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Presence and Localization of a 30-kDa Basic Fibroblast Growth Factor-Like Protein in Rodent Testes*

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ABSTRACT. We have used a recently characterized rabbit antiserum against basic fibroblast growth factor (bFGF), which recognizes various forms of bFGF, to examine the presence and localization of bFGF in the testes of adult rats and mice and the 5-day-old rat. In Western blots of testicular homogenates of adult rats and mice and immature rats, immunoreactive single bands at approximately 30 kDa were detected. Immunocytochemistry revealed specific staining restricted to the tubular compartment. In 5-day-old rat testes, prespermatogonia were immunoreactive. The cytoplasm of pachytene spermatocytes was heavily stained in the adult testes of both species. Staining of these cells became evident around stage IV/V, was prominent in stage VII through IX and declined about stage XII/XIII (rat) or X-XI (mouse). Staining was seen in type A spermatogonia and in elongating spermatids in their cytoplasmatic lobes and along their flagellae. Sertoli cells were unstained. We propose that the pluripotential growth factor bFGF could be involved in the regulation of germ cell proliferation and differentiation in the adult and immature testis. (*Endocrinology* **129:** 921-924, 1991)

THE POTENT mitogen and angiogenic agent basic fibroblast growth factor (bFGF) has been isolated from various tissues, including the bovine and human testes (1-4). At present, however, the localization of bFGF and its function in the adult testis are not clear. In the developing testis of the fetal rat, Leydig cells were bFGF immunoreactive (5). Involvement of bFGF in the control of testicular function was proposed in the immature rat, because bFGF inhibits LH-stimulated androgen production by cultured neonatal rat testicular cells (6). In the growing postnatal testis of the rat, the Sertoli cell apparently is able to produce a bFGF-related factor, as concluded from *in vitro* experiments with isolated Sertoli cells from immature rats (4, 7). In the present study we have shown expression of a 30-kDa bFGF-like protein in germ cells of the adult testes of two rodent species, rat and mouse, and, in addition, in the immature rat testis, using a recently characterized, highly specific antiserum against bFGF (8, 9).

Materials and Methods

Animals

All animals were decapitated under deep CO_2 anesthesia. Testes from adult rats (n = 6; Sprague-Dawley; Charles River, Sulzfeld, Germany), 5 day-old rats (n = 4), and adult mice (n = 4; NMRI; breeding colony at the University of Ulm) were either immersed in Bouin's fixative for 8 h and subsequently embedded in paraffin, or rapidly frozen until sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed. Tubules and interstitial tissues from the testes of two rats were also dissected under a microscope and frozen. Frozen, freshly isolated mouse Leydig cells were a generous gift from Dr. K. Seidl (Hamburg, Germany).

Western blot analyses

Samples were thawed, homogenized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% saccharose and 2% SDS, sonicated and boiled for 5 min under reducing conditions, and used for SDS-polyacrylamide gel electrophoresis, as described previously (10). After transfer onto nitrocellulose, proteins were probed with anti-bFGF (1:500; overnight) and detected with radioactive [^{125}I]protein-A. The characteristics of the antiserum were described in detail previously (8, 9). The antiserum was generated in rabbits immunized against purified bovine pituitary bFGF. Immunoglobulin G (IgG) fractions were purified and proved suitable for both immunocytochemistry and Western blotting (8, 9). Depending on the tissue type, this antiserum recognizes in Western blots several immunoreactive forms of bFGF in immunopurified extracts, including 18-, 24-, 30-, and 46-kDa forms (9).

Immunocytochemistry

Immunocytochemistry was carried out on deparaffinized sections (8 μm) with the peroxidase-antiperoxidase technique (11),

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as described previously (12). In brief, endogenous peroxidase activity was quenched by incubation of the sections with H_2O_2 (0.03%), methanol (10%), and Tris-buffered saline (TBS; 50 mM Tris-HCl buffer and 150 mM NaCl, pH 7.6) for 5 min. Subsequently, sections were incubated with 2% normal swine serum (NSS) in TBS for 30 min and then overnight with the anti-bFGF serum (1:250–1:2000 in TBS-2% NSS). Incubation with a secondary antiserum, swine antirabbit IgG (1:50 in TBS-2% NSS; Dakopatts, Hamburg, Germany) and then incubation with a rabbit peroxidase-antiperoxidase complex (1:100; Dakopatts) followed. Immunoreaction was visualized with a freshly prepared solution of 3,3′-diaminobenzidine-tetrahydrochloride dihydrate solution (0.5% diaminobenzidine-tetrahydrochloride dihydrate in 50 mM Tris-HCl buffer, pH 7.6; Aldrich, Milwaukee, WI) and 0.1 H_2O_2. Controls consisted of 1) incubation with buffer (TBS-2% NSS) instead of the antiserum, 2) incubation with normal rabbit serum instead of the specific antiserum, and 3) incubation with preabsorbed antiserum [50 μg recombinant bFGF (Boehringer, Mannheim, Germany)/ml antiserum (1:500) for 3 h at room temperature]. Sections were viewed with a Zeiss (Oberkochen, Germany) or Nikon (Garden City, NY) microscope.

Results

Western blots

In the testes from both adult species (Fig. 1) and in the testes from immature rats (not shown) immunoreactive bands at approximately 30 kDa were seen. No staining was observed if rat interstitial tissue and freshly isolated mouse Leydig cells were analyzed, but a 30-kDa band was observed in the rat seminiferous tubules samples (not shown).

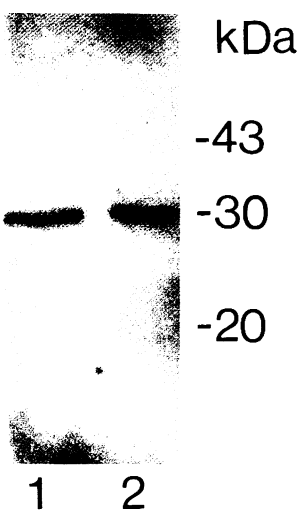

FIG. 1. Western blot analysis of rat and mouse testes probed with anti-bFGF. Mol wt ranges (kilodaltons) are indicated at the *right*. Fifteen micrograms of rat testicular protein per lane were loaded on the gel in lane 1, and the same amount of mouse testicular protein was loaded in lane 2. Detection of anti-bFGF was carried out with [^{125}I]protein-A.

Immunocytochemistry

Specific staining for bFGF was restricted to the tubular compartment of all testes (Figs. 2–4). Thus, in the 5-day-old rats, only the cytoplasm of prespermatogonia (gonocytes) was immunoreactive (Fig. 2A). In adult rats and mice, strong staining was seen primarily within the cytoplasm of pachytene spermatocytes (Figs. 2B–4). Staining of these cell types began about stage IV or V, became prominent in stage VII through IX, and declined about stage XII/XIII (rat) or X-XI (mouse). Staining was observed in type A spermatogonia of the rat and

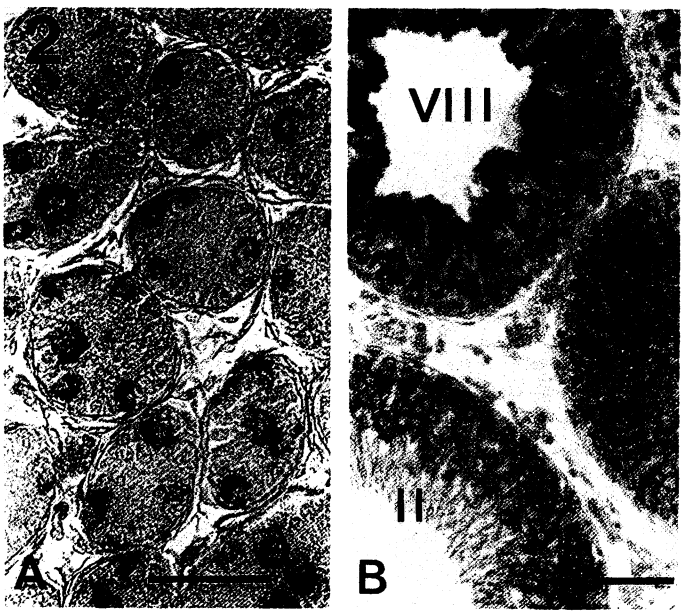

FIG. 2. Immunocytochemical localization of bFGF in 5-day-old rat testis and adult rat testis. Note that staining is restricted to the tubular compartment of 5-day-old rat testes (A) and adult rat testes (B). *Bars* = 50 μm.

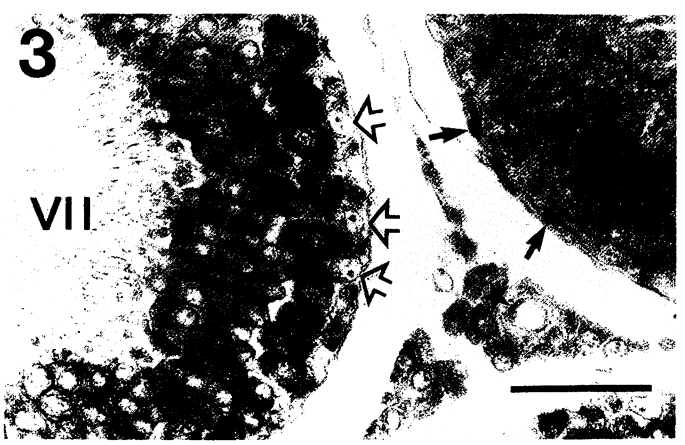

FIG. 3. Immunocytochemical localization of bFGF in adult rat testis. Note immunoreactive pachytene spermatocytes in the stage VII tubule, immunoreactive type A spermatogonia in the otherwise unstained stage I tubule (*arrows*), unstained Leydig cell (*star*), and unstained Sertoli cells (*open arrows*). *Bar* = 50 μm.

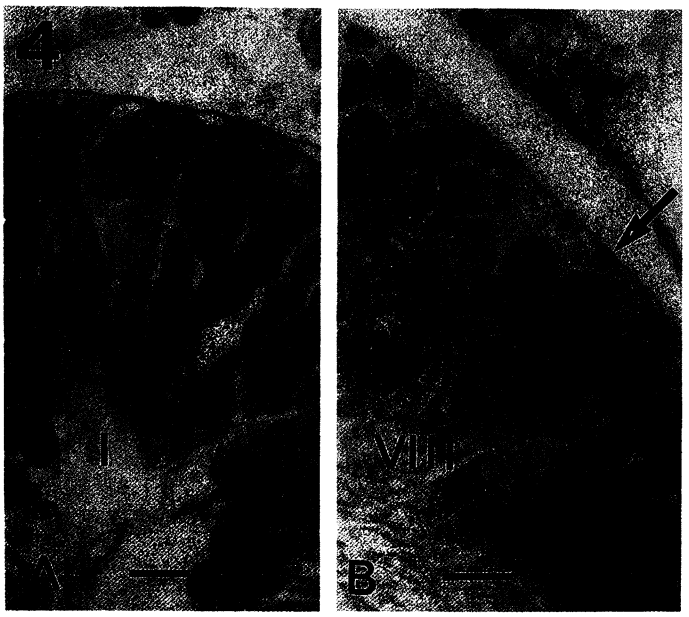

FIG. 4. Immunocytochemical localization of bFGF in adult mouse testis. Note immunoreactive pachytene spermatocytes in the stage VIII tubule (B) and immunoreactive type A spermatogonia (*arrow*). A, Germ cells of the stage I tubule. All Sertoli cells as well as Leydig cells (*star*; B) are unstained. *Bars* = 20 μm.

mouse (where it was punctuate) and in spermatids within the cytoplasmic lobe and along the spermatid flagellae (fine granular staining; Figs. 2–4). Cytoplasm from Sertoli cells was always negative. In all controls the specific staining described above was absent (not shown).

Discussion

These data show for the first time the presence of a 30-kDa bFGF-like factor in the cytoplasm of germ cells in two adult rodent species and in germ cells of the immature rat testis; thus, this study largely extends previous reports on the presence of bFGF in bovine and human testes (1, 2).

Although it is generally thought that bFGF is a 18-kDa molecule, there is evidence for more than one molecular form of bFGF. Several higher mol wt forms (ranging from 20–25 kDa) have been described so far (13–15), and a number of less well characterized bFGF-immunoreactive proteins with higher molecular masses (including 24-, 27-, 29-, 30-, and 46-kDa forms) have been reported by various groups (8, 9, 16–18). Interestingly, a 29-kDa form of bFGF was the predominant form in the pituitary and various brain regions of the rat (17). Thus, bFGF may be synthesized initially as a higher mol wt precursor (18). The 30-kDa bFGF-like protein found in rodent testes could represent such a precursor. This is not only indicated by the fact that it was recognized by a well characterized antibody, which reacts with various forms of bFGF, including 18-, 24-, 30-, and 46-kDa forms, that were found in other tissues (adrenal, ovary, and pituitary) (8, 9), but also by the fact that preabsorbtion of the antiserum with recombinant bFGF abolished the specific staining.

In the present study immunoreactivity for bFGF was not seen in either the Sertoli or Leydig cells of either species. Thus, the situation in adult and prepubertal testes is strikingly different from that in the fetal testis, where bFGF immunoreactivity was reported in the cytoplasm and on the surface of Leydig cells and was associated with the extracellular matrix (5). In contrast, in the postnatal (25-day-old) testis, the Sertoli cell apparently is able to produce a factor with bFGF activity *in vitro* (4). It is well known that bFGF is mitogenic for cells originating from mesoderm and neuroectoderm (3) and in many *in vivo* models acts multifunctionally (5). Thus, one may speculate that bFGF expression is associated with proliferation and differentiation of testicular cells; *e.g.* during fetal life, when Leydig cells proliferate, these cells may be able to produce bFGF. Later, during the first 2 postnatal weeks, the number of Sertoli cells increases (19), and thus, it cannot be ruled out that Sertoli cells, isolated from postnatal immature testes, might express bFGF as well (4). In the present study we were unable to detect immunoreactive bFGF in Sertoli cells, but show that intense bFGF immunoreactivity was restricted to germ cells, not only in the adult but also in the immature testis of the 5-day-old rat. This may indicate that the site of bFGF expression could change during development. Alternatively, it is possible that Sertoli cells in culture start to produce bFGF, because it is known that target cells for bFGF are able to produce this molecule *in vitro* (5). Sertoli cells appear to be target cells for bFGF, which is able to stimulate phenotypic expression of this cell type *in vitro*, *e.g.* FSH receptors and plasminogen activator (20). This has given rise to the thought that bFGF, like other growth factors [*e.g.* acidic fibroblast growth factor, seminiferous growth factor, insulin-like growth factor-I, interleukin-1, nerve growth factor (NGF), and transforming growth factor-α and -β], belongs to a growth factor family that regulates spermatogenesis (7, 21).

Although at present the mechanism by which bFGF leaves the cell is unclear, because bFGF lacks a signal sequence that could mediate the secretion of this molecule (3), bFGF can get out of the cell and interact with its receptor on target cells (22). Extracellular bFGF is mainly associated with extracellular matrix (16). Thus, the intense bFGF immunoreactivity in the cytoplasm of germ cells leads us to believe that intracellular bFGF immunoreactivity in germ cells detected by the antiserum represents the site of highest concentrations and, thus, most likely depicts the cellular origin of the bFGF-like protein (pachytene spermatocytes, type A spermatogonia

in the adult; prespermatogonia in the immature rat). Interestingly, the localization of bFGF immunoreactivity overlaps in part that of NGF in adult rat and mouse testes (23). Thus, germ cells, starting with primary spermatocytes to mature spermatozoa, were NGF immunoreactive, while spermatogonia were unstained for NGF.

In the mature testis, only the germinal epithelium undergoes constant proliferation and meiotic divisions. Our data show a 30-kDa bFGF-like factor in testes of two adult rodent species, predominantly in pachytene spermatocytes. These cells grow rapidly in preparation for the two successive meiotic divisions. During this period of meiotic recombination, synthetic processes are at a maximum (24). Furthermore, bFGF immunoreactivity in type A spermatogonia (highly mitotic active cells) and spermatids (cell types during remodeling of their shape) indicates additional involvement of bFGF during these processes. We, therefore, propose that bFGF, like other growth factors (21), could be involved in the regulation of germ cell proliferation and differentiation in the testis. Further studies on the presence of receptors for bFGF will answer the question of whether bFGF in the tubular compartment of the testes acts in a paracrine way on Sertoli cells and/or regulates the development of germ cells in an autocrine manner.

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