The potential role of transforming growth factor-β (TGFβ) as a mediator of cell-cell interactions within the seminiferous tubule was investigated through an examination of the local production and action of TGFβ. Sertoli cells and peritubular (myoid) cells were isolated and cultured under serum-free conditions. Secreted proteins from Sertoli cells and peritubular cells were found to contain a component that bound to TGFβ receptors in RRA. Reverse-phase chromatography of Sertoli cell and peritubular cell secreted proteins fractionated a protein with similar biochemical properties as TGFβ. This fractionated protein also contained TGFβ bioactivity in its ability to inhibit growth of an epidermal growth factor-dependent cell line. Both peritubular cells and Sertoli cells contained a 2.4 kilobase mRNA species that hybridized in a Northern blot analysis with a TGFβ cDNA probe. TGFβ gene expression was not detected in freshly isolated germ cells. TGFβ alone was not found to influence Sertoli cell nor peritubular cell proliferation with cells isolated from a midpubertal stage of development. The effects of hormones and TGFβ on Sertoli cell differentiation and function were assessed through an examination of transferrin production by Sertoli cells. TGFβ had no effect on transferrin production nor the ability of hormones to influence transferrin production. The presence of peritubular cells in a coculture with Sertoli cells also did not affect the inability of TGFβ to act on Sertoli cells. Although Sertoli cell function did not appear to be influenced by TGFβ, peritubular cells responded to TGFβ through an increase in the production of a number of radiolabeled secreted proteins. TGFβ also had relatively rapid effects on peritubular cell migration and the promotion of colony formation in culture. Cocultures of Sertoli cells and peritubular cells responded to TGFβ by the formation of large cell clusters with ball-like structures. Data indicate that TGFβ may play an important role in influencing the differentiation and migration of peritubular cells. Observations demonstrate the local production of TGFβ within the seminiferous tubule by Sertoli cells and peritubular cells and suggest that TGFβ may have a role as a paracrine- and autocrine factor involved in the maintenance of testicular function (Molecular Endocrinology 3: 625–634, 1989)

INTRODUCTION

The somatic cells of the seminiferous tubule, Sertoli cells and peritubular myoid cells, have a critical role in the maintenance and control of the process of spermatogenesis. Sertoli cells form the tubule and provide the cytoarchitectural support for the developing germinal cells. Peritubular cells surround the tubule and are separated from the Sertoli cells by a basement membrane. This complex extracellular matrix is produced cooperatively by Sertoli cells and peritubular cells (1). The environmental cell-cell interaction mediated by this complex basement membrane is important in maintaining structural integrity for the tubule and in promoting structural differentiation of the cells (2, 3). A complex array of regulatory agents are 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 also postulated to play an important role in maintaining cellular function, growth, and differentiation (4). One example of a potentially important cellular interaction involves the production of a paracrine factor, P-Mod-S (5), by peritubular cells under androgen regulation (5, 6) that has a significant effect in modulating Sertoli cell functions involved in the control of germ cell development (7). This type of observation has led to the postulate that a number of additional locally derived paracrine factors may be important in the regulation of testicular cell function, growth, and differentiation.
A factor that has marked effects on cellular differentiation and growth is transforming growth factor β (TGFβ) (8). TGFβ generally inhibits the growth of epithelial cell types and stimulates the growth of some mesenchymal cell types (9). TGFβ also can influence cellular differentiation (8), promote the production of extracellular matrix proteins (10), and act as a chemotactic agent for some cell types (11). TGFβ is a highly conserved protein and produced by many cell types (12). TGFβ is produced in an inactive form (13) that can be activated with acid treatment or proteolysis. TGFβ acts via unique cell surface receptors (14) that appear to be ubiquitous (15). Different forms of TGFβ have been identified, TGFβ1 and TGFβ3, that are highly homologous and have similar, although not identical, biological activities (16). In addition, a third gene product has been identified and designated TGFβ3 (17), but the protein product for this TGFβ3 gene remains to be identified. The term TGFβ will be used in the current manuscript when the production or action of a specific form of TGFβ is unknown or the actions of multiple forms are possible. TGFβ has been implicated in cell-cell interactions in a number of tissues, including the ovary (18), to regulate cell growth and differentiation. The current study was designed to investigate the possible local production of TGFβ in the seminiferous tubule and investigate the actions of TGFβ on cellular function and growth.

RESULTS

The possible production of TGFβ was initially examined with a TGFβ RRA. Peritubular cell and Sertoli cell secreted proteins were prepared from concentrated serum-free conditioned medium and will be referred to as PSP and SSP, respectively. To activate any latent or inactive forms of TGFβ the secreted protein preparations were exposed to pH 2.0 acid conditions a minimum of 2 h before neutralization and assay. Both Sertoli cell and peritubular cell secreted proteins displaced radiiodinated TGFβ, from its receptor, Fig. 1. The relative amount of binding activity was greater in PSP than in SSP. These observations indicate that both PSP and SSP contain a component that can specifically bind to the TGFβ receptors. The amount of TGFβ present in SSP and PSP was estimated to be 2.5 and 5 ng/mg protein, respectively, using a TGFβ1 standard in the RRA. To confirm the secretion of TGFβ, a preliminary study was done with a TGFβ1 RIA previously described (19), that is cross reactive for both TGFβ1 and TGFβ3. Both SSP and PSP displaced radioiodinated TGFβ, from the antibody used in this TGFβ1 RIA (unpublished observation). Therefore, peritubular cells and Sertoli cells appear to secrete a TGFβ-like substance.

To further characterize this TGFβ receptor binding activity both PSP and SSP were separated by reverse phase chromatography. Any latent or inactive forms of TGFβ were presumably activated by the pH 2.0 acid conditions of the chromatography. TGFβ biological activity was assessed by the ability of individual fractions to inhibit growth of an epidermal growth factor (EGF)- dependent cell-line. A major discrete peak of growth inhibitory 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 inhibitory peaks were the same, 22 min. A minor peak of inhibitory activity was also detected at a 50-min retention time for both PSP and SSP, Figs. 2 and 3. The presence of these growth inhibitory peaks indicates that both PSP and SSP contain TGFβ biological activity. Authentic porcine TGFβ1 was found to have a retention time between 20 and 22
min while TGFβ2 was more hydrophobic and had a retention time between 26 and 28 min (data not shown). SSP primarily contained TGFβ activity which eluted with a similar retention time as TGFβ1 with negligible activity eluting at the retention time 26 to 28 min of TGFβ2. Fig. 2. PSP contained TGFβ activity which eluted with the 20–22 min retention time of TGFβ1, however, PSP also contained a significant amount of activity which eluted at the 26-28 min retention time of TGFβ2. Fig. 3. Results imply that SSP contains TGFβ activity which coelutes with TGFβ1, while PSP contains TGFβ activity which coelutes with both TGFβ1 and TGFβ2. These observations support the postulate that peritubular cells and Sertoli cells produce TGFβ.

Gene expression of TGFβ1 was examined to help confirm active synthesis and secretion of TGFβ by peritubular cells and Sertoli cells. Polyadenylated RNA was obtained from freshly isolated and cultured peritubular cells and Sertoli cells. Northern blot analysis of this RNA with a TGFβ1 cDNA probe previously described (12) is shown in Fig. 4. Hybridization was detected on a single mRNA species from both peritubular cells and Sertoli cells. The size of the mRNA species was similar in peritubular cells and Sertoli cells and comparable to a 2.4 kilobase (kb) species previously shown in a AKR-2B embryonic mouse cell-line (19). Fig. 4. In approximately half of the RNA preparations examined the Sertoli cell transcript was slightly smaller than the 2.4 kb transcripts in peritubular cells and AKR-2B cells. The reason for this smaller size is currently unknown and may be generated due to variable processing by the Sertoli cell or an artifact of the isolation procedure. The relative amount of hybridization was reduced in freshly isolated cells which implies mRNA levels increase during the culture of the cells, data not shown. No hybridization was detected with germ cell RNA. Combined results imply that the TGFβ1 gene is expressed in peritubular cells and Sertoli cells but not germ cells.

The actions of TGFβ1 on Sertoli cell function were assessed through examining effects on transferrin production by Sertoli cells. Sertoli cells were cultured in the absence or presence of various hormones and TGFβ1, Fig. 5. TGFβ1 was not found to affect basal levels of transferrin production nor the ability of different concentrations of FSH to stimulate transferrin production. The ability of a combination of FSH, insulin, retinol, and testosterone to stimulate transferrin production was also not affected by TGFβ1. Fig. 5. Observations indicate that TGFβ1 affects neither transferrin production by Sertoli cells nor the ability of FSH or other regulatory agents to stimulate transferrin production.

Peritubular cells produce a paracrine factor that influences Sertoli cell function (7) and the presence of peritubular cells augments the actions of hormones on Sertoli cell function (6). Therefore, the effects of TGFβ1 on coculture of peritubular cells and Sertoli cells were examined. Sertoli cell cultures were seeded with approximately a 25% contamination with peritubular cells and transferrin production examined. As previously described the basal level of transferrin production was increased when peritubular cells were present from 18–35 ng/μg DNA, Figs. 5 and 6. TGFβ1 was not found to affect transferrin production in these cocultures in the absence, Fig. 6, or presence of hormones, data not shown. Therefore, TGFβ1, does not appear to affect
Fig. 5. Transferrin Production by Sertoli Cells Cultured in the Absence (■) or Presence (□) of TGFβ1 (10 ng/ml)

Sertoli cells were also cultured in the absence (control) or presence of FSH, 1 and 100 ng/ml, and a combination of FSH, insulin, retinol, and testosterone, FIRT. Data is presented as nanograms of transferrin/μg Sertoli cell DNA and is the mean ± SEM for three different experiments done in triplicate.

Fig. 6. Transferrin Production by Monocultures of Sertoli Cells and Cocultures of Sertoli Cells and Peritubular Cells, 3:1 Ratio, Respectively

Cells were cultured in the absence (□ and ■) or presence (□ and ◻) of TGFβ1 (10 ng/ml). Data is presented as nanograms of transferrin per μg DNA and is the mean ± SEM for three different experiments done in triplicate.

Fig. 7. Effects of Regulatory Agents on Sertoli Cell and Peritubular Cell Growth

Cells were cultured in the absence (■) or presence of 10 ng/ml TGFβ1 (■), combination of FSH, insulin, retinol, and testosterone (◆) or 10% fetal serum (◆). After 72 h, then examined for DNA content (micrograms of DNA). Data is presented as a cell growth ratio as compared to control cultures. The mean ± SEM is presented for three different experiments done in triplicate.

Sertoli cell function indirectly through the peritubular cells.

The effect of TGFβ1 on Sertoli cell and peritubular cell proliferation is shown in Fig. 7. Cells were treated starting on day 1 of culture for 4 days followed by a DNA assay to determine alterations in cell number. Fig. 7. Sertoli cell growth was not affected by TGFβ1 nor hormones. Peritubular cell growth was also not altered by TGFβ1 but was stimulated with fetal serum. Similar results were also obtained when the incorporation of [3H]thymidine into DNA was examined after an 18-h treatment with TGFβ1 or 10% fetal serum, data not shown. TGFβ1 alone had no effect on [3H]thymidine incorporation into either Sertoli cell or peritubular cell DNA. The combined results indicate that TGFβ1 alone does not influence Sertoli cell or peritubular cell growth with cells isolated at a midpubertal stage of development.

Radiolabeled secreted protein profiles of Sertoli cells and peritubular cells were examined as a qualitative measure of cellular functions. The effects of TGFβ1 on radiolabeled secreted proteins are shown in Fig. 8. Although hormones can stimulate the production of Sertoli cell radiolabeled secreted proteins, data not shown, TGFβ1 had no detectable effects. In contrast, TGFβ1 promoted the apparent production of a number of radiolabeled secreted proteins by peritubular cells, Fig. 8. Radiolabeled proteins at 180, 220, and 300–400 kilodaltons, as well as a number of smaller proteins, were all increased with TGFβ1 treatment. The radiolabeled secreted proteins applied to the polyacrylamide sodium dodecyl sulfate (SDS) gel were derived from the same number of cells, as determined with a DNA assay. These observations indicate that TGFβ1 has major effects on the apparent production of a number of peritubular cell secreted proteins, but has no detectable effect on Sertoli cell secreted proteins.

The actions of TGFβ1 on Sertoli cell and peritubular cell morphology in culture is shown in Fig. 9. Cells were cultured in the presence of 1% calf serum for 1 day, then treated with TGFβ1 for an additional 1–4 days of culture. Sertoli cell morphology was not influenced by the presence of TGFβ1, even after a 4-day treatment. However, peritubular cell morphology and migration was markedly altered by TGFβ1 treatment. After 24 h of treatment peritubular cells started migrating toward
DISCUSSION

TGFβ has been implicated as a paracrine factor in a number of tissues for the regulation of cellular growth and differentiation (9, 16). The current study demonstrates that both Sertoli cells and peritubular cells are sites for the local production of TGFβ within the seminiferous tubule. Both cell types secrete a substance that can bind to TGFβ receptors and inhibit the ability of EGF to stimulate growth of an EGF-dependent cell line. TGFβ has previously been shown to be produced in a latent inactive form that can be activated by exposure to acid conditions (9, 13). Although low levels of receptor binding activity could be detected in nonacid treated secreted protein preparations, maximal TGFβ receptor binding activity and growth inhibitory activity required acid treatment of both PSP and SSP. Therefore, observations imply that as with most cell types a latent form of TGFβ is produced by both cell types that will require activation. Multiple forms of TGFβ are present which are different gene products of apparently functionally related proteins (16, 17). The protein products TGFβ1 and TGFβ2 have been characterized and appear to have some differential effects on specific cell types (16). The protein product of other TGFβ genes such as TGFβ3 have not been identified or characterized (17). Reverse phase chromatography of secreted proteins indicated that Sertoli cells produce a TGFβ activity which coelutes primarily with TGFβ1, while peritubular cells produce TGFβ activity which coelutes with both TGFβ1 and TGFβ2. Observations imply that Sertoli cells produce TGFβ1, while peritubular cells produce both TGFβ1 and TGFβ2. Other forms of TGFβ whose reverse phase chromatographic properties are unknown at present may also be produced and will need to be considered as a limitation to any data interpretation. Investigations with specific antisera for TGFβ1 and TGFβ2 have been initiated and will help elucidate further the specific forms of TGFβ produced.

To further investigate the production of TGFβ the presence of TGFβ1 gene expression was investigated. Sertoli cells and peritubular cells were both found to express the TGFβ1 gene with a 2.4 kb message being identified. Therefore, Sertoli cells and peritubular cells from rat testis synthesize and secrete TGFβ1 as a potential paracrine factor within the seminiferous tubule. Investigations of the possible expression of TGFβ2 and TGFβ3 have been initiated and will help elucidate what additional forms of TGFβ may be produced in the seminiferous tubule. A mixed population of germinal cells from various stages of development was not found to contain the TGFβ1 message. Although germinal cells do not appear to express the TGFβ1 gene, the possible actions of TGFβ on germinal cell development cannot be excluded. Examination of the possible presence of TGFβ receptors on germ cells and effects on cell function remains to be investigated. Detailed analysis with techniques such as in situ hybridization for TGFβ will be informative to confirm data presented in the current study.
The observation that Sertoli cells and peritubular cells produce TGFβ implies the possible involvement of TGFβ in growth regulation within the seminiferous tubule. Neither Sertoli cell nor peritubular cell growth was influenced by TGFβ alone with cells isolated from a mid-pubertal stage of development. This was examined with analysis of both cell proliferation and [3H]thymidine incorporation into DNA. Therefore, TGFβ, does not appear to be a growth stimulator for either cell type at this stage of development. One of the primary functions of TGFβ is its ability to inhibit cell proliferation (9). The ability of TGFβ to inhibit growth stimulation of Sertoli cell and peritubular cell proliferation remains to be investigated. Sertoli cells have previously been shown to produce an EGF-like substance (20). Recent studies have demonstrated that an EGF-like substance, transforming growth factorα (TGFα), is produced by Sertoli cells and peritubular cells and subsequently can act on peritubular cells and possibly prepubertal Sertoli cells to stimulate cell proliferation (21). The ability of TGFβ to inhibit the actions of EGF/TGFα is a well established biological activity of TGFβ. It is speculated that TGFβ production in the seminiferous tubule will function in part to inhibit the actions of a locally produced EGF/TGFα substance. Sertoli cells are a terminally differentiated nonproliferating cell type in the adult. Sertoli cells lose their ability to proliferate during mid-puberty (22) and this is the stage of development the animals in the current study were obtained. Whether TGFβ may inhibit the stimulatory actions of EGF/TGFα on Sertoli cell growth at an earlier stage of testis development, particularly the prepubertal stage, remains a possibility to be investigated. Peritubular cells continue to proliferate at any stage of testis development and may require a more dynamic growth regulation than Sertoli cells. TGFβ may have an active role as a growth inhibitor for peritubular cells to counter the stimulatory actions of a locally produced EGF/TGFα substance. Germ cells undergo a rapid rate of cell proliferation, but little information is currently available concerning control mechanisms. The possible role of TGFβ in germ cell growth is currently unknown. Further understanding of general growth regulation in the testis will provide insight into the role of TGFβ in growth regulation in the seminiferous tubule. The local production of TGFβ by Sertoli cells and peritubular cells, as well as the production of EGF/TGFβ substances (20, 21), suggests that TGFβ may have a role as a growth regulator in the tubule.
TGFβ also has the ability to regulate cell differentiation for a number of cell types (9, 16). Whether this effect on cell differentiation is an indirect effect of its ability to inhibit cell growth remains to be investigated. The postulate is made that the inhibition of cell growth by TGFβ may simply allow the cell to express a more differentiated state and be more responsive to hormone regulation. The effect of TGFβ on Sertoli cell function and differentiation was examined using transferrin production as a functional marker for the cell. Transferrin production by Sertoli cells (23) is under hormonal regulation (24) and provides a marker protein for Sertoli cell function and differentiation (4). TGFβ was not found to affect transferrin production by Sertoli cells nor the ability of hormones, including FSH, to influence cell function. This result is in contrast to the previous observations made on ovarian cells in which TGFβ1 appears to enhance the ability of FSH to stimulate granulosa cell steroidogenesis (25). Whether TGFβ may influence Sertoli cell functions at an earlier stage of development, i.e., prepubertal, remains a possibility to be investigated. Peritubular cells produce a paracrine factor, termed P-Mod-S, under androgen regulation that can modulate Sertoli cell function (5). Therefore, regulatory agents can influence Sertoli cell function indirectly through actions on peritubular cells (4). For this reason the effect of TGFβ1 on cocultures of Sertoli cells and peritubular cells was examined. The presence of peritubular cells had no effect on the inability of TGFβ1 to influence transferrin production by Sertoli cells. Combined results imply that TGFβ1 has minimal effects on the functions of Sertoli cells isolated at midpuberty or the ability of hormones to influence Sertoli cell function. Therefore, TGFβ does not appear to play a major role in the regulation of adult Sertoli cell function or differentiation. Further information, however, regarding the effects of TGFβ on the developmental regulation of Sertoli cell function and differentiation is required.

To assess the effects of TGFβ on general cellular function, radiolabeled secreted proteins were examined. Although regulatory agents such as FSH, insulin, and retinol influence the secretion of radiolabeled proteins by Sertoli cells, TGFβ1 had no effect on Sertoli cell secretory products. Peritubular cells, however, did respond to TGFβ1 through an increase in the apparent production of 180, 220, and 300–500 kilodalton proteins. Specific peritubular cell secreted proteins with these same molecular weights have previously been identified as collagen (1), fibronectin (26), and proteoglycans (27), respectively. Therefore, TGFβ may increase the production of extracellular matrix components by peritubular cells. Previously TGFβ has been shown to increase extracellular matrix production by several cell types (10). Synthesis and secretion of extracellular matrix components by peritubular cells are involved in the formation of the basement membrane of the seminiferous tubule. This complex extracellular matrix between peritubular cells and Sertoli cells is essential to maintain the structural integrity of the tubule and cytoarchitecture of the epithelium (2, 3). TGFβ, therefore, may have a role in the production and maintenance of this extracellular matrix through its actions on peritubular cells. Peritubular cell-Sertoli cell interactions mediated via extracellular matrix may be regulated in part through the local production of TGFβ. The observation that TGFβ can promote the production of a number of secreted proteins implies that TGFβ can influence peritubular cell differentiation and function. Regulation of peritubular cell function is postulated to be important for the maintenance of testicular function and the process of spermatogenesis (4).

TGFβ has also been shown to influence cell migration and colony formation for a number of cell types (11). Several fibroblast cell types respond to TGFβ as a chemotactic agent (9, 16). Although Sertoli cells did not alter cell morphology or migration in response to TGFβ1, peritubular cells had a profound response to TGFβ1. Within 24 h peritubular cells initiated migration toward cell colonies and eventually formed large clusters, balls, of cells. The ability of TGFβ1 to influence peritubular cell migration may in part be due to potential effects on the production of extracellular matrix components which would allow the cell to migrate and form contacts with other cells more readily. Observations presented suggest that TGFβ may potentially act as a chemotactic agent for the peritubular cell, but this possibility remains to be quantified. Cocultures of peritubular cells were also found to form large cell clusters in response to TGFβ1. Both Sertoli cells and peritubular cells appeared to be 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 previously been described in long-term, 14–20 days cocultures of Sertoli cells and peritubular cells (28). It is speculated that the local production of TGFβ 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 TGFβ present. Observations presented in the current study suggest that TGFβ may have a role in influencing the migration and possible chemotaxis of peritubular cells within the seminiferous tubule. The ability of TGFβ to influence the development of a number of tissues has previously been described (9, 16, 29). An interesting possibility to investigate will be whether TGFβ may play a role in recruiting peritubular cells from the nondifferentiated fibroblast population in the interstitium of the tests. In response to TGFβ these potentially nondifferentiated fibroblasts may in part differentiate to become peritubular cells, produce extracellular matrix and migrate to form the exterior wall of the seminiferous tubule. Further investigation of the developmental and hormonal regulation of TGFβ production in the seminiferous tubule will be required to investigate this hypothesis.

Interactions between peritubular cells and Sertoli cells provide an example of mesenchymal-epithelial cell interactions (4). Cellular interactions between mesen-
chymal cells and epithelial cells are present in most tissues and is postulated to be critical for tissue development and function (30). The ability of TGFβ to mediate important mesenchymal-epithelial cell interactions is a possibility that is suggested by data presented in the current study. Further investigation of peritubular cell-Sertoli cell interactions mediated via TGFβ will provide insight into the regulation of testicular cell growth and differentiation, as well as develop a better understanding of mesenchymal-epithelial cell interactions.

The current study demonstrates the local production of TGFβ in the seminiferous tubule by Sertoli cells and peritubular cells. TGFβ, does not appear to act as a growth stimulator for either cell type at a midpubertal stage of development. The ability of TGFβ to act as a growth inhibitor for growth stimulators within the tubule is postulated to be a potential function of TGFβ. With cells isolated at the midpuberty stage, TGFβ was not found to affect Sertoli cell transferrin production nor the ability of hormones to affect transferrin production. However, the ability of TGFβ to modulate cell growth and differentiation at earlier stages of testis development remains to be examined. TGFβ was found to influence peritubular cell function and migration. The data imply that TGFβ may have a role in the maintenance of peritubular cell differentiation and possibly chemotaxis. Clearly the local production and action of TGFβ within the seminiferous tubule implies that TGFβ may be a paracrine-autocrine factor participating in cell-cell interactions required to maintain testis function and the process of spermatogenesis.

MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestion (31) with a modified procedure previously described (26). 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 then plated in 24-well (1 ml/well) multwell plates at approximately 5 x 10^5 cells per well or in 150-mm culture plates. Cells were maintained at 32°C in a 5% CO2 atmosphere in Ham’s F-12 medium (Gibco). Sertoli cultures were treated as described in Results at the time of plating and retrieved 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 harvested for RNA isolation or DNA assay. Sertoli cell cultures were treated as outlined in Results with test substances or with FSH (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM), and testosterone (1 μM). Data presented were obtained with 10 ng/ml TGFβ (R and D Systems, Minneapolis, MN), but were also confirmed with a concentration of 2.5 ng/ml. The contamination of the Sertoli cell preparations with peritubular cells was determined to be less than 1% as previously described (26) with the use of fibronectin production and desmin cytoskeletal elements as unique functional markers for the peritubular cells. Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (7). Peritubular cells were plated in medium containing 10% calf serum 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 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. The peritubular cell preparations were found to be a highly purified cell population with the use of cytochemistry for alkaline phosphatase activity and desmin cytoskeletal elements.

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 30 min in serum-free medium. The cells released into the medium were harvested and residual tubule fragments 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 were made 25 μM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine and then centrifuged at 1000 x 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 using a membrane with 3000 molecular weight exclusion limit. Cells used for morphological analysis were fixed with formalin and stained with hematoxylin and eosin.

Electrophoresis and Fluorography

Sertoli cell and peritubular cell cultures were maintained for 48 h starting on day 5 of serum-free culture in glycine and methionine-free media containing 5 μCi/ml [3H]glucose and [35S]methionine. The radiolabeled media were collected, centrifuged, and used for analysis. Proteins were electrophoretically analyzed on SDS 7.5-15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (32) buffer system. The gels were fluorographed with diphenylloxazole in acetic acid as previously described (33).

Transferrin RIA

Transferrin production by Sertoli cells was assayed by a RIA described previously (24). An aliquot of the culture media was incubated with rabbit anti-rat transferrin antibody (Cooper Biomedical, Malvern, PA) and iodinated transferrin for 1 h at 37°C followed by 1 h incubation with goat anti-rabbit immunoglobulin G antibody (Sigma). Complexed antibody was then precipitated with polyethylene glycol (Sigma), pelleted by centrifugation, and radioactivity in the pellets determined. All data was normalized per microgram of Sertoli cell DNA at the time of media collection and expressed as nanograms of transferrin per micrograms of DNA.

DNA and Protein Assays

DNA was measured fluorometrically with ethidium bromide (34), as previously described (7). At the end of the culture period, the media was removed, ethidium bromide buffer (EBB) (20 mM sodium chloride, 5 mM EDTA, 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, 100 μM heparin in EBB) and 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 quantify 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 (35).
TGFβ, RRA

TGFβ, from porcine platelets (36) was obtained from R and D Systems (Minneapolis, MN) and used in a RRA previously described (8, 14). TGFβ was radiiodinated with a modified chloramine-T procedure (37), and binding assays were carried out using dishes at a density of $1 \times 10^4$ to $2 \times 10^5$ AKR-2B cells per well. After 24 h, the cells were washed with binding buffer (Dulbecco's PBS, pH 7.4, containing 0.1% BSA and 5 mM MgCl₂). Then, 1 ml binding buffer containing 0.2 ng 125I-labeled TGFβ (15,000 cpm) with various levels of competitors was added to each well. Nonspecific binding was determined in the presence of 1 μg 50% pure unlabeled TGFβ. After 2 h of incubation at room temperature the cells were washed three times with binding buffer, and the bound radioactivity was extracted with PBS containing 0.5% Triton X-100.

TGFβ Growth Assay

Inhibition of EGF stimulated growth of an EGF-dependent cell line was used as an assay for TGFβ bioactivity. Growth of an EGF-dependent cell line, MK cells, was performed as previously described (38). Samples were incubated in the presence of EGF 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.

Cell Growth Assays

Sertoli cells and peritubular cells were plated at 25% confluence in 24-well culture plates and incubated for 24 h in 1 ml culture medium containing 1% calf serum, followed by 48 h culture in 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 [35S]methionine, and the cells were incubated for 4 h. The amount of [35S]methionine incorporated into DNA was then determined, as previously described (18). Alternatively, cells were cultured for 72 h in the presence of growth regulators followed by a DNA assay to determine alterations in cell number.

RNA Isolation and Northern Analysis

Polyadenylated RNA was obtained from Sertoli cells and peritubular cells. Cells were extracted with 5 ml guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris, pH 7.5, 8% (vol/vol) β-mercaptoethanol then precipitated with 1 ml LiCl 18 h at −20 C. The resulting pellet was reconstituted in 4 ml 1% SDS, 1 mM EDTA, 0.1 mM NaCl, 10 mM Tris-Cl, and homogenized if necessary. The sample was then digested with 50 μg/ml proteinase K for 30 min at 37 C, then adjusted to 0.4 mM NaCl, and subsequently incubated with 0.1 g oligo(dG)·oligo(dC) at 37 C overnight, then washed in three 30-min incubations of 0.2% (wt/vol) SDS and 0.15 M NaCl, 16 mM sodium citrate as previously described (19).

Reverse-Phase HPLC

Reverse-phase chromatography was performed as previously described (7) on an analytical VyDAC-C4 column. Unless otherwise stated, the column was equilibrated in 0.1% (vol/vol) trifluoroacetic acid, 0.9% (vol/vol) ethylene glycol, 1 mM triethylamine, pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a 60-min linear gradient from 25–60% acetonitrile. Fractions were collected (5 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.

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