High Frequency of Fibroblast Growth Factor (FGF) 8 Expression in Clinical Prostate Cancers and Breast Tissues, Immunohistochemically Demonstrated by a Newly Established Neutralizing Monoclonal Antibody against FGF 8

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Abstract

Fibroblast growth factor (FGF) 8, also known as androgen-induced growth factor, was originally isolated from an androgen-dependent mouse mammary Shionogi carcinoma SC-3 cell line, in which it was shown to have androgen-regulated properties. We previously demonstrated that Fgf 8 transcripts were detected in several human prostate and breast cancer cell lines and that recombinant FGF 8 was mitogenic to an androgen-sensitive prostate cancer LNCaP cell line. In this study, to characterize the roles of FGF 8 in clinical hormone-responsive cancers, we established a monoclonal antibody against FGF 8. In Western blots, this antibody specifically interacted with a FGF 8b isoform that was identical between mouse and human but was not identical to other murine 8a and 8c isoforms. In a cell growth assay using SC-3 cells, the newly established anti-FGF 8 antibody blocked androgen- and FGF 8-stimulated growth but not basic FGF-stimulated growth. Immunohistochemical analyses by use of the established anti-FGF 8 antibody demonstrated that FGF 8 was frequently expressed in human prostate cancers, appearing in 40 of 43 cases (93%), whereas both prostatic hyperplasia specimens and normal prostate tissues included in biopsy specimens were negative for FGF 8 expression. On the other hand, FGF 8 was detected in normal ductal and lobular epithelial cells in breast tissues. FGF 8 was also frequently expressed in various breast diseases, including fibroadenomas (3 of 5 cases, 60%), intraductal papillomas (3 of 3 cases, 100%), ductal hyperplasias (3 of 6 cases, 50%), and breast cancers (8 of 12 cases, 67%). Androgen receptors were also immunohistochemically detected in FGF 8-positive prostate cancers (40 of 40 cases, 100%) and FGF 8-positive breast diseases (17 of 19 cases, 89%). These findings strongly suggest that FGF 8 is involved in hormone-related tumorigenesis of the prostate and breast.

Introduction

FGF is a family of peptide growth factors that are involved in various biological processes, including tumorigenesis, embryogenesis, and angiogenesis (reviewed in Ref. 1). FGF 8, a member of the FGF family, has also been shown to play a central role in cell proliferation in both tumorigenesis and embryogenesis. During embryogenesis, FGF 8 induces the formation of various organs, such as the craniofacial organs, central nervous system, and limbs (2–4). On the other hand, FGF 8 was originally identified from a mouse mammary Shionogi carcinoma SC-3 cell line (5). Because the expression of FGF 8 is markedly induced in SC-3 cells in response to androgenic stimuli, FGF 8 is known to contribute to the androgen-dependent growth of SC-3 cells (5). In addition, we have clearly demonstrated oncogenic features of Fgf 8, which suggest its involvement in human tumorigenesis (6). As might be expected, our previous in vitro study showed that Fgf 8 transcripts are detected in several human prostate and breast cancer cell lines and that recombinant FGF 8 is mitogenic to an androgen-sensitive human prostate cancer LNCaP cell line (7). These observations prompted us to investigate the expression of FGF 8 in clinical specimens of prostate and breast tissues. For this purpose, we established a mAb against FGF 8. By use of this antibody, a high frequency of FGF 8 expression was demonstrated in clinical specimens of prostate cancers and breast tissues, strongly suggesting that FGF 8 is involved in hormone-related tumorigenesis of the human prostate and breast.

Materials and Methods

Chemicals. Recombinant FGF 8 was obtained from isolated CHO cells transfected with Fgf 8 cDNA, as described previously (7). Recombinant bFGF was purchased from PeproTech Inc. (Rocky Hill, NJ). The other chemicals were of analytical grade.

Cells. The SC-3 cell line used in the present study was derived from an androgen-responsive mouse mammary SC115 tumor. The method for cloning has been described previously (5).

Cell Growth Assay. SC-3 cells were plated on a 96-well plate (3 X 103 cells/well) in 0.1 ml of DMEM:Ham’s F-12 (1:1, v/v), with 2% fetal bovine serum treated with dextran-coated charcoal. The next day, the medium was changed to 0.1 ml of serum-free test medium [DMEM:Ham’s F-12 (1:1, v/v) containing 0.1% BSA]. After 48 h of incubation, MTT was added into the medium at a final concentration of 1 mg/ml. After another 4 h of incubation, formazan substrates were collected, resolved with DMSO, and then measured at an absorbance of 570 nm, with reference at 690 nm.

Synthesis of FGF 8 Fragment. A peptide with identical sequences from amino acid residues 23 to 46 of human and mouse FGF 8b, with an additional cysteine residue in the COOH terminus, was synthesized by an automated peptide synthesizer. The synthesized peptide was conjugated with thyroglobulin from keyhole limpets to potentiate antigenicity.

ELISA. The synthesized FGF 8 peptide was conjugated to thyroglobulin, and either the conjugated FGF 8 peptide or a control peptide was plated onto a 96-well plate at a concentration of 10 μg/ml per 50 μl of each well. After overnight plating at 4°C, the peptide was serially washed, blocked, and then interacted with supernatants of hybridoma cells or nonimmune mouse serum for 2 h. After interaction with peroxidase-labeled antinose immunoglobulin and 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid substances, immune complexes were assayed at an absorbance of 415 nm.

Production of a mAb against Human and Mouse FGF 8. Five-week-old female BALB/c mice were immunized four times by injection with 100 μg of the keyhole limpet hemocyanin-conjugated FGF 8 peptide. The antibody titers of serum of the mice were determined by ELISA, as described above. The spleen was removed 3 days after the final injection of the antigen, and 1 X 108...
anti-FGF 8 antibody activity were screened by ELISA and cloned twice by polyethylene glycol 1000. Cultured hybridoma cells in the wells showing anti-FGF 8 antibody activity were screened by ELISA and cloned twice by limited dilution to obtain monoclonal cell lines. Finally, one stable clone (KM1334) was obtained. The 5 × 10^20 - 20 × 10^6 cells were injected i.p. into 8-week-old female BALB/c nude mice, and mAbs were purified from the ascites fluid. Immunoglobulin class and subclass of KM1334 were determined with a mouse mAb isotyping kit.

**Preparation of Recombinant FGF 8 Isoforms.** cDNAs of Fgf 8 isoforms 8a, 8b, and 8c [nomenclature from MacArthur et al. (8)] were obtained from expression cDNA libraries of androgen-stimulated SC-3 cells, as described previously (5). The pcDLsRa296-ligated cDNAs were transfected into COS 1 cells using a Lipofectamine method (Life Technologies, Inc., Rockville, MD), according to the manufacturer’s instructions. After 72 h of incubation, 20 ml of supernatants were collected and purified with heparin-Sepharose affinity chromatography, as described previously (5).

**Western Blots and Silver Staining.** The purified samples were precipitated with trichloroacetic acid, separated by 10–20% gradient SDS-PAGE, electropheretically transferred to a polyvinylidene difluoride membrane, blocked with 5% Blotto, and incubated with anti-FGF 8 antibody (20 ng/ml). A secondary rabbit antimouse IgG conjugated with horseradish peroxidase (DAKO A/S, Glostrup, Denmark) was used at a dilution of 1:25,000 with ECL Plus detection, according to the manufacturer’s instructions (Amersham Life Science, Amersham, United Kingdom). The same samples were also separated by SDS-PAGE and stained with a silver staining kit (Kanto Chemicals, Tokyo, Japan).

**Prostate and Breast Samples.** All of the prostate and breast samples were chosen at random from patients treated at Jichi Medical School Hospital. The prostate samples consisted of 43 needle biopsy specimens of prostatic cancer and 3 transurethral resection samples of prostatic hyperplasia. The breast samples consisted of 32 excisional biopsy specimens of breast tissues, including 12 specimens of cancer, 12 of mastopathy (6 with ductal hyperplastic lesions), 5 of fibroadenoma, and 3 of intraductal papilloma.

**Immunohistochemistry.** Preliminary in situ hybridization studies revealed that fetal ganglion tissues strongly expressed Fgf 8 transcripts (data not shown). Immunohistochemical conditions for formalin-fixed, paraffin-embedded specimens were set using sections of fetal ganglion tissues. Sections from formalin-fixed, paraffin-embedded specimens were boiled in PBS in a microwave for 10 min, interacted with the established anti-FGF 8 antibody at 4°C overnight, and then stained with horseradish peroxidase. Immunopositive staining was detected with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO), and the sections were counterstained with weak Mayer’s hematoxylin. For negative control, nonspecific mouse IgG1 (Sigma) was used instead of anti-FGF 8 antibody. Human AR was immunohistochemically stained with a polyclonal AR antibody (Novocastra Laboratories, Claremont, United Kingdom), according to the manufacturer’s instructions, with some modifications. Paraffin sections were autoclaved for 5 min at 121°C to retrieve AR antigenicity, immunoreacted with a human AR antibody at 4°C overnight, and then stained by an avidin-biotin horseradish peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA).

**Results and Discussion**

Here, we presented the establishment of a mAb against FGF 8. In Western blots, this antibody specifically interacted with an FGF 8b isoform that was identical between mouse and human but was not identical to other murine 8a and 8c isoforms (Fig. 1A). The relative molecular mass of FGF 8 isoforms derived from transfected COS 1 cells was Mr ~32,000 in Western blots, which was identical to that of native FGF 8 proteins purified from SC-3 cells (5). Mitogenic assays using androgen-dependent SC-3 cells showed that the antibody completely blocked mitogenic effects induced by recombinant FGF 8 (50 ng/ml) but not those induced by bFGF (1 ng/ml; Fig. 1B). The antibody also blocked testosterone-induced growth of SC-3 cells, which was previously shown to be mediated by FGF 8 (5) at higher concentrations (Fig. 1C). Because FGF 8 was the sole mitogenic growth factor secreted from SC-3 cells (5) and both 8a and 8c isoforms had significantly low activities (9), it was likely that the

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**Fig. 1. Specificity and neutralizing effects of the established anti-FGF 8 antibody.** A, Western blots of recombinant FGF 8 isoforms. Recombinant FGF 8 isoforms were prepared from supernatants of transfected COS 1 cells by heparin-Sepharose affinity chromatography. Western blots were prepared using Amersham’s ECL Plus reagents and anti-FGF 8 antibody. Human AR was immunohistochemically stained with a polyclonal AR antibody (Novocastra Laboratories, Claremont, United Kingdom), according to the manufacturer’s instructions, with some modifications. Paraffin sections were autoclaved for 5 min at 121°C to retrieve AR antigenicity, immunoreacted with a human AR antibody at 4°C overnight, and then stained by an avidin-biotin horseradish peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA).

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androgen-induced growth of SC-3 cells was mainly mediated by the FGF 8b isoform and blocked by the 8b-specific antibody. Recently, we established a stable cell line transfected with Fgf 8 cDNA into SC-3 cells (10). In this cell line, the level of DNA synthesis was equal with that of testosterone-stimulated SC-3 cells in the absence of androgen. However, this cell line could not grow in a serum-free medium for a long duration unless it was stimulated with androgen, indicating that androgen may have additional pathways on regulating cellular growth. Although higher concentrations of the antibody required for blocking testosterone-induced growth of SC-3 cells is probably due to continuous production of FGF 8 from testosterone-stimulated SC-3 cells, it is possible that other androgenic action is involved in the growth of SC-3 cells.

Because there was no difference in localization among FGF 8 isoforms (11), we next performed immunohistochemical analyses using the established antibody. FGF 8 was immunohistochemically detected in 40 cases (93%) of prostate cancers (Fig. 2A and Table 1). In contrast, no expression of FGF 8 was shown in the three cases of prostatic hyperplasia or the normal prostatic tissues included in the needle biopsy specimens. There was no significant correlation between FGF 8 expression and histological cancer gradings (Table 1). Using an in situ hybridization method, Leung et al. (12) briefly reported that 71% of human prostate cancers expressed Fgf 8 transcripts and that the levels of its expression were correlated with histological cancer gradings. Although there was no correlation between FGF 8 expression and histological cancer gradings in our study, both studies clearly demonstrated highly frequent and cancer-preferential expression of FGF 8 in clinical prostatic specimens. The discrepancies between these results might be due to differences in the ethnic or epidemiological backgrounds of the examined specimens. Because this study dealt with clinical cancer specimens, cases of clinical stage A, which represent incidental carcinoma, were not included. However, FGF 8 was detected at a high frequency in all clinical stages (Table 1), suggesting that FGF 8 is expressed at early stages in prostatic carcinogenesis. Investigations for FGF 8 expression in precancerous lesions such as prostatic intraepithelial neoplasia are now being considered. Because FGF 8 had androgen-regulated characters in vitro, the status of AR was immunohistochemically examined. All cancer specimens, including FGF 8-negative cases, immunohistochemically contained AR at high levels (Fig. 2B and Table 1). Although further studies will be needed to clarify the relation between FGF 8 expression and the status of androgen sensitivity in prostate cancers, it is reasonable to postulate that FGF 8 expression may be enhanced by androgen in AR-positive cancer cells.

In the breast tissues, it is notable that FGF 8 was frequently detected not only in cancer cells but also in normal and benign epithelial cells. Both lobular and ductal epithelial cells were positive with FGF 8 (Fig. 2C). Three of six duct hyperplasias (50%), five of five fibroadenomas (100%; Fig. 2D), three of three intraductal papillomas (100%), seven

Table 1 FGF 8 and AR expression in prostate and breast tissues

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* No. of specimens expressing both AR and FGF 8.

b Tumor grades and clinical stages were classified according to Japanese classification for prostate carcinoma. Well, moderate, and poor refer to the differentiation of prostate adenocarcinoma.
FOF 8 EXPRESSION IN HUMAN PROSTATE AND BREAST

of nine invasive ductal carcinomas (78%; Fig. 2E), and two of three invasive lobular carcinomas (67%) were also positive for FGF 8 expression (Table 1). The differences of FGF 8 expression between prostate and breast could not be explained in this study. Because Fgf 8 transcripts are detected in the embryonic urinary system (13), FGF 8 may be expressed at a certain development stage in the prostate. Previously, we reported that high concentrations (100 nm to 1 μM) of 17β-estradiol also induced FGF 8 expression at low levels in SC-3 cells via AR (14). Therefore, local estrogen metabolites might modulate cell growth in breast tissues through FGF 8 via AR. Recently, it was clearly demonstrated that >70% of breast cancer cases immunohistochemically contained AR (15, 16) and that growth-stimulatory or -inhibitory responses to androgen were present in different human breast cancer cell lines (17, 18). One of the breast cancer cell line (MDA-MB-231) showed androgen-inducible Fgf 8 expression (19). The immunohistochemical analyses in this study also showed that 77% of specimens from 26 cases of benign and malignant breast disease contained AR (Fig. 2F and Table 1). Among the FGF 8-positive cases, 89% (17 of 19 cases) possessed AR in breast diseases. These observations suggest that an AR-mediated signaling pathway may also regulate cell proliferation in breast tissues and that FGF 8 would be one of the key molecules in such a mode of AR-mediated cell regulation.

In summary, we have established a mAb against FGF 8, and we have immunohistochemically shown that FGF 8 is highly expressed in human prostate cancers and human breast tissues. Thus, the established neutralizing antibody presented here provides a useful tool for understanding growth mechanisms of these cancers, as well as a possible therapeutic tool for their treatment.

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References

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