Endocrine, vol. 5, no. 3, 247–255, December 1996 0969-711X/96/5:247–255/$7.25

Detection of Keratinocyte Growth Factor (KGF) Messenger Ribonucleic Acid and Immunolocalization of KGF in the Canine Testis

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Keratinocyte growth factor (KGF) was originally discovered in human embryonic lung fibroblasts and is a member of the fibroblast growth factor (FGF) family. Members of the FGF family have been shown to regulate testicular function. However, the recently discovered KGF has not been studied in the testis. KGF has been detected in many other tissues, including the prostate, an organ whose development and function have been associated with presence of the testis. In this study, KGF mRNA was detected in the whole testis using reverse transcription polymerase chain reaction (RT-PCR). The 575-bp KGF-specific product was detected along with a 594-bp β-actin-specific product. To identify the cell types in which KGF mRNA was predominantly expressed, interstitial cells were physically separated from seminiferous tubules. The interstitial cells were then sorted on a discontinuous Percoll gradient and total cellular mRNAs isolated. Using RT-PCR and Southern hybridization with specific cDNA probes, the KGF mRNA was detected in interstitial cells. KGF expression levels were then evaluated semiquantitatively with a competitive RT-PCR assay. KGF expression levels were highest in interstitial cells that equilibrated between 20 and 30% Percoll. Enriched Leydig cells and seminiferous tubules expressed low levels of KGF. Finally, immunohistochemical analysis was performed on canine testes using a rabbit anti-KGF polyclonal antibody. The KGF protein was localized predominantly to peritubular cells of the canine testis. These results suggest that KGF is synthesized in the canine testis.

Key Words: Dog; testis; keratinocyte growth factor.

Introduction

Keratinocyte growth factor (KGF) is the seventh member of the fibroblast growth factor (FGF) family that was initially detected in human embryonic lung fibroblasts (Rubin et al., 1989). KGF has since been detected in a variety of organs, including the prostate (Yan et al., 1992; Lin et al., 1994) and breast (Koos et al., 1993). KGF is an epithelial cell-specific growth factor of stromal origin (Rubin et al., 1995) and exhibits mitogenic activity in cultured canine prostatic epithelial cells (Canatan et al., 1996).

To date, no known reports have established the production of KGF by testicular cells. Other FGFs, however, have been detected in bovine (Ueno et al., 1987; Story et al., 1988), rodent (Lahr et al., 1992), and human (Story et al., 1988) testes. FGF can influence testicular function. FGF inhibited LH-stimulated testosterone biosynthesis in neonatal rat testicular cells, possibly by inhibiting 17α-hydroxylase activity (Fauser et al., 1988). Basic FGF (bFGF) can also inhibit 5α-reductase (Murono and Washburn, 1990a) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Murono and Washburn, 1990b) activities in immature rat Leydig cells. Acidic FGF (aFGF) can also regulate 3β-HSD and 5α-reductase activities, and LH receptor levels in immature rat Leydig cells (Murono et al., 1993). In immature pig testis, bFGF stimulated ^{3}H-thymidine incorporation of cultured Sertoli cells (Jaillard et al., 1987). bFGF can also regulate steroidogenesis in cultured porcine Leydig cells (Raeside et al., 1988; Sordoillet et al., 1988; Avallet et al., 1991).

Since KGF can stimulate prostate growth and is induced by androgens, it has been proposed as an "andromedin" in the prostate (Yan et al., 1992). We have previously reported the ability of KGF to stimulate prostate epithelial proliferation (Canatan et al., 1996). However, if KGF can also be produced by distant organs, such as the testis, and be carried to the prostate, KGF may also possess endocrine as well as paracrine effects in the prostate. Soluble forms of the FGF receptor family have already been identified and have been postulated to act as carriers for FGFs (Hanneken et al., 1994; Kishi et al., 1994; Wang and Thomas, 1994).

Received April 22, 1996; Revised July 1, 1996; Accepted August 5, 1996.
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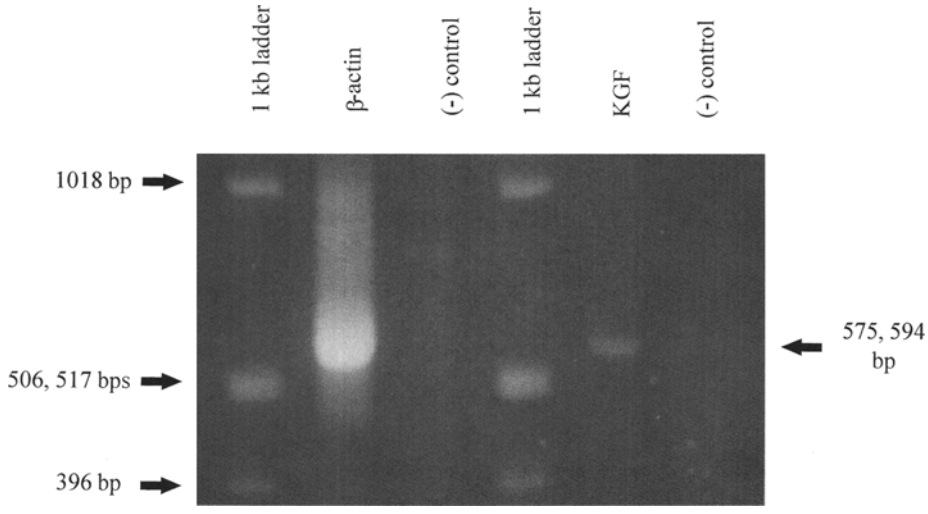

Fig. 1. RT-PCR analysis of total RNA isolated from a dog testis. Oligos 3 and 4 were used for analysis of the β-actin housekeeping gene. Oligos 1 and 2 were used for analysis of KGF. The expected 594- and 575-bp products of β-actin and KGF, respectively, were amplified from total RNA isolated from the canine testis. No products were evident in the negative controls.

To establish the testis as a potential source of KGF, the KGF transcript and protein must be identified in the testis. The current study reports identification of the KGF transcript and immunological localization of the KGF protein in the canine testis.

Results

RT-PCR Analysis of Whole Canine Testis

The results for RT-PCR using KGF-specific primers on total RNA isolated from whole testes are shown in Fig. 1. An expected 575-bp KGF product was detected. The β-actin housekeeping gene was also detected (594 bp). Oligos 3 and 4 were used for analysis of the β-actin housekeeping gene. Oligos 1 and 2 were used for analysis of KGF. The negative control was performed by substituting distilled water for total RNA. No products were evident in the negative controls.

hCG Responsiveness of Percoll-Enriched Interstitial Cells

Interstitial cells were physically isolated from seminiferous tubules and differentially separated on a discontinuous Percoll gradient. Four visible bands of cells were obtained that localized to interfaces between Percoll layers of different concentrations: 10–20% (fraction 1), 20–30% (fraction 2), 30–40% (fraction 3), and 40–50% Percoll (fraction 4). The corresponding densities for 10, 20, 30, 40, and 50% Percoll are 1.028, 1.040, 1.051, 1.062, and 1.074 g/mL, respectively. In order to determine in which population the Leydig cells were enriched, we measured the abilities of the cell populations to produce testosterone after stimulation with 1 IU/mL hCG (Table 1). Mean testosterone production (pg/mL/4 h) of crude interstitial cells, fraction 1, fraction 2, and fraction 3 were 697.5, 0.0, 5.5, and 99.3, respectively. Mean testosterone production (pg/mL/4 h) of crude interstitial cells, fraction 1, fraction 2, and fraction 3 after 4 h of hCG stimulation were 1770.1, 2.3, 90.5, and 4613.2, respectively. Crude interstitial cells were stimulated to increase testosterone secretion by 2.5-fold after hCG priming. The Leydig cells appear to be enriched in fraction 3, which exhibited an almost 50-fold elevation in testosterone production following hCG stimulation. Fractions 1 and 2 produced minimal testosterone in vitro, and fraction 4 produced undetectable levels of testosterone, and was therefore not reported in Table 1.

Table 1
Mean Testosterone Production
by Various Interstitial Cell Populations After hCG Stimulation

	Testosterone, pg/mL/4 h; mean ± SD	
Cell type	No treatment	hCG treatment, 1.0 IU/mL
Unfractionated interstitial cells	697.5 ± 163.4	1770.1 ± 173.6
Percoll fraction 2	55.5 ± 3.1	90.5 ± 9.5
Percoll fraction 3	99.3 ± 18.7	4613.2 ± 484.7

[a]Interstitial cells were physically isolated from seminiferous tubules and differentially separated on a discontinuous Percoll gradient. Four visible bands of cells were obtained, which localized to interfaces between Percoll layers of different concentrations: 10–20% (fraction 1), 20–30% (fraction 2), 30–40% (fraction 3), and 40–50% Percoll (fraction 4). Crude interstitial cells were stimulated to increase testosterone secretion by 2.5-fold after hCG priming. The Leydig cells appear to be enriched in fraction 3, which exhibited an almost 50-fold elevation in testosterone production following hCG stimulation. Fraction 1 produced minimal testosterone in vitro, and fraction 4 produced undetectable levels of testosterone and is therefore not included.

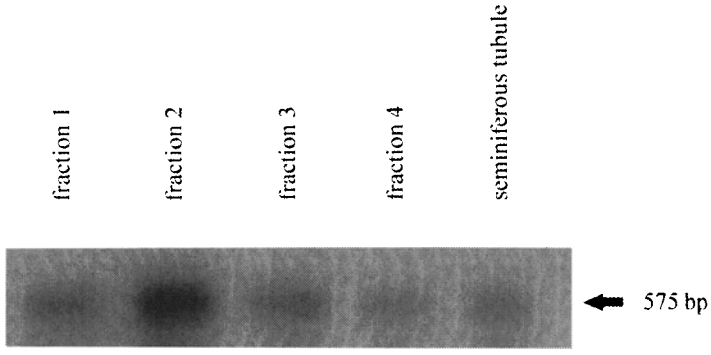

Fig. 2. Southern hybridization analysis of various testicular cell isolates. Interstitial cells were separated from seminiferous tubules and were isolated on a discontinuous Percoll gradient. Each fraction represents cells isolated from different interfaces of the discontinuous Percoll column. Fraction 1: 10–20% Percoll; fraction 2: 20–30% Percoll; fraction 3: 30–40% Percoll; fraction 4: 40–50% Percoll. The expected 575-bp KGF product was identified in all interstitial fractions and in the seminiferous tubules.

RT-PCR and Southern Blot Analysis of Seminiferous Tubule and Percoll-Enriched Interstitial Cells

To characterize further the expression pattern of KGF in the dog testis, seminiferous tubules were physically separated from interstitial cells, and different populations of testicular interstitial cells were isolated on a discontinuous Percoll gradient. Testicles from mature dogs were used for analysis. RT-PCR followed by Southern hybridization was performed for KGF in the various testicular fractions. Figure 2 shows that the expected 575-bp KGF fragment was detected in predominantly the interstitial fraction that was enriched between 20 and 30% Percoll (fraction 2). KGF mRNA expression levels seem to be low in Leydig cell-enriched fraction (fraction 3) and the seminiferous tubules.

Quantitation of KGF mRNA Expression Levels Among the Testicular Fractions

To quantitate the expression levels of the KGF mRNAs in the various interstitial fractions and the seminiferous tubule, a semiquantitative competitive RT-PCR was performed on the same samples. Figure 3A shows that the expected 575-bp KGF product and the 321-bp competitive template were amplified in all interstitial fractions and in seminiferous tubules. PCR was performed only on the KGF competitive template without any testicular cDNAs in the lane labeled KGF Mimic template. The KGF product lane represents PCR analysis on fraction 3 without the addition of the KGF competitive template.

Figure 3B shows that the expected 983-bp GAPDH product and the 630 bp competitive template were amplified in all interstitial fractions and in seminiferous tubules. Amplification of the GAPDH housekeeping gene was used to normalize KGF expression levels among the testicular fractions. PCR was performed only on the GAPDH competitive template without any testicular cDNAs in the lane labeled GAPDH Mimic template. The GAPDH product lane represents PCR analysis on fraction 3 without the addition of the GAPDH competitive template.

The results from the competitive RT-PCR study are depicted in Fig. 3C. KGF expression levels were calculated by normalizing the ratio of KGF product to KGF template with the ratio of GAPDH to GAPDH template. Fraction 2 expressed the highest level of KGF mRNA. The hCG-responsive cells (fraction 3) are presumably Leydig cell-enriched and express low levels of KGF mRNA. The seminiferous tubules also express low levels of KGF mRNA.

Immunohistochemical Localization of KGF in the Canine Testis

Immunolocalization of the KGF protein in canine testis is shown in Fig. 4. The pictures demonstrate that KGF was predominantly localized to peritubular cells (Figs. 4A,B). No KGF was identified in Leydig cells. Some presumably degenerative germ cells and a blood vessel (Fig. 4A, upper right) also stained for KGF. The negative control (Fig. 4C) utilized preimmunized rabbit IgG. An adjacent section was stained with hematoxylin and eosin for histological evaluation (Fig. 4D).

Discussion

The present study demonstrates that KGF is expressed in the interstitial cells of the canine testis. The transcript was amplified by RT-PCR, and its fidelity was confirmed by Southern hybridization with a ^{32}P-labeled cDNA probe. Semiquantitative analysis of the RT-PCR products was performed with competitive RT-PCR. The KGF transcript was predominantly localized to the interstitial cells isolated between 20 and 30% Percoll (fraction 2). Immunohistochemistry localized the KGF protein to peritubular cells.

Our findings suggest that KGF may influence testicular function in the canine testis. Many other growth factors, including other members of the FGF family, have demonstrated abilities to regulate testicular development, growth, and steroidogenesis (Bellve and Zheng, 1989; Lamb, 1993; Saez, 1994). Since KGF acts predominantly on epithelial cells to stimulate proliferation, KGF may stimulate Sertoli cell growth and/or function in the testis. This would corre-

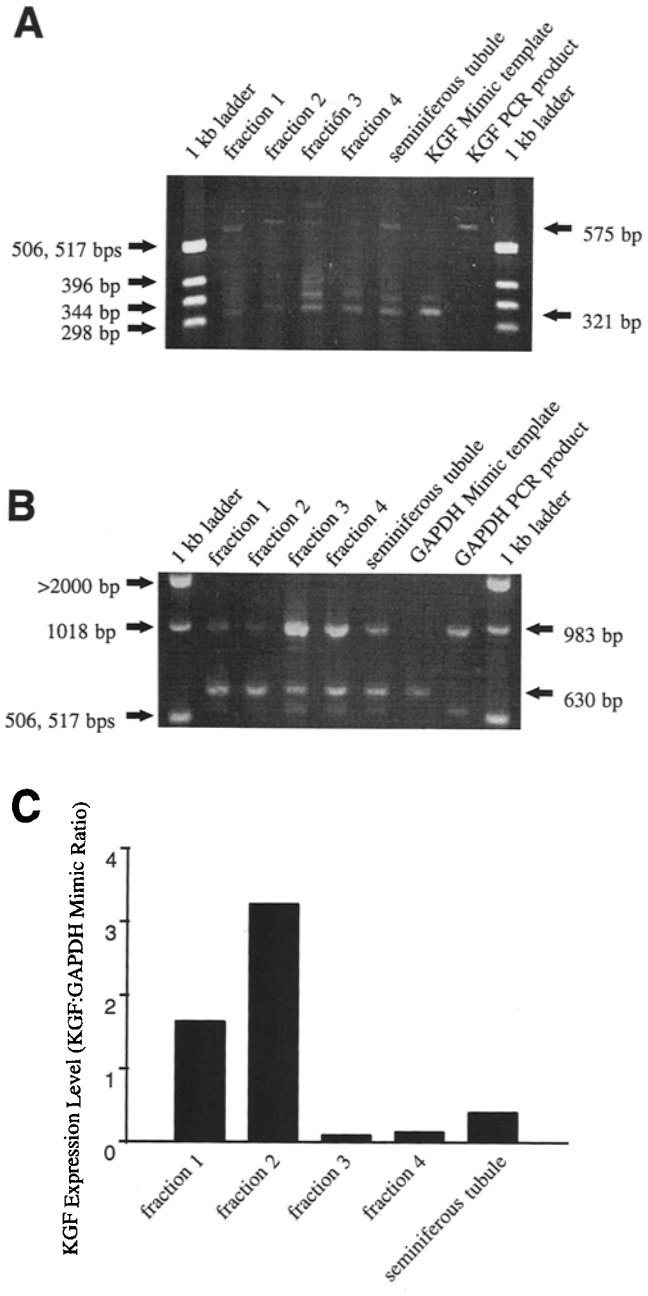

Fig. 3. Interstitial cells were separated from seminiferous tubules and were isolated on a discontinuous Percoll gradient. Each fraction represents cells isolated from different interfaces of the discontinuous Percoll column. Fraction 1: 10–20% Percoll; fraction 2: 20–30% Percoll; fraction 3: 30–40% Percoll; fraction 4: 40–50% Percoll. **(A)** Competitive RT-PCR semiquantitative analysis of KGF in various testicular cell isolates. The expected 575-bp KGF product and the 321-bp KGF competitive template were identified in all testicular cell samples. PCR was performed only on the KGF competitive template without any testicular cDNAs in the lane labeled KGF Mimic template. The KGF product lane represents PCR analysis on fraction 3 without the addition of the KGF competitive template. **(B)** Competitive RT-PCR semiquantitative analysis of GAPDH in various testicular cell isolates. The expected 983-bp GAPDH product and the 630-bp GAPDH competitive template were identified all testicular cell samples. PCR was performed only on the GAPDH competitive template without any testicular cDNAs in the lane labeled GAPDH Mimic template. The GAPDH product lane represents PCR analysis on fraction 3 without the addition of the GAPDH competitive template. **(C)** KGF expression levels in various testicular cell isolates. KGF expression levels were normalized by GAPDH levels. KGF mRNA expression levels were highest in interstitial cells isolated in fraction 2, whereas Leydig-enriched cells (fraction 3) and seminiferous tubules expressed low levels of KGF mRNA.

spond to the present belief that KGF is a paracrine mediator of mesenchymal–epithelial interactions (Rubin et al., 1995).

The peritubular cells consist of basal lamina, collagen myoid cells, and fibroblastic cells (Connell, 1976). The peritubular cells and the seminiferous tubules form a functional unit that necessitate cellular interactions. In fact, some suggest that the peritubular cells may mediate the action of androgens on Sertoli cells (Skinner et al., 1991). Testosterone regulates functions of the Sertoli/peritubular complex, such as spermatogenesis, vascularization, and production of testicular interstitial fluid (Sharpe et al., 1990). Factors that mediate the actions of androgens have been termed "andromedins," and KGF has been suggested to be an "andromedin" in the rat prostate (Yan et al., 1992). KGF, a putative growth factor originating from the mesenchymal-derived peritubular cells, may also be a candidate stromal-to-epithelial-cell andromedin in the canine testis. Other researchers have recognized the potential role of growth factors in cell-to-cell interaction between peritubular cells and its neighboring cells (Skinner et al., 1991).

We propose that KGF is a potential endocrine factor that may influence function of other organs; such as the prostate. KGF can stimulate proliferation of prostate epithelial cells (Canatan et al., 1996) and inhibit 5α-reductase activity of cultured prostate cells (unpublished data) in the dog. The speculation that nonsteroidal testicular factors also regulate prostate development was recently supported by a study published by Juniewicz et al. (1994). Castration followed by replacement of testosterone and estradiol altered developmental progression of BPH in the dog (Juniewicz et al., 1994). The possibility that KGF may also be blood-borne is supported by the identification of soluble forms of FGF receptor 2 (FGFR2) in the medium of human cultured gastric cancer cells (Kishi et al., 1994). KGF binds to the KGFR, which is a alternative splice variant of FGFR2 (Miki et al., 1992). Soluble forms of other members of the FGF receptor family have been isolated in the blood of humans and calves (Miki et al., 1992). Our laboratory has developed an immunoassay system for quantification of KGF protein. In preliminary studies, we were able to detect KGF in seminal fluid isolated from the distal vas deferens and sera of male dogs (unpublished data). Levels of KGF in the serum of a castrated male dog seem to be quantitatively lower (approx 20% lower) than intact dogs (unpublished data).

Rabbit anticanine KGF polyclonal IgG was raised for immunohistochemical localization of KGF. Using conventional immunohistochemical techniques, we were only able to produce positive staining in testicular samples prepared

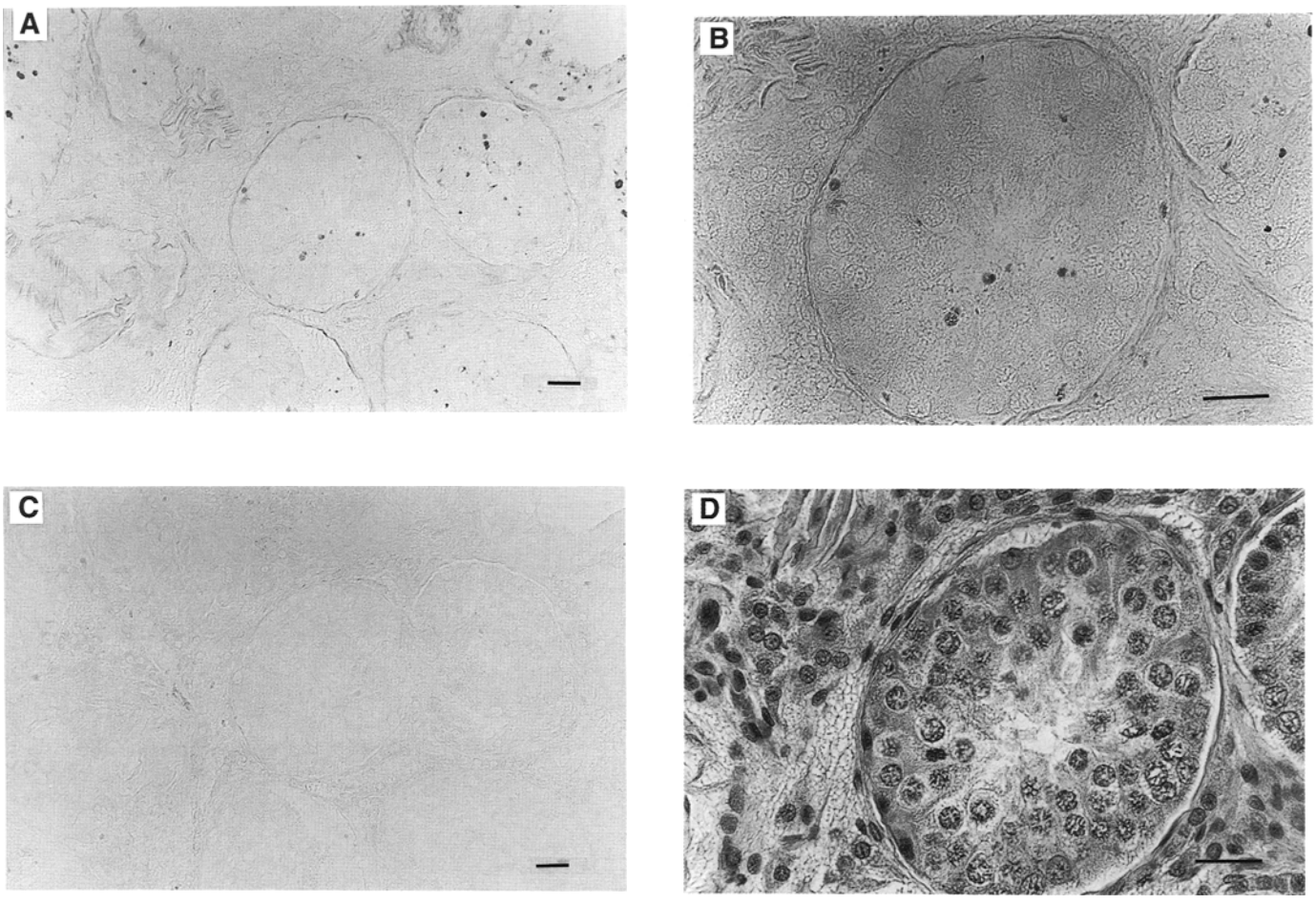

Fig. 4. Immunolocalization of KGF in the canine testis. Rabbit anti-KGF polyclonal IgG were raised against a 21 amino acid peptide specific to KGF. Canine testicular samples were prepared by freeze substitution fixation and paraffin embedded. The KGF protein was localized to peritubular cells adjacent to the basement membrane of the seminiferous tubules (**A** and **B**). When preimmunized IgG was used, no staining was noticeable (**C**). An adjacent section was stained by hematoxylin-eosin for histological evaluation (**D**). Bars = 100 μm.

via freeze substitution. We were not able to produce positive staining with formalin-fixed, paraffin-embedded tissues. Even antigen-retrieval techniques, such as microwaving, could not enhance staining specificity. Tissues prepared with cryostat produced positive staining of interstitial cells, but the integrity of the samples was compromised.

In addition to the peritubular cells, cross-sections of seminiferous tubules revealed immunological staining of degenerative germ cells (Fig. 4). The degenerative germ cells can be identified as pyknotic bodies on H&E stain (Kerr, 1992). The role of KGF in the degenerative process is unknown. However, immunological staining of the degenerative germ cells may suggest overproduction or enhanced affinity of the cells for KGF.

Another consistent finding is the specific KGF localization in blood vessels (Fig. 4). This finding is unsurprising, since endothelial cells produce many FGFs and heparin (Folkman, 1986; Gospodarowic, 1989; Maciag, 1990; Schwartz and Liaw, 1993). Heparin is postulated to provide stability to many FGFs, also known as heparin binding growth factors (Gospodarowic, 1989; Maciag, 1990; Schwartz and Liaw, 1993). Furthermore, FGFs are expressed by vascular smooth muscle and are potent mitogens (Winkles et al., 1987; Gospodarowic et al., 1988; Winkles and Gay, 1991; Alberts et al., 1994). The FGF family of proteins has demonstrated angiogenic activity and may stimulate vascularization during wound healing (Folkman, 1986; Montesano et al., 1986; Schweigerer et al., 1987; Gospodarowic, 1989; Maciag, 1990; Schwartz and Liaw, 1993; Slaving, 1995). Immunolocalization of KGF to blood vessels may indicate both endogenous production of KGF and adherence of KGF to the matrix surrounding the endothelium.

In summary, KGF is expressed predominantly in testicular interstitial cells. The asserted role of KGF as a mediator of mesenchymal–epithelial interactions suggests a paracrine role in the testis. In order to identify KGF as an endocrine factor, we have developed an immunoassay to quantitate KGF levels in canine sera. Within the testis, no specific data on the potential function of KGF exist to our knowledge. The proposed role of KGF as an "andromedin" suggests potential future studies on the ability of androgens to regulate its expression and its actions on Sertoli cells. Since the KGFR is specifically expressed only by epithelial cells

(Rubin et al., 1995), its expression is presumably limited to Sertoli cells. This speculation has not been verified. This is the first known study to identify KGF expression in the testis. This putative "andromedin" may mediate mesenchymal–epithelial interactions, and its discovery in the testis opens many opportunities for future research.

Materials and Methods

Preparation of Isolated Testicular Cell Populations

Fractionation of canine testicular interstitial cells was based on the method of Schumacher et al. (1978) for the isolation of murine Leydig cells. Briefly, testes from adult dogs were decapsulated, cut into small pieces, and incubated in Dulbecco's Modified Essential Medium/Ham's F-12 Nutrient Mixture (DMEM/F12; Sigma Chemical Co., St. Louis, MO) medium containing 0.1% collagenase (Gibco BRL Products, Grand Island, NY), 0.1% bovine serum albumin (Sigma Chemical Co.), 0.01% soybean trypsin inhibitor (Sigma Chemical Co.) and 0.01% DNase I (Sigma Chemical Co.) for 25 min at 37°C with shaking. After seminiferous tubules were allowed to sediment for 5 min on ice, the supernatant containing interstitial cells was filtered (94 μm) and then centrifuged at 250*g* (5 min, 4°C). The pelleted interstitial cells were washed twice in DMEM/F12 and counted in a hemocytometer.

Discontinuous Percoll (Pharmacia Biotech, Inc., Piscataway, NJ) density gradients (10–90%, 45 mL) were prepared by dilutions of a 90% Percoll solution (9:1, Percoll:10-fold concentrated DMEM/F12) with DMEM/F12. Testicular interstitial cells (5 mL containing approx 10^8 cells) were layered on top of the Percoll gradients and then centrifuged for 30 min at 800*g* at room temperature. Four visible bands of cells were obtained, which localized to interfaces between Percoll layers of different concentrations: 10–20% Percoll (fraction 1), 20–30% (fraction 2), 30–40% (fraction 3), and 40–50% (fraction 4). The corresponding densities for 10, 20, 30, 40, and 50% Percoll are 1.028, 1.040, 1.051, 1.062, and 1.074 g/mL, respectively. A fraction of the isolates was used for RNA isolation, whereas the remaining portion was cultured for hCG responsiveness. Red blood cells were visible at the bottom of the centrifuge tube. Cells from each fraction were collected, washed three times with DMEM/F-12, and counted in a hemocytometer. Cell viability was determined by exclusion of trypan blue dye.

To identify the testicular cell fraction containing Leydig cells, the production of testosterone by cells from each fraction in response to human chorionic gonadotropin (hCG; Sigma Chemical Co.) treatment was determined. Cells from each fraction as well as crude interstitial cells were cultured (4 h, 37°C, with shaking) as suspensions (10^6 cells/mL) in tubes containing 1 mL DMEM/F-12 supplemented with 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite in the presence or absence of 1.0 IU/mL hCG, which was found to elicit maximal testosterone production in a pilot study. After incubation, cells were pelleted and the media collected for measurement of testosterone by radioimmunoassay. Leydig cells were determined to be predominantly located in fraction 3 based on response to hCG stimulation.

Radioimmunoassay

Rabbit antiserum to testosterone-7α-bovine serum albumin was obtained from Sigma Chemical Co. and [^{3}H]testosterone ([1,2,6,7,16,17]-^{3}H{*N*}-T, 102.5 Ci/mmol) was purchased from DuPont New England Nuclear Research Products (Boston, MA). Culture medium samples were extracted with diethyl ether (1:7, medium:ether, v:v). Aqueous layers were snap- frozen in a methanol–dry ice mixture, and the organic layers were collected, evaporated, and reconstituted in 1.0 mL of phosphate-buffered saline (PBS) (0.12*M* Na_2HPO_4, 0.04*M* NaH_2PO_4, 0.15*M* NaCl, 0.1% NaN_3) containing gelatin (0.1%). Antiserum and [^{3}H]testosterone were prepared in this buffer so that 0.1 mL contained enough antiserum to bind 30% of added [^{3}H]testosterone (20,000 cpm/0.1 mL/tube). Aliquots of the extracts were incubated with 0.1 mL each of antiserum and [^{3}H]testosterone for 5 h at 20°C followed by 1 h at 4°C in a total volume of 0.6 mL. To separate free and antibody-bound steroids, tubes were then placed in an ice-water bath, and 0.4 mL of cold dextran-coated charcoal solution (0.625% activated charcoal and 0.0625 % Dextran T-70 in buffer) was quickly added to each tube. The tubes were mixed, incubated for 15 min at 4°C, and then centrifuged (4°C, 10 min, 1100*g*). The supernatants were decanted into scintillation vials, and radioactivities of the bound fractions were measured in a liquid scintillation counter after addition of scintillant.

The standard curve used in each assay ranged from 12.5–1000 pg testosterone/mL. Parallelism was confirmed by the lack of difference between the slopes of the standard curve (–2.32) and a line (–2. 31) generated from extracts of a serially diluted pooled culture medium sample that was collected from cultured rat Leydig cells. Sensitivity of the assay, as defined by the value on the standard curve at 2 SD from the zero standard, was 3.1 pg/mL. Crossreactivities of the antiserum as determined by the supplier were: 5α-dihydrotestosterone (23%), 5β-dihydrotestosterone (2%), androstenedione (1.7%), 17α-epitestosterone, 5α-androstane-3β, 17β-diol (1.3%), and <1% for all other steroids tested. Intra- and interassay coefficients of variation were 5.4 and 11.9%, respectively.

Isolation of RNA

Total RNA from canine testes was isolated by the single-step method (Chomczynski and Sacchi, 1987). Briefly, whole testes and isolated testicular cells were homogenized with denaturing solution (4*M* guanidium thiocyanate [Fisher Scientific, Fair Lawn, NJ], 25 m*M* sodium citrate [pH 7.0], 0.1*M* 2-mercaptoethanol and 0.5% sarkosyl). Then 0.1 mL 2*M* sodium acetate (pH 4.0), 1 mL water-satu-

rated phenol, and 0.2 mL chloroform-isoamyl alcohol (49:1) were sequentially added to the homogenate. The mixture was then centrifuged, and RNA was isolated from the supernatant by precipitation with 1 vol 100% isopropanol. The RNA pellet was washed with 75% ethanol, vacuum-dried, and dissolved in diethyl pyrocarbonate-treated water.

Oligonucleotide Primers

In order to amplify the KGF cDNA for reverse transcription polymerase chain reaction (RT- PCR) and competitive RT-PCR, two primers were designed from published human KGF complete mRNA sequence (Finch et al., 1989). The 23-nucleotide sense primer (5'-CAATCTACAATT CACAGATAGGA- 3'; oligo 1) corresponds to nucleotides 339-361. The 18-nucleotide antisense primer (5'-CCATTT AGCTGATGCATA-3'; oligo 2) corresponds to nucleotides 913-896. The predicted length of KGF mRNAs amplified by PCR using oligos 1 and 2 is 575 bp.

In order to amplify the β-actin cDNA by RT-PCR, two primers were designed from published human β-actin complete mRNA sequence (Ponte et al., 1984). The 23-nucleotide sense primer (5'-ACCCACACTGTGCCCATC TACGA-3'; oligo 3) corresponds to nucleotides 519-541. The 23-nucleotide antisense primer (5'-GATCCACAT CTGCTGGAAGGTGG-3', oligo 4) corresponds to nucleotides 1112-1089. The predicted length of β-actin mRNAs amplified by PCR using oligos 3 and 4 is 594 bp.

In order to amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for competitive RT-PCR, two primers were designed from published human GAPDH complete mRNA sequence (Tokunaga et al., 1987). The 26-nucleotide sense primer (5'-TGAAGGTCGGAG TCAACGGATTTGGT-3', oligo 5) corresponds to nucleotides 71-96. The 24-nucleotide antisense primer (5'-CAT GTGGGCCATGAGGTCCACCAC-3'; oligo 6) corresponds to nucleotides 1053-1030. The predicted length of GAPDH mRNAs amplified by PCR using oligos 5 and 6 is 983 bp.

Two primers were designed for production of a MIMIC competitive template (Clontech Laboratories, Inc., Palo Alto, CA) for KGF. The 42-nucleotide sense primer (5'-CAA TCTACAATTCACAGATAGGACAGGTTTCGTGAG CTGATT-3'; oligo 7) and the 38-nucleotide antisense primer (5'-CCATTTAGCTGATGCATATCTGTCAAT GCAGTTTGTAG-3'; oligo 8) can amplify a fragment that is 321 bp when PCR is performed using oligos 1 and 2.

RT-PCR Analysis of Whole Testis and Isolated Testicular Cells

Two micrograms of total RNA from the testes and isolated testicular cells were mixed in the RT buffer (1X first-strand buffer [Gibco BRL Products], 0.01*M* DTT [Gibco BRL], 1 μ*M* dNTPs [Pharmacia Biotech, Inc., Piscataway, NJ], 0.5 μ*M* random hexamer [Pharmacia Biotech, Inc.]) containing 30 U RNase inhibitor (Pharmacia Biotech, Inc.) and 200 U M-MLV reverse transcriptase (Gibco BRL) in a total volume of 40 μL. Complementary DNA (cDNA) was synthesized by first denaturing at 95°C for 10 min and then incubating at 42°C for 2 h, 95°C for 5 min, and 5°C for 5 min. A 20-μL aliquot of the RT mixture was then added to the PCR buffer (1X PCR buffer [Gibco BRL], 1 m*M* $MgCl_2$, 0.1 μ*M* dNTPs, 1 μ*M* of each primers [oligos 1 and 2 for KGF; primers 3 and 4 for β-actin]) containing 2.5 U *Taq* DNA polymerase (Gibco BRL) in a total volume of 100 μL. For the negative control, 20 μL distilled water were added instead of the RT mixture. The PCR mixtures were placed in Perkin Elmer DNA thermal cycler 480 (Norwalk, NJ) and run for 35 PCR cycles (at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min) and a 10-min final extension at 72°C. All PCR products (10-μL aliquots) were run on a 2.5% agarose gel containing 0.5 mg/L ethidium bromide in order to separate and visualize DNA products.

For isolated testicular cells, only 4-μL aliquots of the RT mixture were added to the PCR buffer. Oligos 1 and 2 were used for KGF. The rest of the RT-PCR procedures were identical to the above. All PCR products (10-μL aliquots) were run on a 1.5% agarose gel containing 0.5 mg/L ethidium bromide in order to separate and visualize DNA products.

Competitive RT-PCR Analysis of Isolated Testicular Cells

The RT procedures were performed as mentioned above. The competitive PCR protocol was performed using the Clontech PCR Mimic™ construction kit (Clontech Laboratories, Inc., Palo Alto, CA). Heterologous DNA template was generated for the KGF mRNA using oligos 7 and 8. Heterologous DNA template for the GAPDH mRNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The predicted lengths of the KGF and GAPDH template fragments amplified by PCR are 321 and 630 bp, respectively.

For KGF analysis, a 20-μL aliquot of the RT mixture was then added to the PCR buffer ([1X PCR buffer, 3 m*M* $MgCl_2$, 0.2 μ*M* dNTPs, 1 μ*M* of each primers [oligos 1 and 2], 2×10^{-9}*M* heterologous KGF template) containing 2.0 U *Taq* DNA polymerase in a total volume of 50 μL. For the control, either the RT mixture or template was replaced by distilled water. The PCR mixtures were placed in a thermal cycler and run for 40 PCR cycles (at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min) and a 10-min final extension at 72°C. All PCR products (10-μL aliquots) were run on a 1.5% agarose gel containing 0.5 mg/L ethidium bromide in order to separate and visualize DNA products.

For GAPDH analysis, 4 μL of the RT mixture, oligos 5 and 6, and 1.2 n*M* heterologous GAPDH template were used. Thirty-five PCR cycles were run on a thermal cycler at conditions stated above. A lower number of cycles were performed, since the quantity of GAPDH mRNAs was presumed higher than KGF mRNAs and fewer amplification were needed to produce adequate GAPDH PCR products. All PCR products (10-μL aliquots) were run on a 1.5%

agarose gel containing 0.5 mg/L ethidium bromide in order to separate and visualize DNA products.

Southern Hybridization Analysis

The PCR-generated DNA products were transferred onto nylon membranes following the S & S TurboBlotter alkaline transfer technique (Schleicher & Schnell, Keene, NH). Briefly, DNA products in agarose gels were washed in denaturing solution (3*M* NaCl, 0.4*M* NaOH), transferred onto nylon membranes, neutralized in 0.2*M* phosphate buffer (pH 6.8), and crosslinked with UV light.

^{32}P-labeled KGF probes were synthesized using the DECAPrime II Kit (Ambion, Inc., Austin, TX). A 96-bp oligomer was generated after restriction digestion of a canine KGF cDNA (696 bp; Canatan et al., 1996) with *Hin*fI (Gibco BRL). A 193-bp oligomer was generated after restriction digestion of a canine KGFR cDNA (272 bp, unpublished data) with *Fok*I (Boehringer Mannheim Corp., Indianapolis, IN).

Hybridization was performed using the Paragene HYB-9 hybridization solution (Gentra Systems, Inc., Research Triangle Park, NC). First, the nylon membranes were prehybridized with 1.5–3 mL HYB-9 solution at 64°C for 30 min. Then the denatured probe was added to the hybridization solution, and hybridization was performed at 64°C overnight. The membrane was washed once with 0.2X HYB-9 solution briefly at room temperature and then three times at 64°C for 15 min. For the dot blot and RT-PCR analysis of isolated testicular cells, membranes were exposed to X-ray film (Hyperfilm-MP, Amersham Life Sciences, Inc., Arlington Heights, IL).

Rabbit Anti-KGF Polyclonal IgG Production

A specific region of the canine cDNA has been identified by a special immunological computer-simulation program at the Peptide Laboratory Center at The Ohio State University Comprehensive Cancer Center (Columbus, OH). A 21 amino acid peptide (RTQWYLRIDKRG KVKGTQEMK) corresponding to amino acids 72-92 of the deduced canine KGF protein (Canatan et al., 1996) has been synthesized for production of KGF antibody in the rabbit. Twenty-two promiscuous amino acids were added to the C-terminus of the peptide to increase antigenicity (Kaumaya et al., 1993).

Prior to immunization, blood was collected from New Zealand White male rabbits for use as normal rabbit sera. The synthetic KGF peptide was reconstituted in sterile saline. Ribi Adjuvant System (MPL+TDM+CWS Emulsion, R-730; RIBI ImmunoChem Research, Inc., Hamilton, MT) was reconstituted in 2 mL saline containing 250 µg antigen. Two days after blood collection, the rabbits were immunized with a total of 1.0 mL RAS–antigen mixture in multiple sites (0.05 mL id in 6 sites, 0.2 mL im in each hindleg, 0.1 mL sc in neck region, and 0.2 mL ip). Injections were performed on days 0, 14, and every 28 d thereafter. Blood was collected 10–14 d after each booster starting after the fourth immunization.

Polyclonal IgG was purified using the protein G Sepharose 4 Fast Flow column (Pharmacia Biotech, Inc.). Briefly, rabbit antiserum was passed through the packed gel after 3 bed volumes of binding buffer (20 n*M* sodium phosphate, pH 7.0) were able to maintain a constant flow rate. The bound IgG was eluted with 0.1*M* glycine buffer (pH 2.6), and the fraction was neutralized with 1*M* Tris-HCl (pH 9.0).

Immunohistochemistry

Canine testes were fixed using the freeze-substitution technique published by Taylor (1981). The testis samples were initially placed in isopentane and quickly cooled in liquid nitrogen. Then, the samples were fixed in 0.5% glutaraldehyde at –70°C for 7–14 d. After the fixation period, tissues were dehydrated and embedded in paraffin. Serial sections (6-µm thick) were used for immunohistochemical analysis and hematoxylin-eosin staining.

For immunohistochemical analysis, the sections were deparaffinized in xylene, and hydrated in 100% ethanol, 95% ethanol, distilled water, and PBS (pH 7.1). Nonspecific binding sites were blocked with 5% normal goat serum (NGS; Atlanta Biologicals, Norcross, GA). Then the sections were incubated for 2 h at room temperature with rabbit anti-KGF polyclonal IgG (20 ng/µL) in 1% NGS. The sections were incubated with goat antirabbit IgG (1:20 in PBS, Calbiochem, La Jolla, CA) and streptavidin (Calbiochem) for 30 min each. Finally, the sections were incubated for 10 min in diaminobenzidine-HCl (0.05% in 0.01% H_2O_2). IgGs purified from rabbit blood collected prior to immunization were used as controls. Adjacent sections were stained with hematoxylin-eosin for histological evaluation.

Acknowledgments

The authors would like to thank P. T. Kaumaya, Y. Rikihisa, J. Reiswig, and E. Lee for their technical and intellectual assistance. This study was supported, in part, by NIH grants DK-45916 and CA-16058, and The Ohio State University Canine Research Fund.

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