the information about Ac-ARPCA-NH2/FGF8b interaction into a pharmacophore model to be used for the screening of small molecule databases (44), in the search for novel PTX3-derived FGF8b-binding low molecular weight antagonists suitable for in vivo therapeutic interventions.

VEGF plays a central role in switching on a proangiogenic phenotype in most tumors and inhibition of the VEGF/VEGF receptor system markedly disrupts angiogenic switching and initial tumor growth. However, targeting FGFs in addition to VEGF might show synergistic effects in the treatment of angiogenesis-dependent diseases, including cancer (1, 43). Our preclinical observations indicate that PTX3 inhibits the angiogenic activity of FGF8b and potently suppresses the growth of androgen-regulated tumors that seem poorly vascularized. This occurs despite the fact that PTX3 does not affect the proangiogenic action of VEGF-A on endothelial cells (8) nor its expression by tumor cells (see Fig. 4D and Supplementary Fig. S3A) and may be related to its ability to affect both tumor epithelial and stromal compartments by suppressing the autocrine/paracrine action of FGF8b. Moreover, experimental evidences indicate that drug resistance to VEGF blockade may occur following reactivation of angiogenesis triggered by the compensatory upregulation of the FGF/FGFR system in experimental tumor models (45) and in cancer patients (46), representing a mechanism of escape to anti-VEGF therapy in cancer treatment (43). These observations highlight the need for new antiangiogenic drugs directed against different angiogenic factors. PTX3 or PTX3 derivatives endowed with FGF2/FGF8b antagonist activity may represent novel tools for the treatment of hormonal neoplasms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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