ABSTRACT  Cultured monolayers of bovine pituitary follicular cells, which transport ions, contain high amounts of mitogenic activity for endothelial cells which, on the basis of gene expression analysis, heparin-Sepharose elution profile, bioassay, immunoblotting, radioimmunoassay, and radioreceptor assay, has been identified as basic fibroblast growth factor (bFGF). These data indicate that follicular cells may be a major source of bFGF in the pituitary gland. Considering that bFGF has been proposed to play a role in paracrine regulation of pituitary hormone secretion, the data also suggest that these cells may exert important local regulatory functions.

Of all organs tested, pituitary glands have the highest concentration of basic fibroblast growth factor (bFGF) and contain undetectable levels of its closely related counterpart acidic fibroblast growth factor (aFGF) (1, 2). bFGF is active in vitro on a large variety of cells of mesodermal and neuroectodermal origin (3). In vivo it is an angiogenic factor (4), and it supports limb regeneration in lower vertebrates (5). Recent studies suggest that pituitary bFGF, in addition to having its known effects on cell proliferation and differentiation, may act as a paracrine regulator of hormone secretion (6). However, little is known about the cellular localization of bFGF in the pituitary gland. Although a preliminary report suggested that, at least in rat pituitary, bFGF may be localized in a subpopulation of corticotropes (7), no further evidence has been provided. Also, established cell lines derived from pituitary endocrine cells contain very low or undetectable levels of fibroblast growth factor (FGF) (7).

Recently, we examined the levels of bFGF in various parts of the pituitary, such as the pars distalis (PD) and the pars intermedia (PT). We have observed that the bovine PT contains 3- to 5-fold higher concentrations of bFGF than the PD does. Interestingly, the mammalian PT contains fewer secretory cells than the PD and, with only one known exception (guinea pig), no corticotropes (8). However, it contains an abundance of agranular cells, either associated with follicular structures or interspersed in the cell cords (8, 9). These observations led us to consider the possibility that endocrine cells may not be the primary cell source of bFGF in the adenohypophysis.

We have recently reported the culture of a population of cells isolated from bovine PD as well as PT, that, on the basis of their ultrastructural features and absence of hormone secretion, were identified as follicular cells (FC) (10). Such cells express in culture morphological and electrical properties consistent with cells derived from an ion-transporting epithelium (11). In the present study, we have examined whether pituitary FC might synthesize bFGF.

MATERIALS AND METHODS

Reagents. Tissue culture media, fetal bovine serum, bovine calf serum, 0.15 M NaCl containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV), and antibiotics were obtained from sources previously described (12, 13). Low molecular weight protein standards were from Bio-Rad. All tissue culture dishes were from Falcon, except for large-scale Nunc culture plates (530 cm²), which were obtained from Applied Scientific (San Francisco). Heparin-Sepharose was from Pharmacia. Bovine pituitary bFGF and brain aFGF were purified as previously described (14), and their homogeneity was determined by NaDodSO₄/PAGE, amino acid composition, and NH₂-terminal amino acid sequence analysis (14, 15).

Preparation of FC Cultures. Pituitary FC cultures were prepared and characterized as previously described (10, 11). Confluent cultures, which consisted of homogeneous domeforming cell monolayers (Fig. 1A), were dissociated by exposure to STV containing 0.3% Na₂EDTA (4–5 min, 24°C), and the cells were seeded at a split ratio of 1:10 into large-scale culture plates. Upon reaching confluency, cultures were further passaged or cells were extracted as described below.

Purification of Cell Extracts. FC monolayers (at passages 1–3) were dissociated as described above. The cell suspension was centrifuged (100 × g, 5 min), and the pellet was resuspended in Dulbecco’s phosphate-buffered saline (PBS) and centrifuged again. The pellet obtained was either immediately extracted or stored in liquid nitrogen. For cell extraction, the pellet was resuspended in 2 ml of water containing 0.5% Triton X-100. After 10 min at 4°C, the cell lysate was forced through a 25 gauge needle four times, and the salt concentration was adjusted to 0.4 M NaCl. The cell lysate was then centrifuged (50,000 × g, 30 min, 4°C) and the crude extract was applied to a heparin-Sepharose column (0.5 ml) that had been equilibrated at room temperature with 10 mM Tris-HCl, pH 7.0, containing 0.6 M NaCl. After loading, the column was sequentially eluted with 10 mM Tris-HCl, pH 7.0, containing 0.6, 1, and 3 M NaCl. The flow rate was 21 ml/hr. Fractions (0.7 ml) were collected and aliquots thereof were diluted with 0.2% gelatin in calcium- and magnesium-free PBS and tested for their ability to stimulate cell proliferation. The dry weights of the crude extracts were determined after dialysis and lyophilization of aliquots thereof.

Purification of Tissue Extracts. Bovine pituitaries and stalk–median eminences were collected from freshly slaughtered steers. The PD and the PT were dissected and tissues were homogenized in Teflon microhomogenizers in the

Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; aFGF, acidic FGF; FC, follicular cells; PD, pars distalis; PT, pars intermedia; ACE, adrenal cortex-derived capillary endothelial cells; BBCE, brain cortex-derived capillary endothelial cells.
presence of 0.5% Triton-X 100 in distilled water. The tissue extracts were then forced three times through 25 gauge needles and the salt concentration was adjusted to 0.4 M NaCl. The crude tissue extracts were centrifuged and subjected to heparin-Sepharose affinity chromatography as described for the FC extracts. The dry weights of the crude extracts were determined as described above.

Cell Proliferation Assays. For cell proliferation assays, bovine capillary endothelial cells from brain cortex (BBCE) or adrenal cortex (ACE) or bovine adrenal cortex cells were used. The cells were isolated and maintained as previously described (12, 13). BBCE or ACE cells were plated at a density of $1 \times 10^4$ or $2 \times 10^4$ cells per 35-mm tissue culture dish containing 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM glutamine, gentamicin at 50 μg/ml, and Fungizone at 0.25 μg/ml. Bovine adrenal cortex cells were seeded at a density of $2 \times 10^4$ cells per 35-mm tissue culture dish containing 2 ml of F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, gentamicin at 50 μg/ml, and Fungizone at 0.25 μg/ml. Dishes received bFGF or samples to be tested in 10-μl aliquots. After 4 or 5 days, cultures were treated with trypsin and cell
densities were determined with a Coulter Counter. Values of cell densities represent the means of duplicate determinations, which varied by less than 10%.

**Immunoblot Analysis, Radioimmunoassays (RIAs), and Radioreceptor Assays.** Immunoblot analysis was done as previously described (16), using monoclonal anti-bFGF antibody (McAb 8; 0.87 μg/ml) directed against the NH2 terminus of bFGF (17), instead of rabbit anti-bFGF antibodies (16). Controls were not exposed to the monoclonal antibody.

RIA and radioreceptor assays were done as previously described (16, 18, 19).

**Blot Hybridization Analysis of RNA.** Total RNA was prepared from confluent first-passage FC cultures essentially as previously described (20). Aliquots thereof were separated on 1.2% agarose/formaldehyde gels (21), then examined for the presence of bFGF gene transcripts by hybridization with a 32P-labeled 1.45-kilobase (kb) EcoRI fragment isolated from a bovine cDNA clone (22).

**RESULTS**

To determine whether pituitary FC might contain bFGF-like material, extracts prepared from cultured PD- or PT-derived FC were subjected to heparin-Sepharose affinity chromatography (23). The eluted fractions were then examined for their abilities to stimulate the proliferation of bFGF-dependent target cells. Fig. 1 B and C show the bioactivity profiles obtained with extracts prepared from PD- or PT-derived FC, respectively. In both cases most of the (99%) present in the crude extract were either not retained by the heparin-Sepharose column or were eluted in the 0.6 M NaCl wash. The unabsorbed material had little biological activity (less than 5%), which could be fully accounted for by unadsorbed bFGF when assayed by RIA (15 ng of bFGF per ml). Elution of the columns with 1 M NaCl yielded a minor peak of bioactivity that represented less than 2% of the total activity, while elution with 3 M NaCl yielded one major bioactive peak that contained more than 90% of the total bioactivity present in crude extracts. This chromatographic behavior is similar to that of bFGF (14) and unlike that of aFGF, which is known to elute with 1 M NaCl (14, 24). The 3 M NaCl fractions containing the highest bioactivity were pooled and further examined for the presence of bFGF. They will be referred to as purified cell extract.

The purified cell extract was then subjected to immunoblot analysis with monoclonal anti-bFGF antibodies that recognize the NH2 terminus (residues 1-10) of bFGF. A weak nonspecific Mr 30,000 band was apparent, and a strong immunoreactive band of Mr 16,000 was detected in the same position as bFGF (Fig. 2), strongly suggesting that the purified cell extract indeed contained bFGF or closely related material.

The bFGF present in the heparin-Sepharose purified cell extracts was quantified by bioassay, RIA, and radioreceptor assay. Since similar results were obtained with extracts purified from PD- or PT-derived FC, data are shown only for the PD-purified cells. As shown in Fig. 3, 0.5 μl of the purified cell extract was able to elicit maximal stimulation of ACE cell proliferation. Half-maximal stimulation was observed with 0.05 μl and was equivalent to that provided by 50 pg of bFGF. This suggests that 1 ml of the purified cell extract contains the equivalent of 1 μg of bFGF. Similar results were obtained when the purified extract was tested on adrenal cortex cells.

The purified cell extract was further examined by using a RIA specific for bFGF, which does not monitor the related aFGF or other unrelated molecules (18). As shown in Fig. 4A, increasing concentrations of native bFGF or purified cell extract could displace the bound ligand in a parallel manner. One microliter of purified cell extract was as potent as 1 ng of bFGF in displacing 125I-bFGF, indicating that the purified cell extract contained 1 μg of bFGF per ml. This value is in close agreement with that obtained from the bioassay.

To assess whether the bFGF-like immunoreactive material present in the purified cell extract might be able to interact with the FGF receptor, it was analyzed for its ability to inhibit 125I-bFGF binding to the FGF receptor present on cell membranes of BHK-21 cells, a clone of baby hamster kidney-derived fibroblasts (19). The addition of increasing concentrations of either native bFGF or immunoreactive bFGF-like material resulted in a progressive inhibition of 125I-bFGF binding (Fig. 4B). Ten nanograms of immunoreactive bFGF induced an almost complete inhibition of 125I-bFGF binding, and 2.4 ng of immunoreactive bFGF induced half-maximal inhibition of binding, thus being as potent as 1.8 ng of native bFGF. This indicated that most of the immunoactive material in the purified cell extract contained bFGF.

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**Fig. 2.** Immunoblot analysis of cell extract purified from PD-derived FC by monoclonal anti-bFGF antibody. Lane A, purified cell extract (25 μl). The antibody reacts strongly and specifically with a single band of Mr 16,000 that ran in the same position as bFGF. A weak labeling at Mr 30,000 can also be observed. This labeling was observed also in controls that did not receive the anti-bFGF antibody and therefore is not related to bFGF. Lane B, bovine pituitary-derived bFGF (25 ng in 25 μl of PBS). Lane C, bovine pituitary-derived bFGF (25 ng in 25 μl of 10 mM Tris-HCl, pH 7.0/3 M NaCl). The molecular weight markers used were phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).

**Fig. 3.** Effect of purified cell extract on the proliferation of ACE cells. ACE cells were seeded at the density of 1 × 10⁴ per dish in 35-mm tissue culture dishes. The indicated amounts of purified cell extract or bFGF were added every other day and cells were counted after 5 days. Values are the means (±SD) of three experiments performed in duplicate.
munoactive material contained in the purified cell extract is able to interact with the FGF receptor and is therefore closely related to bFGF.

This was confirmed by the demonstration that polyclonal antibodies directed against bFGF and capable of neutralizing its biological activity were also capable of neutralizing the biological activity of the purified cell extract (Fig. 5), indicating that its growth-promoting activity is very similar or even identical to bFGF. In contrast, such antibodies did not neutralize the bioactivity of aFGF.

Further proof that FC can synthesize bFGF was provided by blot hybridization analysis of total RNA prepared from cultured FC derived from either PD or PT. Two species of mRNA (3.7 kb and 7.0 kb) complementary to the bovine bFGF cDNA were readily detected (Fig. 6). These comigrate with bFGF mRNA prepared from other cell types known to express bFGF mRNA and demonstrate the presence of bFGF transcripts in FC.

To correlate the bFGF content of tissue extracts of PD and PT with the content of cultured FC, PD and PT were homogenized and subjected to heparin-Sepharose chromatography. bFGF content was determined by RIA. As shown in Table 1, FC contain 35-fold more bFGF than the PD and 10-fold more than the PT.

**DISCUSSION**

Of all organs, pituitaries have been shown to contain the highest concentrations of bFGF (500 μg/kg of tissue). Although capillary endothelial cells, which can synthesize bFGF (25), could contribute to the bFGF content of pituitaries, they clearly could not account for the whole bFGF content of this organ, since in other tissues that are heavily vascularized, such as corpus luteum or adrenal gland, the bFGF content is only about 50 μg/kg of tissue. This raises the possibility that in the pituitary gland other cell types as well could produce bFGF. Previous studies have focused on endocrine cells as source of bFGF and preliminary studies suggest that corticotropes could contain bFGF (7).

On the basis of observations that the PT contains 3- to 5-fold more bioactive material than the PD and that it

**Table 1.** bFGF content as determined by RIA in heparin-Sepharose-purified crude extracts of PD, PT, and cultured FC

<table>
<thead>
<tr>
<th>Tissue/cell extract</th>
<th>bFGF-like immunoreactivity,* ng/mg crude extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>PT</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>FC</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

PD, PT, or FC crude extracts were subjected to heparin-Sepharose affinity chromatography. The fractions eluted with 3 M NaCl that displayed the highest bioactivity were pooled and their bFGF content was determined by RIA. Dry weight of crude extracts was determined after dialysis and lyophilization.

*Values are means ± SEM of three determinations.
contains large numbers of FC, we have focused on that cell type as a possible source of bFGF in vivo. Our results indicate that FC contain considerable amounts of material that, by its behavior with respect to heparin-Sepharose chromatography, bioassay, biological activity neutralization studies, RIA, immunoblotting, and radioreceptor assay, is indistinguishable from bFGF. Also, the presence of bFGF transcripts in FC indicates that these cells express the bFGF gene. Quantification of the bFGF content in FC indicates that they can contain as much as $2 \times 10^5$ bFGF molecules per cell. This value is based on the extraction of $1.5 \times 10^8$ FC, yielding 40 mg of crude extract, which, after affinity chromatography on heparin-Sepharose, resulted in the recovery with a 25% yield of 1.4 µg of bFGF or 5.6 µg of bFGF based on 100% yield (140 ng of bFGF per mg of crude extract or 37.5 fg of bFGF per cell).

The available evidence indicates that the expression of bFGF in FC is not a property acquired in vitro but most likely corresponds to the in vivo situation. Although no quantitative data are available for cattle, the mammalian PT consistently contains large numbers of FC, either associated with follicles or interspersed among secretory cells (8, 9). In the sheep, a species phylogenetically very close to the steer, such cells represent the predominant cell population of the PT (8). This clearly fits with the presence in the PT of an even higher concentration of bFGF than in the PD. Also, we observed a decrease in bFGF content in FC cultures with subsequent passages (data not shown), which is consistent with the gradual loss of a differentiated function in culture. Furthermore, the PT and PD tissue homogenates had heparin-Sepharose bioactivity profiles identical to the profile of FC cultures (data not shown).

There is increasing evidence to support the concept that FGF is an extremely versatile factor, capable of inducing proliferation as well as differentiation in a wide variety of cell types of mesodermal and neuroectodermal origin (3). Investigation of the in vivo effects of FGF is just beginning, but it is already clear that it could play an important role in angiogenesis, as well as in regenerative and repair processes (4, 5).

Although the role of bFGF in FC of the PT is unknown, it is worth pointing out that, in view of the intimate and unique relationships of the PT with the hypothalamohypophyseal portal vessels (26), the presence of an angiogenic factor such as bFGF in FC could relate to the development and the maintenance of the differentiated state of the portal vessels.

Recent studies (6, 7) have also indicated that pituitary FGF, independently from its proliferative effects, plays an important role in the differentiation of pituitary cells and in the paracrine regulation of hormone secretion. Such studies show that, after exposure to FGF, cultured lactotropes and thyrotropes become more responsive to thyrotropin-releasing hormone, possibly due to a stimulation of synthesis of prolactin and thyroid-stimulating hormone (7). Within that context, it is interesting to note that recent studies suggest that FC can exert a complex regulatory role in pituitary hormone release by paracrine mechanisms. It is therefore tempting to speculate that at least some of these effects may be mediated by bFGF.

In conclusion, our studies indicate that FC, a morphologically well-characterized population of pituitary cells (27–29), but of enigmatic function, are a major source of a potent and versatile regulatory protein like bFGF. We have recently described (11) another differentiated function of FC, namely polarized ion transport. The coexistence of these two functions strongly suggests that FC play a far more important and dynamic role in pituitary function than previously thought.

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