Immunohistochemical Localization of Basic Fibroblast Growth Factor and Gonadotrophin in the Goat Pituitary Gland

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Abstract: *The present study was designed to investigate the immunohistochemical localization and distribution of basic fibroblast growth factor (bFGF) in the goat pituitary gland. We examined various fixatives for tissue preparation, and confirmed that methyl Carnoy-fixed tissues were associated with a better preservation of bFGF. After fixation, paraffin- embedded tissue sections were prepared, and were stained immunohistochemically with mouse monoclonal antibody against bovine bFGF. Approximately 30% of parenchymal cells in the pars distalis of the goat pituitary gland displayed strong immunoreactivity against the bFGF antibody. Endothelial cells occasionally showed positive immunoreactivity. No immunopositive cells were found in either the pars intermedia of the pituitary gland or posterior lobes. Based on analysis of serial sections stained with antibodies against pituitary hormones (luteinizing hormone and adrenocorticotropic hormone), bFGF-positive cells appear to be a subpopulation of gonadotrophs and or corticotrophs. The present findings indicate that immunohistochemical reactivity for bFGF may be partially attributed to differences in fixatives and that bFGF may play critical roles in the goat pituitary gland.*

Key words: *Basic fibroblast growth factor, Pituitary gland, Goat*

Basic fibroblast growth factor (bFGF) was originally identified as an activity in extracts of bovine pituitary glands [1] and this peptide growth factor is known to be a potent angiogenic factor [2]. The presence of bFGF has been reported in the brain, corpus luteum, kidney, adrenal gland, and retina as well as the pituitary gland [3, 4]. Although it is well established that the anterior pituitary is a rich source of bFGF [5], the specific roles it plays in the pituitary remain to be elucidated. Since the synthesis of bFGF does not include a signal sequence [6], the mechanism by which it is released from the cells is unclear. Previous studies, indicating immunoreactivity for bFGF using a range of fixation and preparative protocols in rat kidney and human eye, suggested that the immunostaining patterns of bFGF are dependent on the immunohistochemical procedures [4, 7]. Also immunohistochemical studies of rat pituitary glands have suggested that bFGF is present in a subpopulation of gonadotrophs and/or corticotrophs [3, 8]. In the anterior pituitary glands of ruminants and other species, the zona tuberalis (ZT) is composed of basophils, e.g. gonadotrophs and thyrotrophs [9–11], but there have been no previous studies relating to ZT or localization of bFGF using immunohistochemical techniques. The objective of the present study was, therefore, to identify the detailed localization and distribution of bFGF-positive cells by immunohistochemical techniques, and to determine the correlation with bFGF-positive cells and parenchymal cells, mainly gonadotrophs, in the goat pituitary.

Received: January 5, 1999
Accepted: February 4, 1999
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Materials and Methods

Animals and tissue preparation

Nine adult female Tokara goats (anestrus) bred on Kyushu University Farm were used. They were killed by exsanguination under deep anesthesia with pentobarbital sodium (Nembutal injection, Dinabot, Osaka, Japan). The pituitary gland was excised and divided into two blocks at the mid-sagittal level. Three pituitary

glands were immersed in methyl Carnoy's fixative (methanol: chloroform: glacial acetic acid, 6:3:1) for 12 h, and three were immersed in Bouin's fixative (picric acid: formalin: glacial acetic acid, 15:5:1) for 12 h, at room temperature (RT; 23–26°C) and embedded in paraffin. The other three were immersion fixed in sublimate-formalin (saturated mercury chloride solution: formalin, 9:1) for 24 h at RT, and then the fixed tissues were washed under running water, dehydrated through an ethanol series, and embedded in paraffin. All paraffin-embedded tissues were cut into sagittal serial sections (4 μm thick).

Immunohistochemistry

For immunohistochemical detection of bFGF, luteinizing hormone-β (LH-β) and adrenocorticotropic hormone (ACTH) in the parenchymal cells of pituitary glands were used. The sections were stained by the avidin-biotin-peroxidase complex (ABC) method [12] using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A). Briefly, the pituitary sections were dewaxed in xylene, rehydrated through an ethanol series and washed in distilled water. To inhibit endogenous peroxidase activity, they were incubated with 0.3% hydrogen peroxide in methanol for 30 min at RT and washed with 0.01 M phosphate-buffered saline (PBS; pH 7.4). Nonspecific binding of the primary antibodies was blocked by preincubation with normal horse serum for the monoclonal antibody or with normal goat serum for the polyclonal antibodies. Then, the sections were incubated with each primary antibody for 1 h at RT. The primary antibodies used in the present study and their working dilutions are listed in Table 1. After incubation, sections were washed well with PBS, and incubated with biotinylated horse anti-mouse IgG antiserum for the monoclonal antibody or with biotinylated goat anti-rabbit IgG antiserum for the polyclonal antibodies for 30 min at RT.

After washing with PBS, they were incubated with avidin-biotin-peroxidase complex for 1 h at RT. The sections were dehydrated through an ethanol series and were mounted with Eukitt (Kindler, Freiburg, Germany).

Morphometrical analysis

The nuclei of the bFGF-immunopositive pituitary parenchymal cells and all nuclei of the parenchymal cells (approx. 1,000 cells/each region; three sections/pituitary) were counted in the anterior, central and posterior regions of the pars distalis. The percentage of the bFGF-positive cells (number of nuclei of bFGF- positive cells $\times$ 100/total number of parenchymal cells) in each region was calculated. The serial sections stained histochemically with different antibodies were processed using the NIH image analysis software on a Macintosh computer to analyze the co-expression patterns of the different pituitary hormones.

Statistical analysis

Immunohistochemical and conventional data were analyzed by ANOVA with the StatView IV program using a Macintosh computer. Differences at a probability of $P<0.05$ were considered significant. Values are presented as means ± SE.

Results

Immunohistochemistry

Immunohistochemical reaction against bFGF on the goat pituitary sections was well preserved when the tissue samples were fixed with methyl Carnoy's fixative (Fig. 1A). When the samples were fixed with Bouin's or sublimate-formalin fixatives (Fig. 1 B and C, respectively), no positive immunoreactivity was detected.

The bFGF-positive staining was observed mainly in the cytoplasm of the pituitary parenchymal cells, and these bFGF-positive cells were interspersed among immunonegative parenchymal cells within the pituitary cell cords (Fig. 2s). These bFGF-immunopositive cells occasionally contained vacuoles (Fig. 2A). The nuclei of the parenchymal cells, however, were occasionally immunopositive for bFGF (Fig. 2B). Immunoreactivity against bFGF was occasionally observed in both the cytoplasm and nucleus of the endothelial cells (Fig. 3A) and the nuclei of interstitial cells (Fig. 3B). These scattered positive interstitial cells were observed in both anterior and posterior regions, however fewer positive

Table 1. Antibodies used for immunohistochemical detection of bFGF-, LH-β- and ACTH-positive cells

Antibody	Species	Dilution	Source
Anti-bovine bFGF (monoclonal)	Mouse	1:80	Upstate Biotechnology Inc. (USA)
Anti-human LH-β (polyclonal)	Rabbit	1:2,000	UCB-Bioproducts S.A. (Belgium)
Anti-ACTH (1-24) KLH/synthetic (polyclonal)	Rabbit	1:2,000	ICN ImmunoBiologicals (USA)

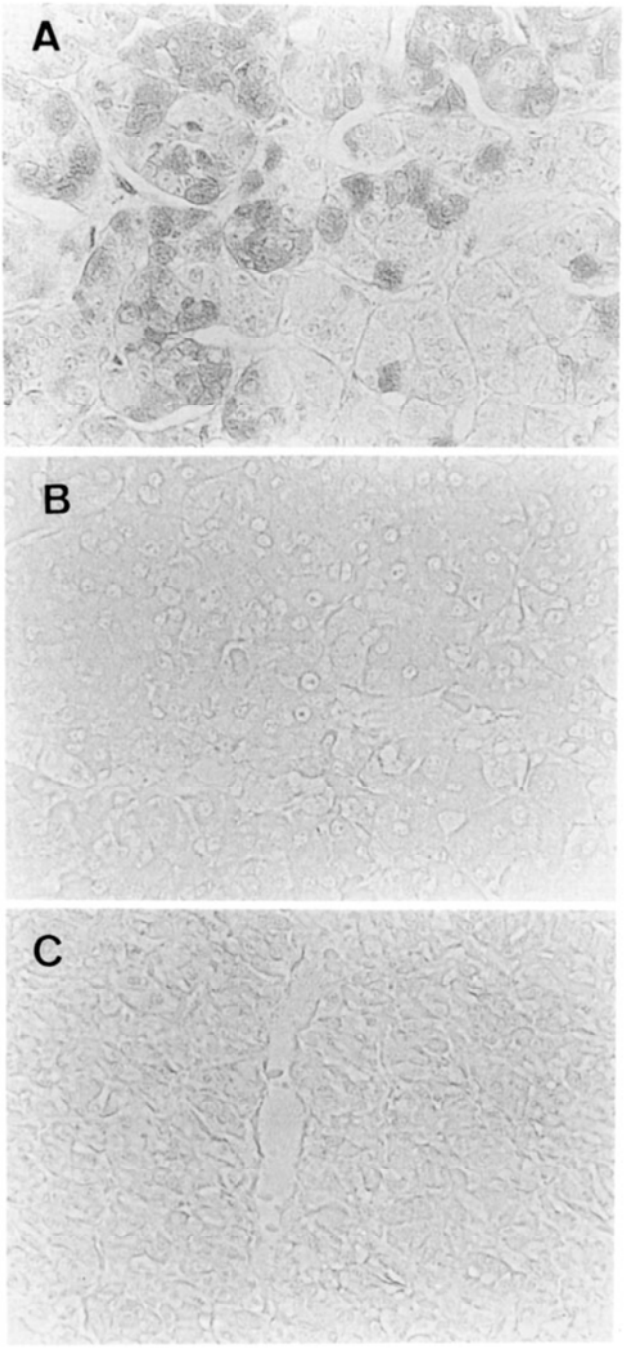

Fig. 1. Light micrographs of the anterior pituitary glands fixed with Carnoy's or Bouin's fixative or sublimate-formalin (A, B and C, respectively). After fixation, paraffin sections were immunohistochemically stained for bFGF. Immunohistochemical reactivity to bFGF was well preserved when the tissues were fixed with Carnoy's fixative (A). bFGF-positive staining was observed mainly in the cytoplasm of the pituitary parenchymal cells, and these cells were interspersed among immunonegative parenchymal cells within the pituitary cell cords. No positive immunohistochemical reactivity was detected on the sections fixed with Bouin's fixative (B) or sublimate-formalin (C). (× 200).

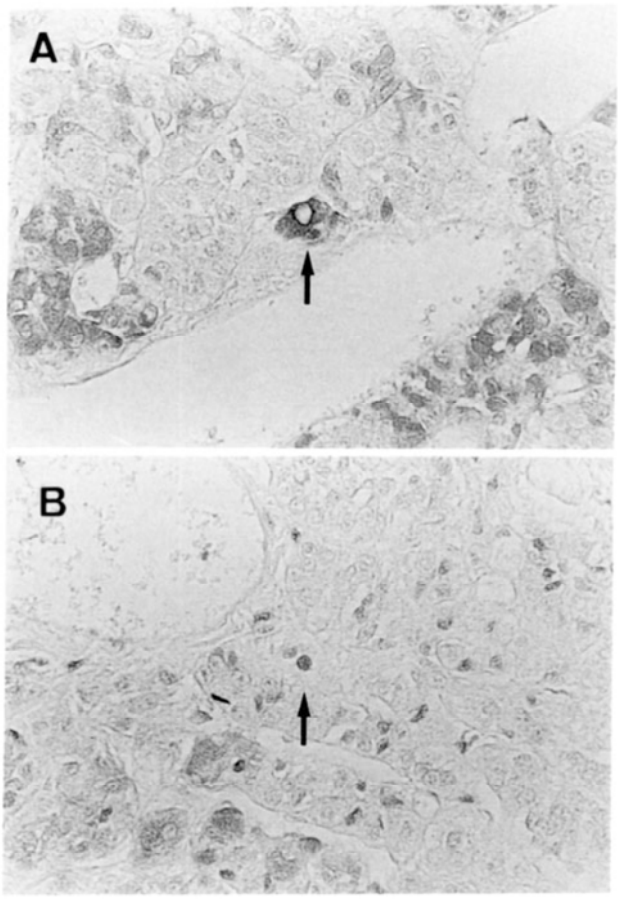

Fig. 2. Light micrographs of the anterior pituitary glands fixed with Carnoy's fixative. bFGF-immunopositive parenchymal cells occasionally contained vacuoles (A; arrow), and the nuclei of the parenchymal cells were occasionally immunopositive for bFGF (B; arrow). (× 200).

cells were observed in the central region (data not shown). Representative results of morphometrical analysis of percentage of bFGF-positive cells in the pars distalis of three goats are shown in Fig. 4. As described above, the pars distalis was divided into three regions, and bFGF-positive cells tended to be more abundant in the anterior and central regions than in the posterior region. No bFGF immunoreactivity was found in either the pars intermedia of pituitary glands or in the posterior lobes (data not shown).

Serial sections were stained for both bFGF and LH-β (Fig. 5A and B, respectively) and bFGF and ACTH (Fig. 5C and D, respectively). As shown in these photographs, bFGF-positive cells were co-localized with gonadotrophs (LH-β-positive cells) and/or with corticotrophs (ACTH-positive cells).

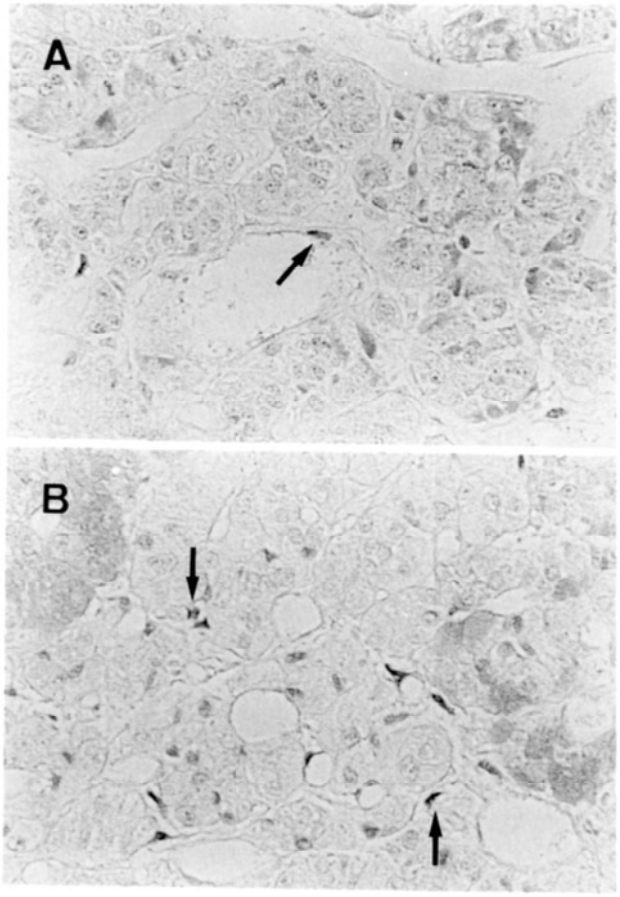

Fig. 3. Light micrographs of the anterior pituitary glands fixed with Carnoy's fixative. Positive immunoreactivity for bFGF was occasionally observed in both the cytoplasm and nucleus of endothelial cells (A; arrow) and the nuclei of interstitial cells (B; arrows). (× 200).

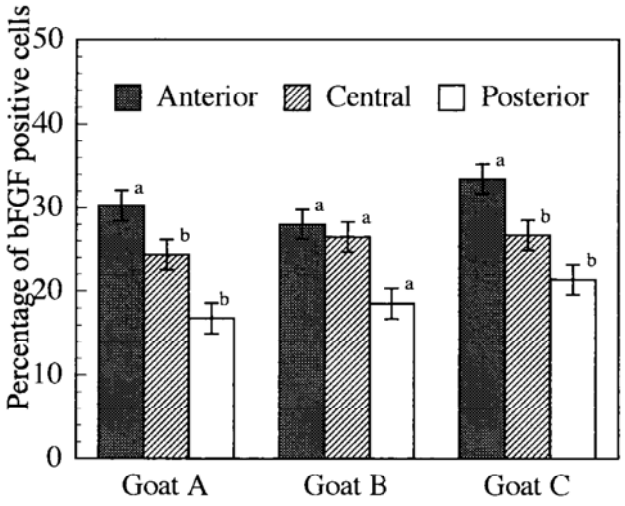

Fig. 4. Percentages of bFGF-positive cells in the pars distalis of the goat pituitary gland. The pars distalis of pituitary glands were divided into three regions as described in Materials and Methods. Data are the means ± SE of triplicate determinations and differences at a probability of $P<0.05$ were considered significant.

Discussion

In the present study, immunohistochemical reactivity for bFGF in the goat pituitary gland could be detected when the tissues were fixed with methyl Carnoy's fixative, while no immuno- reactivity was observed in those tissues fixed with Bouin's or sublimate-formalin fixatives. Marín *et al.* [13] reported that methyl Carnoy's fixation yielded better results than the use of aldehyde-containing fixatives for detection of the immunohistochemical localization of bFGF in the rat pineal gland. The immunolocalization of bFGF is considered to be dependent on several parameters, including the nature of the antibody and the type of tissue fixation process used [4, 7]. Some isoforms of bFGF with different molecular weights have been isolated from the rat kidney [14], and different bFGF mRNA isoforms have also been isolated from different intracellular locations, i.e. in the nucleus or in the peripheral cytoplasm [15]. Such molecular variation in bFGF will cause differences in the sensitivity of immunohistochemical detection with different fixation procedures. In the present study, bFGF immunoreactivity was detected mainly in parenchymal cells of the pars distalis in the goat pituitary gland. Moreover, parenchymal cells which were double positive for bFGF and LH-β and for bFGF and ACTH were detected. We concluded that bFGF-positive parenchymal cells include a subpopulation of gonadotrophs and/or corticotrophs. In previous studies in rats [3, 8], immunoreactivity for bFGF was also observed within the cytosol of both gonadotrophs and corticotrophs. However, Marín *et al.* [16] demonstrated the presence of bFGF in somatotrophs in human pituitary glands. On the other hand, Grothe *et al.* [17] reported that bFGF immunoreactivity was present in all the parenchymal cells of the pars distalis of the bovine pituitary glands. Such species-dependent differences in the distribution of bFGF cells in the pituitary glands are considered to be dependent on the methodological differences including tissue fixation procedures and antibodies used, and further detailed investigations are required for methodological improvement.

In most cases, the immunoreactivity for bFGF was observed within the cytosol of pituitary parenchymal cells.

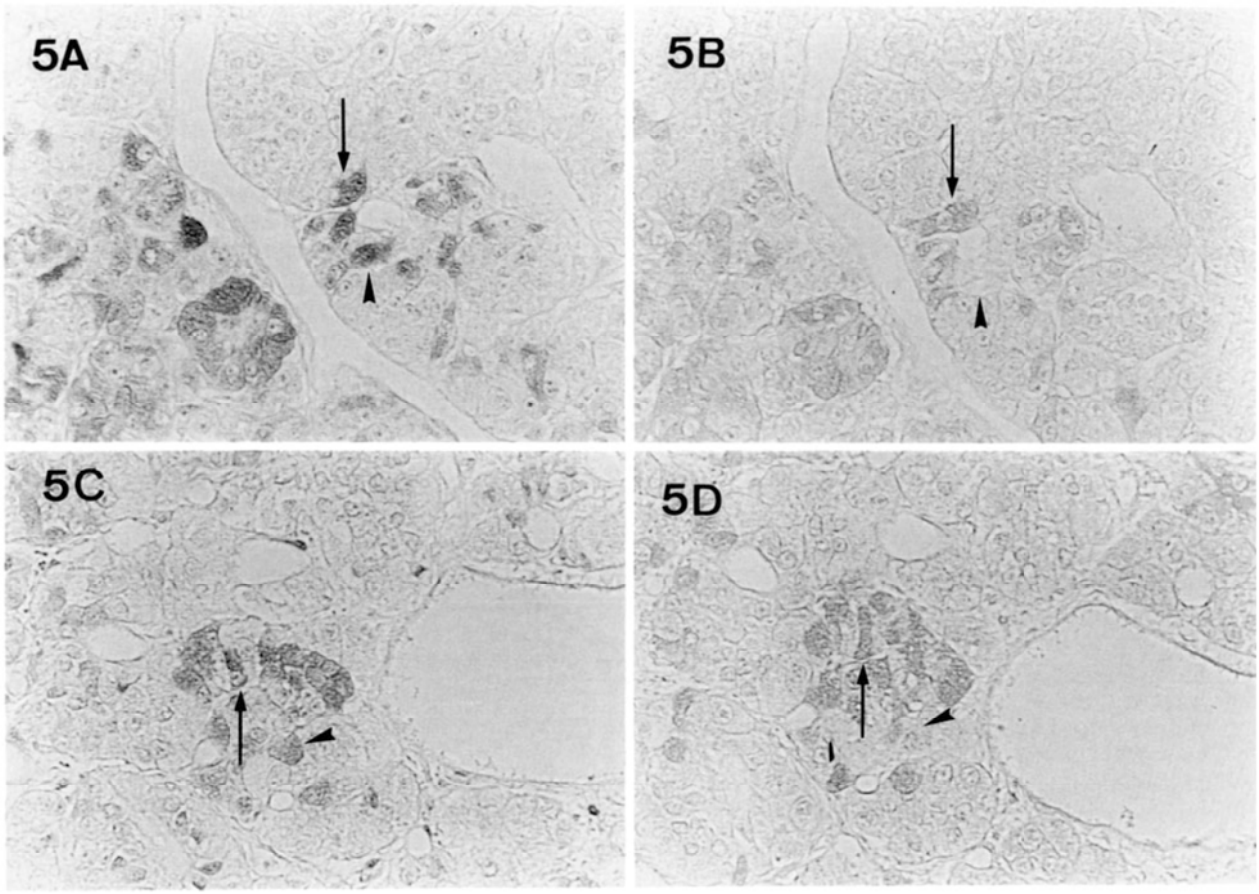

Fig. 5. Serial sections were stained for both bFGF and LH-β (A and B, respectively) and for bFGF and ACTH (C and D, respectively). Positive staining for bFGF was co-localized with LH-β- and (arrows)/or (arrow heads) with ACTH-positive cells. (× 200).

However, bFGF was also demonstrated in the nuclei of parenchymal cells. Similar to our results in previous studies, bFGF was detected immunohistochemically in the nuclei of pituitary parenchymal cells in rats and humans [8, 16]. *In vitro* studies [18, 19] revealed that bFGF is first translated and located in the cytosol, and then translocates into and is accumulated in the nuclei. However the detailed mechanisms of nuclear localization of bFGF in the pituitary parenchymal cells *in vivo* have not yet been clarified.

Several hypotheses have been proposed concerning the physiological roles of bFGF in the pituitary gland. In rat fetuses [8], immunopositivity for extracellular bFGF was observed at sites of capillary penetration adjacent to immature gonadotrophs during the early stages of pregnancy, and then bFGF was detected in the cytosol of a subpopulation of gonadotrophs. bFGF stimulates angiogenesis and plays an important role in tumorigenesis of prolactinomas in rats [20]. *In vitro* studies [3, 21] suggested that bFGF could be involved in the autocrine and/or paracrine regulation of the secretion of different pituitary hormones (prolactin, thyrotropin etc.). These results suggest that the parenchymal cells positive for bFGF synthesize and store bFGF, and contribute to the neovascularization from systemic blood vessels in the pituitary glands with bFGF acting a regulator of differentiation of immature parenchymal cells located close to the blood capillaries.

The ZT, which is composed of basophils, e.g. gonadotrophs and thyrotrophs, is mainly situated between the cranioventral and the central region of the pars distalis in the pituitary glands of ruminants and several other mammals [9–11]. Relatively large blood capillaries are seen in this area as compared to the other regions of the pars distalis. However, in the present study, bFGF immunoreactivity was observed not only in a subpopulation of gonadotorophs but also in corticotrophs, showing no tendency to be more abun-

dant in the ZT of the goat pituitary gland. Moreover, positive immunoreactivity for bFGF was demonstrated in the endothelial cells distributed throughout the pars distalis. We concluded that bFGF is an important factor in the angiogenesis and cytodifferentiation which occur during fetal development.

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