Demonstration of Fibroblast Growth Factor Receptor-1 in Rat Adrenal Gland as Revealed by Reverse Transcription-polymerase Chain Reaction and Immunohistochemistry

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Recent studies have shown that FGF-1 (aFGF) and FGF-2 (bFGF) are expressed in the adrenal glands. In order to understand the roles of FGFs in the adrenal gland, we investigated the expression of the fibroblast growth factor receptor-1 (FGFR1) in rat adrenal gland using reverse transcription-polymerase chain reaction (RT-PCR) method, Western blot and immunohistochemistry. RT-PCR experiment using a primer set to amplify the acidic region of FGFR1 gave two bands of about 426 bp and 160 bp in both the adrenal cortex and medulla. The sizes of the large and small PCR products corresponded well to the three IgG-like domain (long form) and two IgG-like domain (short, or secreted form) FGFR1, respectively. Western blotting analysis using an antibody capable of detecting the acidic region of FGFR1, gave two bands in the adrenal gland with molecular weights of about 130 kDa and 75 kDa, corresponding to the long and the secreted form of FGFR1, respectively. Immunohistochemical study using the antibody demonstrated that FGFR1-positive cells were distributed mainly in the zona glomerulosa of the adrenal cortex and medulla. A few positive cells were scattered in the zona fasciculata and reticularis. In the adrenal medulla, almost all cells were positive for FGFR1. Double immunostaining revealed that FGFR1 colocalized with tyrosine hydroxylase-immunoreactive catecholamine cells in the adrenal medulla.

Key words: FGF, adrenal gland, zona glomerulosa, adrenal medulla, receptor

I. Introduction

Fibroblast growth factors (FGFs) comprise a family of polypeptides that exhibit mitogenic activity toward a wide variety of mesodermal and neuroectodermal cells in many tissues, including the adrenal gland [1, 5, 10]. In the adrenal gland, Grothe and Unsicker proved by immunohistochemistry that basic fibroblast growth factor (bFGF; FGF-2) is localized in the zona glomerulosa and an outer part of the zona fasciculata of the cortex, as well as in chromaffin cells of the medulla [11, 12]. Recently, we have demonstrated that acidic fibroblast growth factor (aFGF; FGF-1) is localized in ethanolamine N-methyltransferase-, an enzyme of adrenaline synthesis, immunoreactive adrenergic cells in rat adrenal medulla [24]. Although such a selective localization implies definite roles for FGF-1 and FGF-2 in the adrenal gland, their roles are far from being totally understood.

In order to understand the roles of FGFs in the adrenal gland, it is important to clarify the localization of FGF receptors, because the FGFs have been thought to exert their biological activities mainly through interaction with specific cell surface receptors in responsive cells. So far, at least four different FGF receptors (FGFRs) have been cloned [18].
These include Flg (FGFR1) [7, 17, 22], bek (FGFR2) [7], FGFR-3 [20] and FGFR-4 [28]. FGFR1 is known to be one of the high affinity receptors for both FGF-1 and FGF-2 [18]. It has been predicted that the FGFR1 gene generates multiple forms of receptor protein by alternative splicing [16, 17]. According to Johnson et al. [17], they are divided into two main forms: a three (the long form) and a two (the short, or secreted form) IgG-like domain in the extracellular region.

Recent in situ hybridization and immunohistochemical studies have demonstrated that the FGFR1 is expressed in several tissues such as the brain [14, 23, 30, 31] and prostate [13]. Concerning the expression of FGFR receptors in the adrenal gland, a previous study using a sensitive ribonuclease protection assay showed that FGFR1 mRNA was detected in the rat adrenal gland [26, 27]. The highest amounts of FGFR1 mRNA were found in postnatal (P) day 1 and day 8 adrenal cortices. A previous immunohistochemical study also demonstrated that FGFR1 protein was observed in adrenal gland [9]. However, the precise localization of FGFR1 in the adrenal gland was not shown. The present study aimed at clarifying the expression and localization of FGFR1 in the rat adrenal gland by reverse transcription-polymerase chain reaction (RT-PCR), Western blots and immunohistochemistry.

II. Materials and Methods

Tissue preparations for immunohistochemistry

The experiment was conducted in strict adherence to the principles of laboratory animal care (NIH, revised 1985) and the standards of animal experiment and animal care of our University. Five male Wistar rats (Clea Japan Inc., Japan) weighing 160–200 g were used. The animals were housed on a 12 hr light/dark schedule and given free access to food and water. Under pentobarbital anesthesia (80 mg/kg), the animals were perfused via the ascending aorta with 10 mM phosphate buffer containing 0.9% NaCl (pH 7.4) followed by a fixative containing 4% paraformaldehyde, 0.2% picrotate and 0.35% glutaraldehyde in 100 mM phosphate buffer (pH 7.4). The animals were kept in crushed ice during perfusion. After perfusion, the bilateral adrenal glands were quickly removed, and immersed for one or two days in a fixative consisting of 4% paraformaldehyde and 0.2% picrotate in 100 mM phosphate buffer (pH 7.4). They were then placed in 100 mM phosphate buffer containing 15% sucrose, frozen with dry-ice and cut into 20-μm-thick sections. The sections were collected in 100 mM phosphate-buffered saline (pH 7.4) containing 0.3% Triton X-100 (PBST).

RNA analysis

Under pentobarbital anesthesia (80 mg/kg), male Wistar rats were perfused via the ascending aorta with 10 mM phosphate buffer containing 0.9% NaCl (pH 7.4) to wash remaining blood out. The adrenal glands were dissected, and the fresh tissues of the glands were quickly divided into cortex and medulla under dissection microscope. Total RNA was isolated from adrenal cortex and medulla according to the acid guanidium thiocyanate-phenol method [6] with a slight modification. In brief, either the adrenal cortex or medulla was homogenized for 45 sec at room temperature in 0.5 ml of Trizol solution (Life Technology, Rockville, MD, USA). After adding 0.4 ml of chloroform to the homogenate, the solution was briefly mixed and left for 5 min at room temperature. The suspension was then centrifuged at 10,000 g for 5 min at 4°C. After centrifugation, the aqueous phase containing RNA was transferred to a new tube. RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol and stored for 1 hr at −20°C. The insoluble RNA was collected again by centrifuging at 10,000 g for 20 min at 4°C. The pellet was washed with 70% ethanol and dried. Finally, the pellet was dissolved in 20 μl of distilled water which had been incubated overnight with 0.02% diethyl pyrocarbonate (DEPC) and autoclaved for 20 min at 121°C. Prior to reverse transcription, 5 μg of the total RNA prepared as above was incubated for 1 hr at 37°C with 10 units RNAsase free DNAsase (Pharmacia, USA) and 20 units of RNAsin (Wako Pure Chemicals Co., Japan) to eliminate any trace DNA contamination. The total RNA was then reverse-transcribed using 500 pmol of random hexamers (Pharmacia, USA) with 80 units of Malonyne murine leukemia virus reverse transcriptase (Life Technology, Rockville, MD, USA) by incubating for 1 hr at 37°C, and the reverse transcriptase was inactivated by adding the same volume of buffer-saturated phenol (Life Technology, Rockville, MD, USA). After centrifugation for 5 min at 10,000 g, the upper phase was recovered into a new tube. The cDNA was precipitated by adding the ethanol and the pellet was washed again with 70% ethanol. Finally, the solution was solved in 20 μl of DEPC-treated water. The cDNA obtained was used in the PCR analysis for FGFR1 mRNA.

Details of the primers for PCR used in this study are summarized in Table 1. The primers for FGFR1 were designed to detect the three and two IgG-like forms of FGFR1 at different sizes. To confirm the technical validity of dissecting the adrenal cortex from the medulla, tyrosine hydroxylase (TH) mRNA was also amplified. Since TH mRNA is known to be expressed only in chromaffin cells of the adrenal medulla, but not in cells of the adrenal cortex. The reaction mixture for PCR consisted of 500 ng of the converted cDNA, 200 ng of each of the primers, 200 μM of each of four deoxynucleotide triphosphates and 2 U Taq polymerase (Wako Pure Chemicals Co., Japan) dissolved in 50 μl of 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 10 mM mercaptoethanol (Wako Pure Chemicals Co.) and 2 mM MgCl₂. The profile of the thermal cycle used was: (1) denaturation at 95°C for 30 sec, (2) annealing at 58°C for 40 sec, and (3) extension at 72°C for 90 sec. We performed the PCR for 30 to 32 cycles. In addition, β-actin mRNA was amplified for 28 cycles under the same thermal profile to assess the variability of mRNA contents. The PCR products obtained were electrophoresed on a 3% agarose gel and stained with ethidium bromide.
Immunochemical procedures

The sections were treated for 30 min at room temperature with 0.5% H2O2 in PBST to destroy endogenous peroxidase-like activity. After rinsing several times in PBST, the sections were incubated for three days at 4°C with a FGFR1 antiserum (FR237, diluted 1:10,000), for 2 hr at room temperature with biotinylated anti-rabbit IgG (diluted 1:1,000, Vector Labs., USA), and for 1 hr at room temperature with the avidin-biotin-peroxidase complex (diluted 1:4,000, Vector Labs., USA). All the immunochemicals used were diluted with PBST. After each incubation, all sections were washed several times with PBST. Color was developed by reacting the sections for 10 min with a mixture containing 0.02% 3,3′-diaminobenzidine, 0.0045% H2O2 and 0.6% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The stained sections were mounted on glass slides and cover-slipped with Entellan (Merck, Germany).

For immunohistochemical controls, some sections were stained with a pre-immune rabbit serum or with a FGFR1 antiserum which had been absorbed with an excess of the FGFR1 peptide.

Production and characterization of the FGFR1 antiserum (FR237) has been reported before [23]. The antiserum recognizes the acidic region of FGFR1, which is a region common to all forms of FGFR1.

Double immunofluorescent labeling

The double immunofluorescent labeling was performed according to previous reports [19, 24] with slight modification. In brief, sections were incubated with PBST containing anti-FGFR1 rabbit polyclonal antibody (R237, 1:10,000) and anti-tyrosine hydroxylase mouse monoclonal antibody (0.5 μg/ml, Chemicon International, Temecula, CA, USA) for 3 days at 4°C. After washing with PBST several times, they were incubated with Texas Red labeled anti-mouse IgG (raised in goat, Cappel, diluted at 1:100) and Alexa488 labeled anti-rabbit IgG (raised in goat, Molecular Probe, diluted at 1:500) for 4 hr at 4°C. Fluorescence was observed by confocal laser-scanning microscope (BioRad, Hercules, CA, USA).

Western blotting

For Western blot analysis, fresh tissues of rat adrenal glands were homogenized in five volumes of ice-cold 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, phenylmethyl-sulfonyl-fluoride (100 μg/ml), leupeptin (1 μg/ml), and pepstatin (1 μg/ml) as well as the complete™ protease inhibitor cocktail (Complete Mini; Boehringer Nuannheim Biochemicals, Mannheim, Germany). The homogenate was centrifuged at 9,000 g for 20 min at 4°C. The supernatant was collected as a crude cytosolic fraction. The pellet was again homogenized in the same buffer containing 1% Triton-X 100 at 4°C. The suspension was kept on ice for 1 hr, and then centrifuged again at 9,000 g for 20 min at 4°C. The supernatant was collected as a crude soluble membrane fraction. About 50 μg of the soluble membrane fraction was electrophoresed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Japan, Japan). The membrane was incubated for 30 min with 25 mM Tris-HCl containing 150 mM NaCl (TBS) and 5% skim milk at room temperature, and further incubated overnight with the FGFR1 antiserum (FR237, diluted 1:10,000) in TBS containing 1% skim milk at room temperature. After washing with TBS containing 0.1% Tween 20, the membrane was reacted for 1 hr with alkaline peroxidase-labeled anti-rabbit IgG polymer (Histofine, diluted 1:500, Nichirei, Tokyo) in TBS containing 1% skim milk at room temperature. The labeling was visualized by incubating with a mixture containing 0.02% 3,3′-diaminobenzidine, 0.0045% H2O2 and 0.6% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). Precision pre-stained markers (BioRad, Hercules, CA, USA) were used as a marker for molecular size.

III. Results

Figure 1A shows PCR products amplified by using primers for detecting mRNA of β-actin, TH and FGFR1 in the adrenal cortex and medulla. The primers for actin mRNA gave a single band of 259 bp with a comparable staining intensity in both cortex (lane 1) and medulla (lane 2). The primers for TH mRNA revealed a single band of 646 bp in the medulla (lane 4), but not in the cortex, confirming a sufficient separation of the medulla from the cortex. The primers for FGFR1 mRNA gave two bands of 426 bp (arrow) and 160 bp (arrowhead) in both the adrenal cortex and medulla. The sizes of the large and small PCR products

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<th>Table 1. PCR primers used in this study</th>
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<td>Gene</td>
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* 426 bp in three IgG-like forms of FGFR1, ** exclude actin intron E
corresponded well to those of three (the long form) and two (the short, or secreted form) IgG-like domain of FGFR1, respectively.

The Western blotting analysis using a soluble membrane fraction of rat adrenal gland showed two bands in the adrenal gland with the molecular weights of about 130 kDa and 75 kDa, corresponding to the long and the short and secreted form of FGFR1, respectively (Fig. 1B).

Immunohistochemical examination for FGFR1 in rat adrenal gland revealed positive staining distributed mainly in the zona glomerulosa of the cortex and the adrenal medulla (Figs. 2, 3). The capsule of the adrenal gland was also stained (Figs. 2, 3). In other cortical layers, the zona fasciculata and reticularis, a few positive cells were scattered (arrows in Fig. 2B). In the adrenal medulla, almost all cells were positive for FGFR1 (Fig. 2C). These staining structures were abolished by immunohistochemistry using the FGFR1 antiserum pre-absorbed with the FGFR1 peptide (Fig. 2D).

Figure 3 shows the results of double immunostaining for FGFR1 (red) and TH (green). FGFR1-positive cells in the adrenal cortex were negative for TH (Fig. 2A–C). Since red and green combine to produce a yellow color under dual exposure of the section, cells in the adrenal medulla contained both FGFR1 and TH (Fig. 3E, F).

IV. Discussion

The present RT-PCR and Western blot analyses clearly demonstrated that both FGFR1 mRNA and its product protein was expressed in both the adrenal cortex and medulla. The results were in good agreement with previous reports [9, 26]. It has been shown that the FGFR1 gene is capable of yielding multiple forms of receptor protein by alternative splicing [16]. In human, five forms of FGFR1 have been reported [17]. In mouse tissues, the long (three IgG-like domain) and short (two IgG-like domain) forms were expressed in several tissues [4]. In rat kidney, Kim et al. [21] reported three forms of FGFR1: α- (three IgG-like domain), β- (two IgG-like domain) and γ-form (intracellular form). Yazaki et al. [32] reported the three and the two IgG-like domain forms in various rat tissues including the brain, lung, kidney and small intestine. We also reported that the long and the secreted forms of FGFR1 were expressed in rat brain [23]. However, little information is available concerning the variants of FGFR1 in rat adrenal glands. Our Western blot analysis indicated that the rat adrenal gland mainly produced two forms with molecular weights of about 130 kDa and 75 kDa, corresponding to the long and the secreted forms of FGFR1, respectively [22, 23].

The present study by immunohistochemistry further indicated that FGFR1-positive staining was mainly localized in the zona glomerulosa and the medulla of the adrenal gland as well as the capsule. The localization of FGFR1 in the zona glomerulosa and the capsule is supported by a previous autoradiographic study showing that the binding of FGF-2 was detected in the zona glomerulosa and the capsule [3]. Our results indicate that the binding of FGF-2 is caused by FGFR-1. It is well known that fibroblasts in the capsule express FGFR1 and proliferate by FGF-2, while the precise role of FGFR1 in the zona glomerulosa remains unknown.
Interestingly, however, FGF-2 was also detected in the rat zona glomerulosa [11, 12]. Moreover, the expression pattern of FGFR1 mRNA matches that of FGF-2 mRNA in developing and adult rat adrenal glands [26]. In culture, FGF-2 is so far the most potent mitogen of adrenocortical cells [15]. The concomitant localization of FGF-2, reported previously, and its receptor FGFR1, shown here, in cells of the rat zona glomerulosa supports the proposed role of FGF-2 as an autocrine growth factor for adrenocortical cells [2, 3].

As to yet another source of the ligand FGF-2 for FGFR1, it is possible that FGF-2 may be co-released together with vasopressin into the blood stream to act on their distant target, the zona glomerulosa of the adrenal gland, since the zona glomerulosa functioning in water metabolism is under regulatedly the pituitary gland, which involves vasopressin-containing neurons in the hypothalamic supraoptic and paraventricular nuclei that have been shown to possess FGF-2 [8, 25]. Further physiological roles of FGF-2 and FGFR1 remain to be elucidated, particularly in relation to the water balance in the body.

Fig. 2. Immunohistochemical staining for FGFR1 in rat adrenal gland. A: Positive staining is seen in the zona glomerulosa of the cortex and the adrenal medulla. B: High magnification of the zona glomerulosa of the cortex. A few positive cells are scattered in the zona fasciculata (arrows). C: High magnification of the zona glomerulosa of the adrenal medulla. Almost all cells appear to be stained positively. D: A control section stained with the FGFR1 antiserum preabsorbed with the FGFR1 peptide. No positive staining was observed except for the non-specific staining limited to the adrenal capsule (arrow). Bars=100 μm (A and D), and 50 μm (B and C).
The present study demonstrates the expression of FGFR1 in the adrenal medulla. The results are in good agreement with a previous study using the ribonuclease protection assay [26, 27]. The present study further clarifies that FGFR1 colocalized with tyrosine hydroxylase-immunoreactive catecholamine cells in the adrenal medulla. In the adrenal medulla, both FGF-1 and FGF-2 are expressed [11, 12, 24]. Recently, Peng et al. [29] reported that a signaling pathway of FGF-2 and nuclear FGFR1 activated the TH gene in bovine adrenal medullary cells. The colocalization of FGFR1 with FGF-2 in TH-positive cells indicates that FGFR1 plays a role in regulation of TH gene expression in vivo. In both adrenal cortex and medulla, FGFR1 seem to be localized in both the cell surface and cytoplasm. The precise localization of FGFR1 at the subcellular level should be clarified by immuno-electron microscopy.

In summary, the present study demonstrates that the long and the secreted forms of FGFR1 are expressed in both adrenal cortex and medulla. The double-immunofluorescent labeling for FGFR1 (red) and TH (green) in rat adrenal gland is shown in Figure 3. A and D show localization of FGFR1 (red). C and F demonstrate localization of TH (green). B and E show an overlaid view of images. In double-labelled cells, red and green combine to yield a yellow color. FGFR1-positive cells in the adrenal cortex were negative for TH, while cells in the adrenal medulla displaying a yellow color contain FGFR1 and TH. Bar=100 μm (A–C) and 25 μm (D–F).
the adrenal cortex and the adrenal medulla in rats. In the adrenal cortex, FGFR1 is mainly localized to cells in the zona glomerulosa. In the adrenal medulla, FGFR1 is colocalized with tyrosine hydroxylase-immunoreactive catecholamine cells.

V. Acknowledgments

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VI. References