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Transforming Growth Factor-α Gene Expression and Action in the Seminiferous Tubule: Peritubular Cell-Sertoli Cell Interactions*

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ABSTRACT. The local production and action of an epidermal growth factor (EGF)-like substance within the seminiferous tubule was investigated as a potential mediator of cell-cell interactions. Peritubular (myoid) and Sertoli cells were isolated and cultured under serum-free conditions. Proteins secreted by Sertoli and peritubular cells were found to contain a component that bound to the EGF receptor in a RRA. Separation of secreted proteins by reverse phase chromatography fractionated a protein that contained EGF bioactivity in its activity to stimulate growth of an EGF-dependent cell line. Biochemical properties examined for both Sertoli and peritubular cell EGF activities were similar with each other, but distinct from murine EGF. Northern blot analysis with an EGF cDNA probe did not detect EGF gene expression in peritubular, Sertoli, or germ cells. The possible production of an EGF-like substance such as transforming growth factor-α (TGFα) was investigated with a molecular probe to human TGFα. Both peritubular and Sertoli cells contained a 4.5-kilobase mRNA species that hybridized in a Northern blot analysis with a human TGFα cRNA probe. An immunoblot with a TGFα antisera confirmed the production of TGFα by the detection of a protein in both Sertoli and peritubular cell secreted proteins. TGFα gene expression was not detected in freshly isolated germ cells. Scatchard analysis revealed the presence of high affinity EGF receptors on peritubular cells and the absence of such receptors on Sertoli or germ cells. TGFα was found to stimulate peritubular cell proliferation, but had no effect on Sertoli cell growth. The effects of hormones and TGFα on Sertoli cell function and differentiation were assayed through an examination of transferrin production by Sertoli cells. TGFα had no direct effect on transferrin production or the ability of hormones to influence Sertoli cells. However, the presence of peritubular cells in coculture with Sertoli cells allowed TGFα to stimulate transferrin production. TGFα was also found to have relatively rapid effects on peritubular cell migration and the promotion of colony formation in culture. Cocultures of peritubular and Sertoli cells also responded to TGFα by the formation of large clusters of cells. Observations demonstrate the local production of TGFα by Sertoli and peritubular cells, and action of TGFα on peritubular cells and, potentially, Sertoli cells. The local production and action of TGFα may have a critical role as a paracrine/autocrine factor involved in the maintenance of testicular function (*Endocrinology* **124**: 845–854, 1989)

CELL-cell interactions between peritubular (myoid), Sertoli, and germinal cells have a critical role in the maintenance and control of the process of spermatogenesis (1). Sertoli cells provide the proper microenvironment and cytoarchitectural support for the developing germinal cells. Peritubular cells surround the seminiferous tubule and in cooperation with Sertoli cells produce a basement membrane that provides structural integrity for the tubule (2, 3). A complex array of regulatory agents is required to act on seminiferous tubule somatic cells to maintain tubule and testis function. In addition to externally derived agents such as FSH and androgens, locally derived paracrine factors are postulated to play an important role in maintaining cellular function, growth, and differentiation (1). One example of an important cell-cell interaction characterized in culture involves the production of a paracrine factor, P-Mod-S (4), by peritubular cells under androgen regulation (4, 5). P-Mod-S has a significant effect in modulating Sertoli cell functions involved in the control of germ cell development (6). This type of observation has led to the postulate that a number of locally derived paracrine factors may participate in the regulation of testicular cell function, growth, and differentiation.

Epidermal growth factor (EGF) (7) is speculated to be involved in the maintenance of testicular function (8–12). Due to the negligible levels of EGF in the circulatory system (13), the local production of an EGF-like substance would appear to be required. Preliminary observations have proposed that EGF-like substances may be produced locally in the seminiferous tubule by Sertoli cells (14). Although adult Sertoli cells are a terminally

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differentiated nongrowing cell type, both germ and peritubular cells undergo a continuous rate of cell proliferation. Therefore, the growth of peritubular cells and, possibly, germinal cells may require locally derived growth factors. For these reasons, the potential production and action of EGF or an EGF-like substance in the seminiferous tubule were investigated.

Transforming growth factor-α (TGFα) is a protein that has structural homology with EGF, binds to the EGF receptor (15), and exhibits a spectrum of biological activities similar, although not identical, to that of EGF (16). TGFα is a unique gene product that is produced as a precursor integral membrane protein which is processed into a soluble extracellular protein (17). TGFα was initially isolated from the conditioned medium of virally transformed fibroblasts (18) and has subsequently been shown to be produced by a large number of neoplastic cells (19) and cells of embryonic origin (20). Recent reports have also demonstrated that TGFα is produced by normal adult epithelial cell types, including bovine pituitary cells (21) and human keratinocytes (22). These results imply that TGFα may be a growth regulator in normal adult tissue. Therefore, the current study examines the possibility that TGFα is produced locally in the seminiferous tubule to participate in the regulation of cell proliferation and differentiation.

Materials and Methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (23) with a modified procedure previously described (24). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco, Grand Island, NY) to remove interstitial cells, followed by a collagenase digestion (1 mg/ml; type I, Sigma, St. Louis, MO) and then a hyaluronidase digestion (1 mg/ml; Sigma). Sertoli cells were plated in 24-well (1 ml/well) multiwell plates at approximately 5×10^5 cells/well or in 150-mm culture plates. Cells were maintained at 32 C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cell cultures were treated as described in *Results* at the time of plating and retreated after 48 h of culture when the medium was replenished. Unless otherwise stated, a 72-h medium collection on day 5 of culture was obtained for analysis, and the cells were harvested for RNA isolation or DNA assay. Sertoli cell cultures were treated as outlined in *Results* section with test substances or with FSH (NIDDK oFSH-16, National Pitiutary Agency; 100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM), and testosterone (1 μM). Data presented were obtained with 10 ng/ml human recombinant TGFα (the generous gift of Rik Derynck, Genentech, CA), but were also confirmed with a concentration of 2.5 ng/ml, and with TGFα from Penninsula Laboratories (Belmont, CA). The level of peritubular cell contamination in the Sertoli cell preparation was judged to be less than 1%, as previously described (24).

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (6). Peritubular cells were plated in medium containing 10% calf serum (Hazelton, Dutchland, PA) and grown to confluence. Cells were then subcultured and plated at 25% confluence. After 3–4 days of culture, subcultured cells were confluent, and cells were either obtained for RNA isolation or the cell cultures were washed for 24 h with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections.

Germ cells were prepared from the testis of adult 60-day-old rats. After removal of the tunica, seminiferous tubules were gently teased apart and chopped. Tubules were incubated for 30 min in serum-free medium. The cells released into the medium were harvested, and residual tubule fragments were gravity sedimented. This crude cell preparation contained predominantly germ cells at various developmental stages, as determined morphologically.

Freshly collected peritubular cell and Sertoli cell serum-free conditioned medium was supplemented with 25 μM phenylmethylsulfonylfluoride and 0.1 mM benzamidine and then centrifuged at 1000 × *g* for 15 min at 4 C to remove cell debris. When required, medium was frozen and stored at −20 C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system (Amicon Corp., Lexington, MA), using a membrane with a 3000 mol wt exclusion limit. Cells used for morphological analysis were fixed with formalin and stained with hematoxylin and eosin.

Electrophoresis and immunoblot procedure

Concentrated conditioned medium (5–10 ml; 100-fold concentrated) was applied to a C18 reverse phase Sep-Pak column (Waters, Milford, MA) and eluted with 75% acetonitrile in 0.1% trifluoroacetic acid. Proteins were electrophoretically separated on sodium dodecyl sulfate (SDS)-7.5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (25) buffer system. The gel was then blotted to nitrocellulose, and specific lanes were immunoblotted as previously described (26) with a TGFα antiserum (Penninsula Laboratories). A 1:100 dilution of primary antisera was used and detected on the immunoblot with an avidin/biotin immunoperoxidase procedure (Vectastain, Vector Laboratories, Burlingame, CA).

Transferrin RIA

Transferrin production by Sertoli cells was assayed by a RIA described previously (27). An aliquot of the culture medium was incubated with rabbit antirat transferrin antibody (Cooper Biomedical, Malvern, PA) and iodinated transferrin for 1 h at 37 C, followed by a 1-h incubation with goat antirabbit immunoglobulin G antibody (Sigma). Complexed antibody was then precipitated with polyethylene glycol (Sigma) and pelleted by centrifugation, and radioactivity in the pellets was determined. All data were normalized per μg Sertoli cell DNA at the time of medium collection and expressed as nanograms of transferrin per μg DNA.

DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (28), as previously described (6). At the end of the culture period, the medium was removed, ethidium bromide buffer (EBB; 20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris, pH 7.5; Sigma) was added to the wells, and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide and 100 U/ml heparin in EBB), diluted 1:2 with EBB buffer, and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. Total protein concentration was measured according to the method of Bradford (29).

RNA isolation and Northern analysis

Polyadenylated RNA was obtained from germ, Sertoli, and peritubular cells. Cells were extracted with 5 M guanidine isothiocynate, 10 mM EDTA, 50 mM Tris (pH 7.5), and 8% (vol/vol) β-mercaptoethanol and then precipitated with 1 M LiCl for 18 h at −20 C. The resulting pellet was reconstituted in 1% SDS, 1 mM EDTA, 0.1 M NaCl, and 10 mM Tris-HCl and homogenized if necessary. Samples were then digested with proteinase-K and applied to an oligodeoxythymidine affinity column. Polyadenylated RNA was separated electrophoretically on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure previously described (30). The human TGFα cRNA probe was obtained from a cDNA that contained the complete coding sequence (31). The insert was subcloned into the plasmid SP65 in the antisense orientation with regard to the transcriptional direction of the SP6 promotor. The cRNA probe was labeled with [^{32}P]UTP as previously described (32). A human EGF cDNA probe (33) was labeled by a random primer extension method (34). A 700-basepair insert of p1B15 (35), a rat cDNA which encodes cyclophilin, was subcloned into the plasmid SP65 promotor to produce a cRNA probe. p1B15 is a gene that appears to be constitutively expressed and was used as a control probe to demonstrate intact RNA. RNA was hybridized to the various probes at 65 C for 20 h (SP6 probes) (22) or 43 C for 40 h (primer extended probe). Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.2% BSA, 0.2% Ficoll, 0.1% SDS, 0.2% polyvinylpyrrolidone, and 50 μg/ml sonicated denatured herring sperm DNA. Posthybridization washes [three 20-min washes in 0.1 × standard saline citrate (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4)-0.1% SDS-1 mM EDTA] were performed at the same temperature as hybridization.

Reverse phase HPLC

Reverse phase chromatography was performed, as previously described (6), on an analytical Vydac-C4 column (Vydac, Hesperia, CA). Unless otherwise stated, the column was equilibrated in 0.1% (vol/vol) trifluoroacetic acid, 0.5% (vol/vol) ethylene glycol, and 1 mM triethylamine, pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a linear gradient from 25–60% acetonitrile. Fractions were collected (2 min) and dried in a Speed-Vac apparatus (Savant, Hicksville, NY). Dried samples were then reconstituted for analysis and, when necessary, stored at −20 C.

Cell growth assays

EGF-stimulated growth of an EGF-dependent cell line was used as an assay for EGF bioactivity. Growth of an EGF-dependent cell line (MK cells) was performed as previously described (13). Samples were incubated on MK cells cultured at 37 C for 72 h. Cell number was then determined to assess EGF growth stimulation. EGF was supplied by the Vanderbilt University, Reproductive Biology Research Center, Tissue Culture Core Laboratory, directed by Dr. G. Carpenter.

Sertoli and peritubular cells were plated at 25% confluence in 24-multiwell culture plates and incubated for 48 h in 1 ml culture medium containing 0.1% calf serum. Then, the cells were treated for 18 h with various concentrations of growth regulators. The medium was replaced with 0.5 ml culture medium containing 1 μCi [^{3}H]thymidine, and the cells were incubated for 4 h. The amount of [^{3}H]thymidine incorporated into DNA was then determined, as previously described (36). Alternatively, cells were treated on day 2 of culture, then maintained for 72 h in the presence of growth regulators, followed by a DNA assay to determine alterations in cell number.

EGF RRA and Scatchard analysis

An EGF RRA was established with a human fibroblast cell line, as previously described (7). Samples were incubated at 4 C for 4 h on human fibroblasts, then [^{125}I]iodo-EGF binding was assessed. All data were obtained with preincubation conditions of sample and [^{125}I]iodo-EGF. Preincubation conditions involve a 4 C incubation with sample for 4 h, followed by removal of the sample and then addition and incubation of tracer for 2 h at 4 C. These conditions reduce the possible generation of a false positive in the assay due to the presence of proteases in the sample. In addition, all conditioned medium samples contained the protease inhibitors phenylmethylsulfonylfluoride and benzamidine.

The possible presence of EGF receptors on cells was investigated with a Scatchard analysis using [^{125}I]iodo-EGF as previously described (7, 37). Confluent cultures were incubated under serum-free conditions with increasing amounts of murine [^{125}I]iodo-EGF in the absence or presence of 200 ng nonradiolabeled EGF for 4 h at 4 C. The amount of [^{125}I]iodo-EGF specifically bound was determined and presented as a ratio of bound/free [^{125}I]iodo-EGF used in the incubation *vs.* the concentration of [^{125}I]iodo-EGF specifically bound (moles per liter).

Both the RRA and Scatchard analysis were performed with the assistance of the Vanderbilt University Reproduction Biology Research Center, Tissue Culture Core Laboratory, directed by Dr. G. Carpenter.

Results

The potential production of an EGF-like substance was initially examined with an EGF RRA. Peritubular and Sertoli cell-secreted proteins were prepared from concentrated serum-free conditioned medium and will be referred to as PSP and SSP, respectively. Both Sertoli and peritubular cell-conditioned media displaced radioiodinated EGF from its receptor (Fig. 1). Preincubation conditions, outlined in *Materials and Methods*, were used to reduce the possible generation of a false positive in the assay due to the presence of proteases. A 75–100% displacement of [^{125}I]iodo-EGF was obtained with 5–10 mg secreted proteins, which approach saturating concentrations of proteins. The relative amount of binding activity was consistently greater in PSP than in SSP. These observations indicate that both PSP and SSP contain a component that can specifically bind to the EGF receptor.

To further characterize this EGF receptor-binding activity, both PSP and SSP were separated by reverse phase chromatography. EGF biological activity was assessed by the ability of individual fractions to stimulate growth of an EGF-dependent cell line. A major discrete peak of growth stimulatory activity was detected in the profiles for both SSP (Fig. 2) and PSP (Fig. 3). The retention times for both the SSP and PSP major stimulatory peaks were the same (12 min). Authentic murine EGF had a retention time of 4 min (data not shown). Therefore, the hydrophobicity of the EGF activity in SSP and PSP was different from that of authentic EGF. These observations imply that peritubular and Sertoli cells produce an EGF-like substance, which may be distinct from EGF.

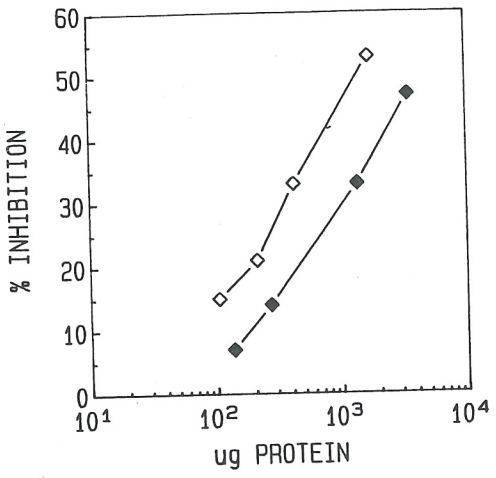

FIG. 1. EGF RRA on SSP (♦) and PSP (◇). The percent binding inhibition of [^{125}I]iodo-EGF was determined with increasing concentrations of secreted protein. Data presented are the mean of a replicate determination and are representative of three separate experiments on three separate secreted protein preparations.

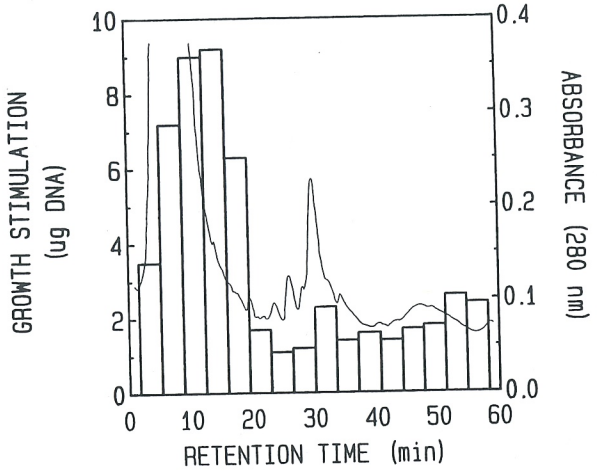

FIG. 2. Reverse phase chromatography of SSP. The ability of individual fractions to stimulate MK cell growth is expressed as growth stimulation (*bar graph*), as determined through quantitation of micrograms of DNA present after treatment. Protein elution was monitored at 280 nm (*histogram*). Data presented are from a representative experiment of three separate experiments.

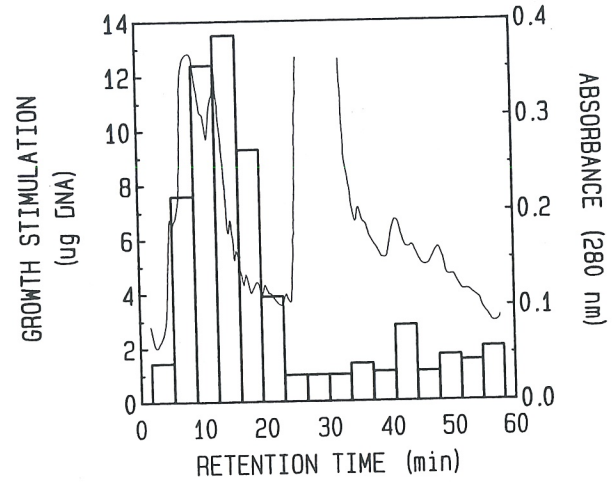

FIG. 3. Reverse phase chromatography of PSP. The ability of individual fractions to stimulate MK cell growth is expressed as growth stimulation (*bar graph*), as determined through quantitation of micrograms of DNA present after treatment. Protein elution was monitored at 280 nm (*histogram*). Data presented are from a representative experiment of three separate experiments.

To determine whether TGFα may be responsible for the EGF-like activity, TGFα gene expression was examined in peritubular and Sertoli cells. Polyadenylated RNA was obtained from freshly isolated and cultured peritubular and Sertoli cells. Northern blot analysis of this RNA with a human TGFα cRNA probe (22) obtained from a cDNA containing the complete coding region for TGFα is shown in Fig. 4. This TGFα cRNA probe does not recognize EGF (22) and has greater than 90% sequence homology with rat TGFα (16). Hybridization was detected with a single mRNA species from both peritubular and Sertoli cells. The size of the mRNA

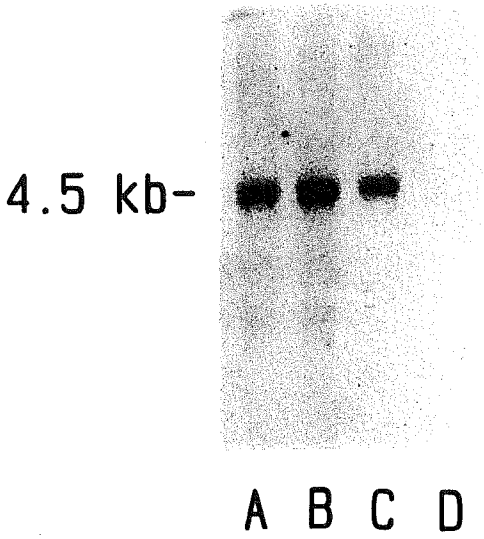

FIG. 4. Northern blot analysis for TGFα gene expression. Polyadenylated RNA (5 μg) was isolated from a SW620 cell line (A), peritubular cells (B), Sertoli cells (C), and germ cells (D). RNA was specifically hybridized with a [^{32}P]cRNA probe for TGFα. Data presented are from a representative experiment of three separate experiments.

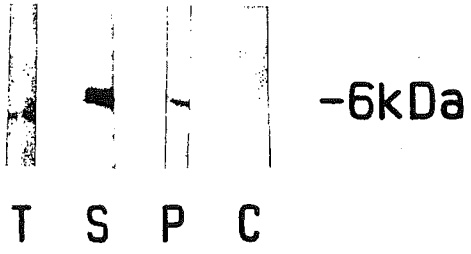

FIG. 5. TGFα immunoblot of TGFα (T), SSP (S), and PSP (P). A control (C) with nonimmune rabbit serum was performed on SSP and PSP that were combined and electrophoretically separated. Detection of the TGFα antibody was performed with an immunoperoxidase procedure. The lanes presented are representative of two different experiments.

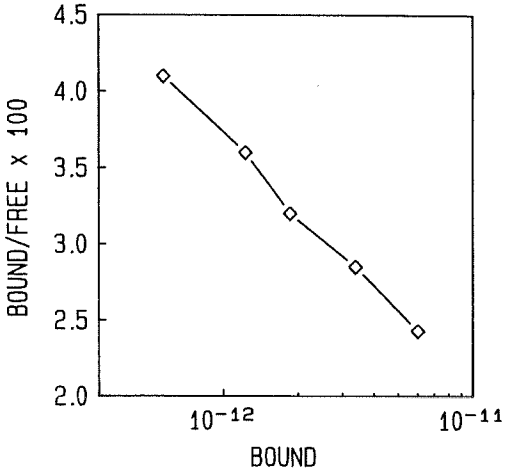

FIG. 6. Scatchard analysis of the EGF receptor on peritubular cells. The amount of [^{125}I]iodo-EGF bound to the cells, expressed as bound/free ratio *vs.* the concentration (moles per liter) bound, expressed as bound. Data are from a representative experiment of three different experiments.

species was similar in the two cell types and comparable to a 4.5-kilobase (kb) species detected in a SW 620-transformed colon cell line, as previously described (38) (Fig. 4). The relative amount of hybridization was reduced in freshly isolated cells, which implies that mRNA levels may increase during the culture of the cells, as previously described (38). No hybridization was detected with germ cell RNA. Northern analysis with a molecular probe (1B15) to cyclophilin, which is a constitutively produced protein (35) that constitutes approximately 1% of the total mRNA in the cell, demonstrated that the RNA analyzed was intact and that hybridization was observed for all RNA samples (data not shown). Northern blot analysis with a human EGF cDNA probe, previously described (33), showed no detectable hybridization with peritubular, Sertoli, or germ cell RNA. However, a 5.2-kb mRNA species was detected with polyadenylated RNA from rat kidney (data not shown). These observations imply that the TGFα gene, and not the EGF gene, is expressed in peritubular and Sertoli cells. An immunoblot procedure (39) with an antisera to TGFα (Penninsula Laboratories) detected a 6K protein in PSP and SSP that had a similar, although not identical, migration as authentic 5K TGFα (Fig. 5). This observation provides additional evidence that TGFα is responsible in part for the EGF-like activity detected in peritubular and Sertoli cell-conditioned medium.

The observation that Sertoli and peritubular cells produce TGFα initiated an investigation of the potential sites of action of an EGF-like substance in the seminiferous tubule. The presence of EGF receptors was investigated with a Scatchard analysis using [^{125}I]iodo-EGF as previously described (7, 37). Peritubular cells were found to contain high affinity EGF receptors with apparent K_d (±SEM) of $3.3 \times 10^{-10} \pm 0.5 \times 10^{-10}$ M and approximately 12,000 ± 2,400 binding sites/cell (Fig. 6). The possible presence of a class of low affinity EGF-binding sites remains a possibility to be investigated. The values calculated for binding affinity and binding sites per cell are approximate due to a number of variables associated with this type of analysis, as previously discussed (37). Sertoli cells isolated from 20-day-old rats were not found to contain high affinity EGF receptor-binding sites at various conditions of temperature (4 or 37 C) and incubation periods (4 or 18 h). Preparations of germ cells at various stages of development also were not found to contain high affinity EGF receptor-binding sites by Scatchard analysis. Therefore, EGF receptors can be detected on peritubular cells, but not on Sertoli or germ cells.

The effect of TGFα on cell proliferation is shown in

Fig. 7. Primary cultures of peritubular and Sertoli cells were maintained in 0.1% calf serum for 48 h. Cells were then treated on day 2 of culture with EGF (10 ng/ml), TGFα (10 ng/ml), or 10% calf serum for 3 days, followed by a DNA assay to determine effects on cell number. Sertoli cell growth was not affected by any treatment (Fig. 7). Peritubular cell growth was stimulated by both EGF and TGFα treatment, with the level of stimulation similar to that by calf serum. Similar results were obtained when the incorporation of [^{3}H]thymidine into DNA was examined after an 18-h treatment with TGFα, EGF, or calf serum starting on day 2 of culture (data not shown). These results indicate that TGFα stimulates the proliferation of peritubular cells, but has no influence on Sertoli cell growth.

The actions of TGFα on Sertoli cell function were assessed by examining effects on transferrin production by Sertoli cells. Sertoli cells were cultured in the absence or presence of EGF and TGFα. TGFα did not affect the basal level of transferrin production (Fig. 8). The ability of a combination of regulatory agents previously shown to maximally stimulate transferrin production (40) (FSH, insulin, retinol, and testosterone) was also not affected by TGFα (data not shown). These observations indicate that TGFα/EGF does not affect transferrin production by Sertoli cells or the ability of FSH or other regulatory agents to stimulate transferrin production.

Peritubular cells produce a paracrine factor that influences Sertoli cell function (4, 6), and the presence of peritubular cells augments the actions of hormones on Sertoli cell function (5). Therefore, the effects of TGFα on a coculture of peritubular and Sertoli cells were examined. Sertoli cell cultures were seeded with approximately 25% contamination with peritubular cells, and transferrin production was examined. As previously described, the basal level of transferrin production was increased, with peritubular cells present from 18–28 ng/μg DNA (Fig. 8). Interestingly, TGFα and EGF both significantly increased the levels of transferrin production in these cocultures ($P < 0.01$, by Student's t test; Fig. 8). TGFα had no effect on the ability of hormones to stimulate transferrin production (data not shown). Therefore, TGFα appears to affect Sertoli cell function indirectly through the peritubular cells.

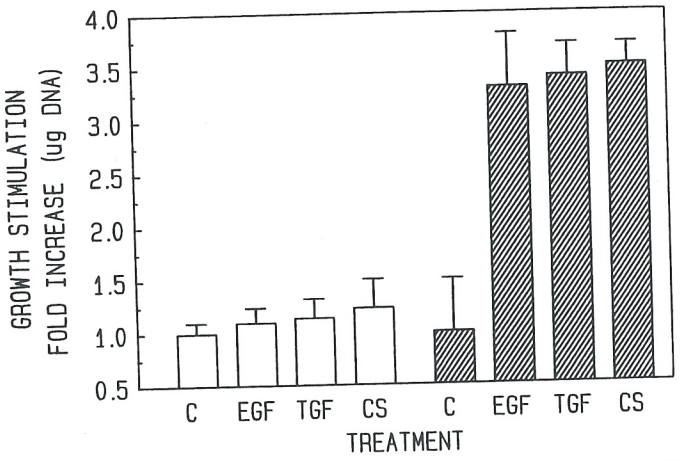

FIG. 7. Effects of regulatory agents on Sertoli and peritubular cell growth. Sertoli cells (□) and peritubular cells (▨) were cultured in the absence (C) or presence of 10 ng/ml TGFα, 10 ng/ml EGF, or 10% calf serum (CS), for 72 h starting on day 2 of culture, then examined for the presence of DNA (micrograms). Data are presented as a fold increase compared to control values. The mean ± SEM are presented for three different experiments, performed in triplicate.

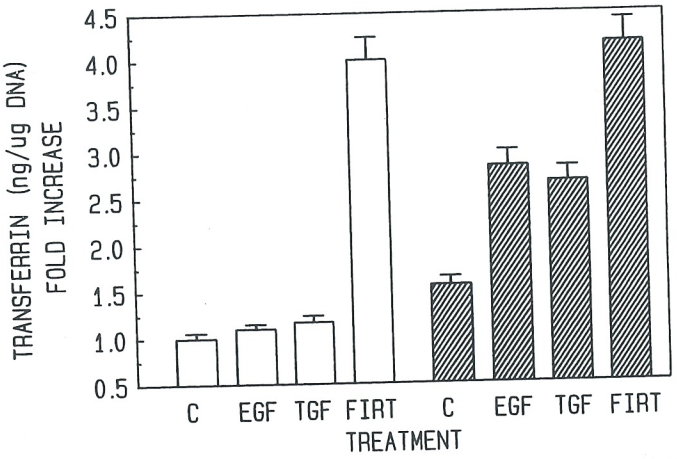

FIG. 8. Transferrin production by cultures of Sertoli cells (□) and cocultures of Sertoli and peritubular cells (3:1 ratio; ▨). Cells were cultured in the absence (C) or presence of 10 ng/ml TGFα, 10 ng/ml EGF, or a combination of FSH, insulin, retinol, and testosterone (FIRT). Data are presented as nanograms of transferrin per μg DNA and expressed as fold increase above control values (mean ± SEM from three different experiments, performed in triplicate).

The actions of TGFα on Sertoli and peritubular cell morphology in culture are shown in Fig. 9. Cells were cultured in the presence of 1% calf serum for 1 day, then treated with TGFα (10 ng/ml) for an additional 1–4 days of culture. Sertoli cell morphology was not influenced by the presence of TGFα, even after a 4-day treatment. However, peritubular cell morphology and migration were affected by TGFα treatment. After 24 h of treatment, peritubular cells started migrating toward small islands of cells and, after 48 h of treatment, formed small clusters of cells (Fig. 9). After 72–144 h of treatment large colonies of cells were formed that had long cellular extensions for attachment to the plastic substratum. Longer treatment with TGFα increased the size of the cell colonies and reduced the ability of the colonies to remain attached to the culture substratum. Cocultures of peritubular and Sertoli cells (25:75 ratio) were also affected by TGFα. Cells migrated to cell colonies and had a tendency to form irregularly shaped cell clusters (Fig. 9). Longer treatment with TGFα resulted in the formation of large cell clusters that maintained attachment to the culture substratum. The effects of TGFα on

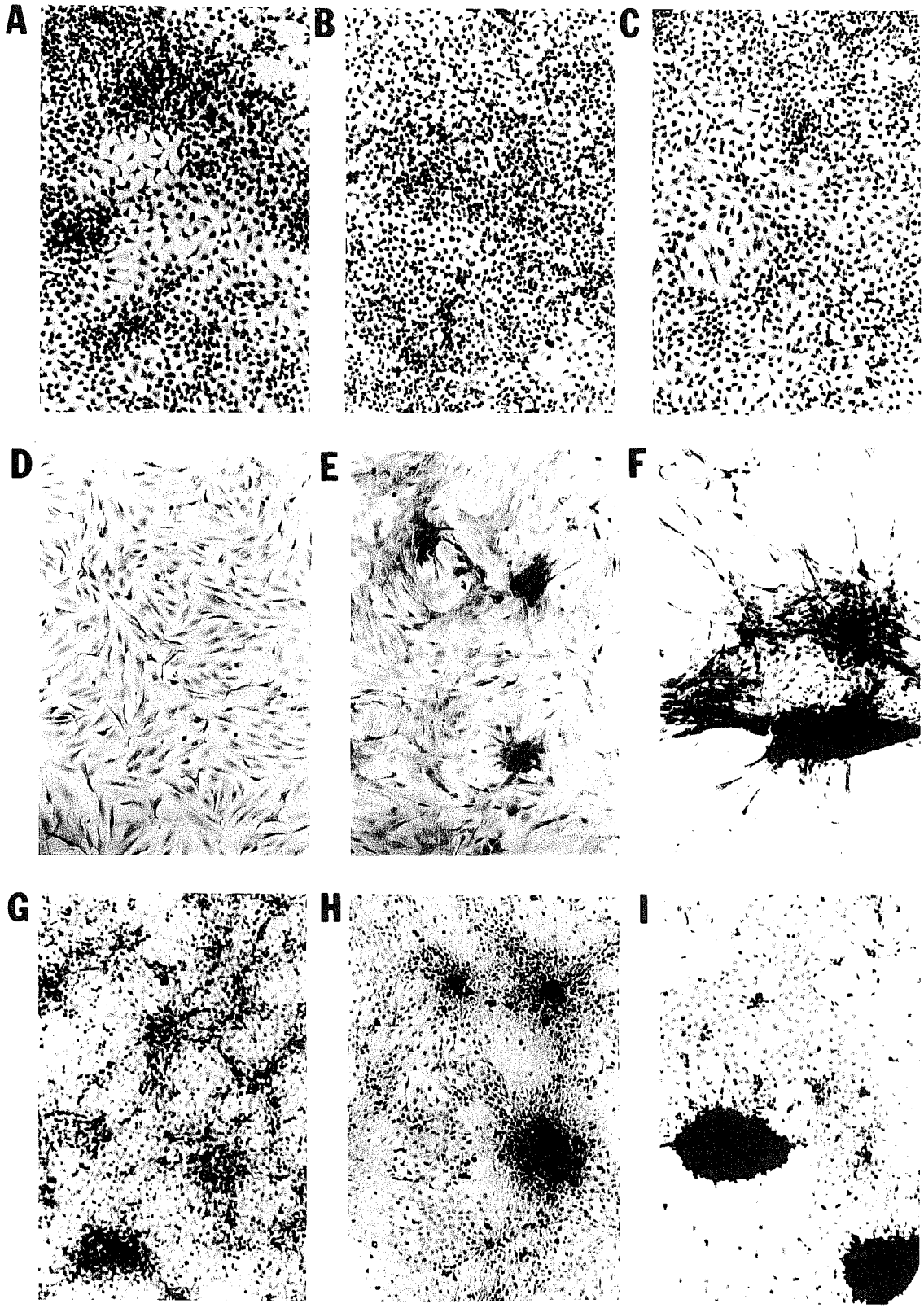

FIG. 9. Morphological analysis of Sertoli cells (A–C), peritubular cells (D–F), and cocultures of Sertoli and peritubular cells (3:1 ratio; G–I). Cells were cultured in the absence (A, D, and G) or presence of TGFα (10 ng/ml) for 24 h (B, E, and H) or 96 h (C, F, and I). Cells were fixed and stained with hematoxylin and eosin on day 3 (B, E, and H) or day 5 (A, C, D, F, G, and I) of culture. Data are presented at a ×135 magnification and are representative of a minimum of three different experiments performed on three different cell preparations.

cell morphology and migration were relatively rapid and were initiated within 24 h and complete within 72–144 h. Although effects could be observed in the absence of serum, optimal effects required 1% calf serum. Higher concentrations of serum appeared to interfere with the morphological effects of TGFα. These observations indicate that TGFα has a significant effect in promoting peritubular cell migration and the formation of unique cellular structures in culture. Unique cell clusters were also formed in cocultures of peritubular and Sertoli cells in response to TGFα treatment. In contrast, TGFα has no detectable effect on Sertoli cell morphology.

Discussion

TGFα is an EGF-like substance produced by a number of neoplastic cells (19) and recently has been shown to be produced by normal tissues (21, 22). Therefore, TGFα may play an important role in growth regulation for many EGF-responsive normal tissues. Both Sertoli and peritubular cells secrete a substance that binds to the EGF receptor and stimulates growth of an EGF-dependent cell line. The hydrophobicity of these EGF activities were distinct from that of authentic murine EGF. These results support the previous observations that Sertoli cells produce an EGF-like substance with unique biochemical properties distinct from those of EGF (14). The potential production of an EGF-like substance such as TGFα was previously speculated (14) and investigated in the current study. Whether the EGF-like substance previously reported to be produced by Sertoli cells (14) is TGFα remains to be determined. TGFα was expressed by Sertoli and peritubular cells. Secretion of TGFα by Sertoli and peritubular cells was confirmed with an immunoblot procedure of secreted proteins using a TGFα antiserum. However, TGFα gene expression was not detected in a mixed population of germinal cells from various stages of development. Therefore, Sertoli and peritubular cells appear to be sites of TGFα synthesis in the rat testis. Northern blot analysis with a human EGF cDNA probe did not detect EGF message in Sertoli, peritubular, or germ cells. Further analysis with more specific molecular probes may be necessary to unequivocally determine the absence or presence of EGF gene expression. These observations imply that peritubular and Sertoli cells, but not germ cells, synthesize and secrete the EGF-like substance TGFα as a potential autocrine/paracrine factor within the seminiferous tubule.

The local production of TGFα by peritubular and Sertoli cells initiated an investigation of the potential sites of action for TGFα/EGF within the seminiferous tubule. Peritubular cells contained high affinity EGF receptor-binding sites. Germ cells isolated at various stages of development did not contain a high affinity EGF receptor. These observations imply that germ cells do not provide a site for TGFα/EGF action with the techniques and reagents used in the current study. Sertoli cells also did not contain high affinity EGF receptor-binding sites. Cells isolated for the current study were obtained from 20-day-old rats, which are at a midstage of puberty. In the rat, puberty is initiated at 10 days of age and is complete at 35–40 days of age. It is possible that the expression of EGF receptors on Sertoli cells may be developmentally regulated. These observations imply that potential sites for TGFα action in the seminiferous tubule are peritubular and, possibly, Sertoli cells, but apparently not germ cells.

The ability of TGFα to promote cell proliferation through the EGF receptor is a well established bioactivity of TGFα (16). Sertoli cells in the adult testis are a terminally differentiated nongrowing cell type (41). Cell proliferation occurs at the early stages of testis development, then terminates. The current study demonstrates that Sertoli cells from the midstage of puberty do not proliferate in response to TGFα/EGF or hormones. Further investigation is required to elucidate whether Sertoli cells at early stages of testicular development may respond to TGFα/EGF. Peributular cells maintain a continuous level of cell proliferation in the adult testis. In contrast to Sertoli cells, observations made in the current study demonstrate that TGFα can stimulate peritubular cell growth. The local production of TGFα in the seminiferous tubule may act as a growth regulator for peritubular cells.

The effect of TGFα on Sertoli cell function and differentiation was examined using transferrin production as a functional marker for the cell. Transferrin production by Sertoli cells (40) is under hormonal regulation (27) and provides a marker protein for Sertoli cell function and differentiation (1). TGFα did not affect transferrin production by Sertoli cells or the ability of hormones to influence cell function. Whether TGFα may directly influence Sertoli cell functions at an earlier stage of development, *i.e.* prepubertal, is currently unknown. Peritubular cells produce a paracrine factor, termed P-Mod-S, under androgen regulation that can modulate Sertoli cell functions such as transferrin production (4). Therefore, regulatory agents can influence Sertoli cell function indirectly through actions on peritubular cells (5). For this reason the effect of TGFα on cocultures of Sertoli and peritubular cells was examined. The presence of peritubular cells allowed TGFα to influence transferrin production by Sertoli cells. Results imply that TGFα can indirectly affect Sertoli cell function through actions on the peritubular cells. Whether this cell-cell interaction is mediated via the paracrine factor P-Mod-S and whether TGFα stimulates P-Mod-S production remain

to be elucidated. Previous observations of direct effects of EGF on Sertoli cell function (42) may have been in part the result of peritubular cell contamination and will require further investigation. These results imply that TGFα may indirectly influence seminiferous tubule cell function and differentiation through cell-cell interactions between peritubular and Sertoli cells.

In the course of examining the actions of the TGFα/EGF on peritubular and Sertoli cells, effects on cell morphology and migration were observed. Although Sertoli cells did not alter cell morphology or migration in response to TGFα, peritubular cells had a profound response to TGFα. Within 24 h peritubular cells initiated migration toward cell colonies and eventually formed large clusters of cells. These observations suggest that TGFα may act as a chemotactic agent for the peritubular cell, but this possibility remains to be elucidated. Cocultures of peritubular and Sertoli cells formed large cell clusters in response to TGFα. Both Sertoli cells and peritubular cells were present in these cell colonies, which is speculated to be due to the ability of Sertoli cells to readily attach to peritubular cells. Peritubular cells are postulated to be a primary participant in the formation of the cell colony. The formation of ball-like cell clusters has been previously described in long term (14–20 days) cocultures of Sertoli and peritubular cells (43). The speculation is made that the local production of TGFα by the cells may play a role in the formation of these ball-like cell structures previously described. The delayed response previously observed may have been due to the low levels of TGFα present. Observations presented in the current study suggest that TGFα may have a major role in influencing the migration of peritubular cells within the seminiferous tubule. Therefore, TGFα may be important in the formation and organogenesis of the seminiferous tubule. Observations not presented demonstrate that Sertoli and peritubular cells also produce TGFβ, which did not influence cell proliferation or function, but has similar effects on peritubular cell morphology as those of TGFα (Skinner, M. K., unpublished observation, manuscript submitted). TGFβ has previously been shown to influence cell migration, chemotaxis (44), and development (45). Whether the actions of TGFα on peritubular cell morphology or migration may be indirectly mediated through effects on TGFβ production remains to be investigated. Peritubular cells have a continuous growth and turnover at all stages of seminiferous tubule development. TGFα may, therefore, play a role in recruiting peritubular cells from the nondifferentiated fibroblast population in the interstitium of the testis. TGFα could act as a growth factor to stimulate cell proliferation and migration toward the tubule. In response to TGFα, these potentially nondifferentiated fibroblasts may proliferate and migrate to form the exterior wall of the seminiferous tubule. Further investigation of the developmental and hormonal regulation of TGFα production in the seminiferous tubule will be required to investigate this hypothesis.

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