Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 82-88

DETECTION OF ACIDIC FIBROBLAST GROWTH FACTOR mRNA IN THE RAT OVARY USING REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION AMPLIFICATION

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Received September 14, 1989

SUMMARY: We have examined the expression of acidic fibroblast growth factor (aFGF) in the rat ovary using reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from hypothalami of adult rats and whole ovaries or isolated granulosa cells of gonadotropin-primed immature rats. The RNA was reverse transcribed and amplified by PCR using oligonucleotide primers specific for rat aFGF. RNA from hypothalamus or whole ovary yielded a dominant DNA band corresponding in size to the aFGF segment spanned by the two primers (301 base pairs, bp). Its identity with the aFGF sequence was confirmed by restriction enzyme analysis. The aFGF product was also obtained from two of four granulosa cell RNA preparations; when obtained, the intensity of the signal was less than that from whole ovary, indicating that the major sites of aFGF expression are outside the granulosa layer.

Acidic and basic FGF (bFGF), also known as heparin-binding growth factors I and II (HBGF I and II), respectively, are potent mitogens for cells of mesodermal and neuroectodermal origin, including endothelial cells (1). Because of their direct effects on endothelial cell growth, migration, and protease synthesis, and because of their ability to induce new blood vessel growth in various *in vivo* assays, they may play a role in angiogenesis (1,2). The ovary is one of the few sites in the adult animal where angiogenesis normally occurs (3). While it has been shown that bFGF is produced in the ovary (4-6), there is no information concerning aFGF expression there. Until recently, aFGF was thought to be confined to the nervous system, but has now been found to be expressed in some nonneural tissues and cells as well (1,7,8).

The study of expression of the FGF's, as well as other growth factors, has been hampered by the lack of sensitivity of conventional methods to detect their short-lived mRNA's (9). It is now possible to convert mRNA to cDNA by RT and selectively amplify specific sequences by PCR so that they can be visualized after fractionation by gel electrophoresis (10). Using this method, Rappolee and colleagues were able to detect the expression of several growth factors by

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; aFGF or bFGF, acidic or basic fibroblast growth factor; HBGF, heparin-binding growth factor; GT, guanidine thiocyanate; BSA, bovine serum albumin; M-MLV, Moloney murine leukemia virus; bp, base pairs.

0006-291X/89 $1.50

small numbers of macrophages (9) and mouse embryos (11). Using RT-PCR, we have examined the expression of aFGF mRNA in the rat ovary at a time when follicular development is occurring and endothelial cells, as well as several other cell types, are rapidly proliferating.

MATERIAL AND METHODS

Animal and Cell Models

All studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To obtain ovaries and isolated granulosa cells, Sprague-Dawley rats [Crl:CD(SD)BR strain, Charles River, Wilmington, MA] were primed with 20 IU of PMSG (sc) at 25-27 days of age. They were killed 48 h later and the ovaries aseptically removed and placed in Hepes-buffered Medium 199 (Gibco, Grand Island, NY). Extraneous tissues were quickly trimmed away. Whole ovaries were immediately homogenized in cold guanidine thiocyanate (GT) buffer [4 M GT, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% sodium N-lauroylsarcosine, and 8% β-mercaptoethanol; 10 ml/g tissue] using a rotor/stator homogenizer. Granulosa cells were harvested by needle puncture followed by the application of gentle pressure, as previously described (12). The isolated cells were pelleted and resuspended in GT buffer (2 ml/10^6 cells). Hypothalami were obtained from young adult male rats and homogenized in cold GT buffer (10 ml/g).

Extraction of RNA

All reagents used in these studies were designated as being suitable for use in molecular biology studies or, alternatively, of the highest grade available. All equipment was treated and reagents prepared using standard methods to eliminate RNase activity (13). Total RNA was isolated from tissues and cells using the method of Cathala et al. (14). Samples in GT buffer were homogenized with five strokes of a Potter-Elvehjem tissue grinder to shear DNA, and transferred to a 50 ml polycarbonate centrifuge tube. Six volumes of 4 M LiCl were added and the sample incubated overnight at 0-2 °C. The resulting precipitate was pelleted by centrifugation (12,100 x *g* for 30 min) and the supernatant removed. The pellet was dissolved in 2.5 ml of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.5% sodium dodecyl sulfate. Proteinase K (Boehringer Mannheim, Indianapolis, IN) was added to a concentration of 150 μg/ml and the samples incubated at 43 °C for 30 min. The digest was extracted once with phenol and again with phenol/chloroform/isoamyl alcohol (25:24:1). One tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol were added and the samples incubated at -20 °C. The RNA was pelleted by centrifugation (10,000 x *g* for 30 min), dissolved in water, and the concentration determined by absorbance at 260 nm.

RT

First strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (15,16, and Technical Bulletin 8025-1, BRL, Gaithersburg, MD). One μg of total RNA in 1 μl of water was reverse transcribed, except in the case of the rat hypothalamus where 150 ng was used. The RNA was incubated at 37 °C for 60 min with 100 U (0.5 μl) of cloned M-MLV reverse transcriptase (BRL) and the following reagents: 1) 5X reaction buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 50 mM dithiothreitol, and 15 mM $MgCl_2$], 2 μl; 2) 0.2 μg oligo-$dT_{12\text{-}18}$ (Pharmacia, Piscataway, NJ), 0.5 μl; 3) 1 μg bovine serum albumin (BSA; Boehringer Mannheim), 1 μl; 4) dNTPs (0.5 mM each dATP, dCTP, dGTP, and dTTP; BRL), 2.0 μl; and 5) water to 10 μl. The tube was then heated to 93 °C for 5 min and flash cooled to 4 °C. Fifty U of fresh MMLV-reverse transcriptase was added and the incubation repeated for a second 60 min period (16). The sample was cooled to 2 °C and stored on ice or frozen until used in a PCR. For each RT, a blank was prepared using all the reagents except the RNA sample; an equivalent volume of water was substituted for the latter. This RT blank was used to prepare the PCR blank.

Oligonucleotide Primers

The oligonucleotide primers were synthesized in the Biopolymer Laboratory of the Department of Microbiology and Immunology, University of Maryland School of Medicine using an Applied Biosystems (Foster City, CA) Model 380B DNA synthesizer. The oligonucleotides were used without further purification. The sequences of the primers are shown in Fig. 1. Each pair of primers was selected based on the criteria described by Rappolee et al. (16); principally, each pair spanned an intron and included one or more restriction enzyme sites. The aFGF primers spanned the rat aFGF sequence from bases 419-719, as numbered by Goodrich et al. (17). This segment contains a ScaI site (AGT/ACT) at bases 499-504 and RsaI sites (GT/AC) at bases 484-

A. Rat aFGF Primers

1. 5′-CAGGAGCGACCAGCACCTTCA-3′ 419-439

2. 3′-TAGAACAAGGAGGGGGAGGGC-5′ 699-719

B. Rat β-Actin Primers

1. 5′-ATCGTGGGCCGCCCTAGGCA-3′ 100-119

2. 3′-GGGGAGACTTGGGATTCCGGT-5′ 323-343

Fig. 1. The sequence of PCR primers for rat aFGF and rat β-actin. (A) For aFGF, primer 1 is identical to bases 419-439 of the rat cDNA and primer 2 is complementary to bases 699-719 (17). (B) For rat β-actin, primer 1 is identical to bases 100-119 of the cDNA and primer 2 is complementary to bases 323-343 (18).

487 and 500-503. The β-actin primers encompassed the rat cytoplasmic β-actin sequence (18) from bases 100-343, as described previously (6).

PCR

PCR was carried out according to GeneAmp DNA Amplification Reagent Kit instructions (Perkin Elmer Cetus, Norwalk, CT) with modifications as described by Rappolee et al. (16). The following reagents were added to a 500 µl polypropylene microcentrifuge tube (Brinkmann Instruments, Westbury, NY): 1) 10X reaction buffer [100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl and 0.01% gelatin (w/v)], 5 µl; 2) 10 mM $MgCl_2$, 5 µl; 3) water, 15.8 µl; 4) dNTP mixture (1.25 mM each of dATP, dCTP, dGTP, or dTTP; GeneAmp kit or BRL), 8 µl; 5) 1 U of cloned *Thermus aquaticus* DNA polymerase (Amplitaq, Perkin Elmer Cetus), 0.2 µl; 6) BSA, 0.1%, 5 µl; 7) primer mixture (50 pmoles of each primer in water), 10 µl; and 8) 1 µl of the RT sample. The RT sample was always added last to avoid cross-contamination of samples. The 10X buffer is included in the GeneAmp kit; the additional $MgCl_2$ was added to raise the concentration to 2.5 mM (16). The components were concentrated in the bottom of the tube by centrifugation and overlaid with 50 µl of mineral oil (Sigma, St. Louis, MO). PCR incubations were carried out in an MJ Research (Cambridge, MA) Programmable Thermal Controller. During each PCR cycle (30 to 50), the samples were heated to 94 °C to denature template complexes (120 sec initially and 30 sec during all subsequent cycles), cooled to either 50, 60, or 65 °C to anneal template and primers (30 sec), and heated to 72 °C to allow extension to occur (90 sec). The final 72 °C incubation was extended for an additional 7 min to maximize strand completion, as recommended in the GeneAmp kit instructions. The samples were then rapidly cooled to 2 °C and kept on ice or frozen until analyzed. Each time a PCR was carried out, a blank was prepared using all reagents, including either β-actin or aFGF primers, except the RT sample, for which 1 µl of the RT blank was substituted. In all trials, the blank lane was devoid of any bands, except for primer complexes that run near the gel front (19), confirming that all reagents were free of template.

Gel Electrophoresis

A sample of the PCR mixture (9 µl) was added to 1 µl of 10X loading buffer (30% Ficoll, 0.2 M EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanole FF) and fractionated by electrophoresis in a constant 60 V field in a 0.75 mm thick, nondenaturing, 8% polyacrylamide gel (7 x 8 cm format). Standard size markers were φX174 RF DNA/HaeIII fragments (0.37 µg/lane; BRL). The sizes of the markers are 1,353, 1078, 872, 603, 310, 271/281, 234, 194, 118, and 72 bp. Gels were stained for 30 min in ethidium bromide (0.5 µg/ml), destained in water for 15 min, and examined on a 312 nm uv transilluminator. Gels were photographed with a Quick Shooter camera and hood (IBI, New Haven, CT) and Polaroid 665 film.

Restriction Enzyme Analysis

Restriction enzymes (ScaI and RsaI) and matched 10X buffers were obtained from Amersham (Arlington Heights, IL). A PCR sample (10 µl) was ethanol precipitated, redissolved in 5 µl of water and incubated for 1 h at 37 °C with enzyme as per manufacturer's instructions. The digest, along with an untreated sample, was then electrophoresed and stained as described above.

RESULTS

Because the brain, including the hypothalamus, is a relatively rich source of FGF's (1,2, 20,21), RNA from rat hypothalamus was used as a positive control in these studies. RT-PCR amplification of hypothalamic RNA using the aFGF-specific primers yielded one major product which was located between the 310 and 281 bp standards, corresponding to the expected size of 301 bp (Fig. 2). In addition, three minor bands, two larger and one smaller than the dominant product, were generated. As expected, treatment of the putative aFGF PCR product with ScaI yielded two fragments (Fig. 2). The larger fragment was located just below the 234 bp standard, corresponding to the expected length of 218 bp; the smaller fragment was located just above the 72 bp standard, corresponding to the expected length of 83 bp. Whether the minor bands represent aFGF-related sequences or nonspecific amplification products is not known at this time.

RT-PCR of whole ovarian RNA yielded one major band and four minor bands (Fig. 3); the major band and three of the minor bands were identical in size to those obtained from hypothalamic RNA. Treatment with ScaI completely digested the major product into two fragments of the expected size (Fig. 3). To reduce nonspecific amplification, the number of PCR cycles was reduced from 50 to 30. This resulted in the generation of only one PCR product which corresponded in size to that expected for the target aFGF sequence (Fig. 3). This product was cleaved by ScaI into the expected fragments (data not shown). Digestion with RsaI, for which there are two sites in the target segment, yielded two fragments which corresponded in size to the expected 218 and 67 bp fragments (data not shown); the intervening 16 bp fragment was not visible. A third band located just above the 72 bp standard was also generated; it most likely represents the combination of the 16 and 67 bp fragments within which the RsaI site may be less efficiently cut. Based on these results - the generation of a single product using aFGF-specific

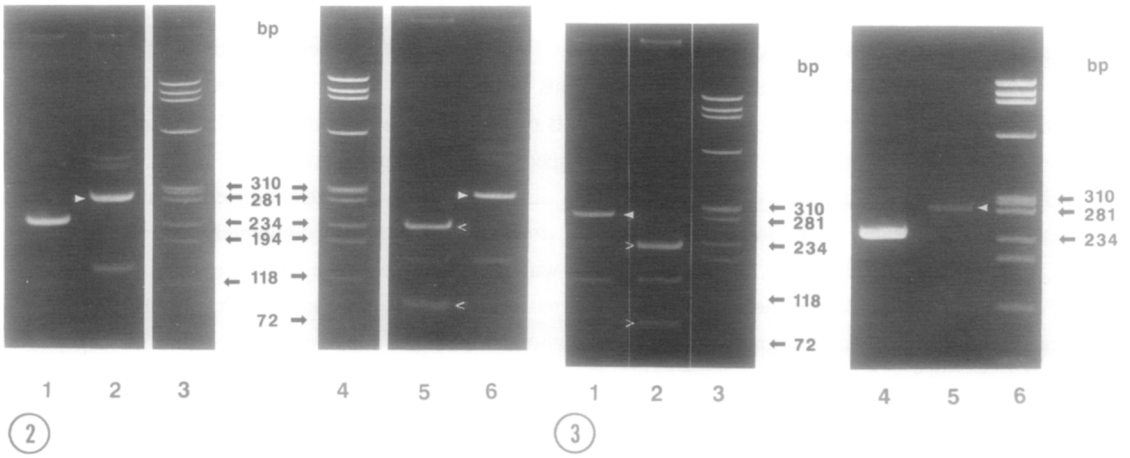

Fig. 2. Detection of aFGF and β-actin mRNA in hypothalamus by RT-PCR. PCR was carried out for 50 cycles with an annealing temperature of 60 °C. Lane 1: β-actin product (expected size is 244 bp). Lanes 2 and 6: aFGF product (solid arrow; expected size is 301 bp). Lanes 3 and 4: DNA size standards. Lane 5: Fragments obtained after digestion of aFGF product with ScaI (open arrows; expected sizes are 218 and 83 bp).

Fig. 3. Detection of aFGF and β-actin mRNA in whole ovary by RT-PCR. Lane 1: aFGF product obtained after 50 PCR cycles with an annealing temperature of 60 °C (solid arrow). Lane 2: ScaI fragments of aFGF product (open arrows). Lanes 3 and 6: DNA size standards. Lane 4: β-actin product (30 cycles, 60 °C). Lane 5: aFGF product (30 cycles, 60 °C).

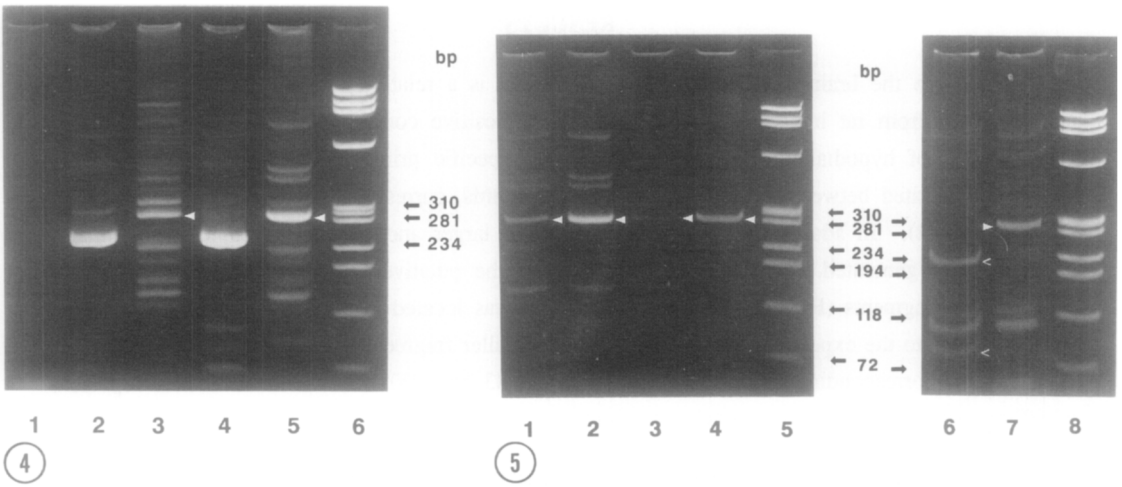

Fig. 4. RT-PCR of whole ovary or isolated granulosa cell RNA (50 cycles, 60 °C annealing temperature). Lane 1: Blank. Lane 2: Granulosa cell β-actin product. Lane 3: Granulosa cell aFGF product (arrow). Lane 4: Whole ovary β-actin product. Lane 5: Whole ovary aFGF product. Lane 6: DNA size standards.

Fig. 5. RT-PCR of granulosa cell RNA. Lane 1: Granulosa cell aFGF product (40 PCR cycles, 60 °C annealing temperature). Lane 2: Ovary aFGF product (40 cycles, 60 °C). Lane 3: Granulosa cell aFGF product (30 cycles, 60 °C). Lane 4: Ovary aFGF product (30 cycles, 60 °C). Lanes 5 and 8: DNA size standards. Lane 6: ScaI fragments (open arrows) of granulosa cell aFGF product (40 cycles, 65 °C) . Lane 7. Uncut granulosa cell aFGF product (40 cycles, 65 °C; solid arrow).

primers, the correct size of the product, and the presence of ScaI and RsaI sites at the predicted locations in the product - it was concluded that the RT-PCR product was the aFGF sequence.

In contrast to whole ovary, RT-PCR of several granulosa cell RNA preparations yielded inconsistent results, suggesting the presence of extremely low levels of aFGF mRNA. Only two of four preparations yielded a distinct band of the expected size; the other two were completely negative for the aFGF product in several trials. When it was obtained, the aFGF band was relatively light and/or accompanied by numerous other bands of similar intensity (Fig. 4). Reducing the number of PCR cycles from 50 to 30 or 40 and increasing the annealing temperature from 60 to 65 °C eliminated most of the nonspecific amplification products (Fig. 5). Three different granulosa cell PCR samples containing a distinct band of the size predicted for the aFGF fragment were treated with ScaI (Fig. 5). Each time, the product was completely digested into fragments of the expected size, indicating identity with the aFGF sequence. In all experiments, the RT-PCR reagent blank was negative, ruling out reagent contamination as a source of the aFGF product. In the case of the granulosa cell preparations which did not contain detectable message, the β-actin product was obtained, confirming that intact, amplifiable mRNA was present.

DISCUSSION

These studies demonstrate for the first time that the aFGF gene is expressed in the ovary. Although the physiological functions of the FGF's are still far from clear, considerable evidence suggests that they are vital for a wide range of developmental and regenerative processes (1,2). The ovary is one of the few sites in the body where developmental changes, including

angiogenesis, continue into adulthood (3). The expression of both aFGF and bFGF (4-6) there is consistent with their playing a role in these events. This is also one of the first clear demonstrations of aFGF expression in a nonneural tissue. Previously, only rat cardiac myocytes (8,22) had been shown to contain both aFGF protein and mRNA. In addition, aFGF is expressed in *vitro* by several tumor cell lines (reviewed in 1), foreskin fibroblasts (7), and vascular smooth muscle cells (7). Bovine kidney (23) and bone matrix (24) also contain an aFGF-like factor.

The sites of aFGF expression in the ovary, which is composed of granulosa cells, oocytes, thecal cells, fibroblasts, endothelial cells, pericytes, vascular smooth muscle cells, macrophages, surface epithelium, and other types of cells, are unknown at present. The failure to obtain amplification of the aFGF sequence in some granulosa cell preparations and the relatively weak amplification and high incidence of nonspecific products in others indicates that they are not a major site of aFGF production. Saiki et al. (10) attributed similar variability in PCR results to low levels of template. When template is rare, the polymerase enzyme may not come in contact with it or fully extend all strands during the first few PCR cycles, resulting in little or no amplification. Priming of more abundant, nontarget sequences during the initial cycles can generate nonspecific products; their build-up can interfere with the specific reaction. The apparent scarcity of transcripts raises the possibility that the aFGF mRNA may not have originated from the granulosa cells at all, but rather from small numbers of other cells, such as leukocytes, that are probably present to a variable degree in granulosa cell preparations. It is possible that oocytes express aFGF mRNA, but their numbers should be relatively constant from one preparation to another. The lack of aFGF expression by granulosa cells themselves would be consistent with our finding that they do not express bFGF (6). The stronger, cleaner aFGF signal generated from an equivalent amount of whole ovarian RNA indicates that the major site of expression is outside the granulosa layer. As discussed earlier, aFGF is expressed by vascular smooth muscle cells and foreskin fibroblasts (7), and comparable classes of cells in the theca and stroma could be a source of aFGF in the ovary.

FGF's are not mitogenic for rat granulosa cells but do affect their function (25). One can conclude from this that rat granulosa cells possess FGF receptors. It seems unlikely, however, that aFGF or bFGF could reach granulosa cells from the thecal or interstitial compartments. Neither the aFGF or the bFGF gene codes for a signal sequence and evidence suggests that neither is freely secreted by producing cells (1,2). Basic FGF is not found in free form outside of cells but is instead bound in the extracellular matrix (26); the same is probably true for aFGF. However, five additional members of the HBGF family, some of which do appear to be secreted, have now been discovered - int-2 (27), hst/KS3 (28,29), FGF-5 (30), FGF-6 (31), and KGF (32). Granulosa cells, as well as other ovarian cell types, may produce and/or respond to one or more of these factors. Granulosa cell-conditioned medium does contain FGF-like activity (33,34), but it seems unlikely that this activity is due to aFGF, which does not appear to be a freely secreted.

The development of RT-PCR is a major advance in the study of growth factor gene expression. Northern blot analysis, the most frequently used method for specific mRNA detection, lacks sufficient sensitivity to detect transcripts in small amounts of tissue or cells, especially when those transcripts have short half-lives, which appears to be the case for many growth factors (1,9). For example, Shimasaki et al. (21) were unable to detect bFGF mRNA in ovaries of 6-week-old, PMSG-primed rats using Northern blot analysis, while we were able to do so using RT-PCR (6).

RT-PCR has the capability to amplify to a detectable level as few as 100 copies of an mRNA (16). This sensitivity makes it particularly suitable for studies of ovarian function, where specific types of tissues or cells can usually be obtained only in limited quantities.

ACKNOWLEDGMENTS: This study was supported by grant CA45055 from the National Institutes of Health. Additional support was provided by a Special Research Initiative Support award to R.D.K. and a Short Term Research Training Program grant to R.H.S. from the University of Maryland at Baltimore. We also thank Dr. Erik Olson for his assistance in these studies.

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