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Cytochemical and Biochemical Characterization of Testicular Peritubular Myoid Cells[1]

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ABSTRACT

Testicular peritubular myoid cells secrete a paracrine factor that is a potent modulator of Sertoli cell functions involved in the maintenance of spermatogenesis. These cells also play an integral role in maintaining the structural integrity of the seminiferous tubule. To better understand this important testicular cell type, studies were initiated to characterize cultured peritubular cells using biochemical and histochemical techniques. The electrophoretic pattern of radiolabeled secreted proteins was similar for primary and subcultured peritubular cells and was unique from that of Sertoli cells. Morphologic differences between Sertoli cells and peritubular cells were noted and extended with histochemical staining techniques. Desmin cytoskeletal filaments were demonstrated immunocytochemically in peritubular cells, both in culture and in tissue sections, but were not detected in Sertoli cells. Desmin is proposed to be a marker for peritubular cell differentiation as well as a marker for peritubular cell contamination in Sertoli cell cultures. Peritubular cells and Sertoli cells were also stained histochemically for the presence of alkaline phosphatase. Staining for the alkaline phosphatase enzyme was associated with peritubular cells but not with Sertoli cells. Alkaline phosphatase is therefore an additional histochemical marker for peritubular cells. Biochemical characterization of peritubular cells relied on cell-specific enzymatic activities. Creatine phosphokinase activity, a marker for contractile cells, was found to be associated with peritubular cells, while negligible activity was associated with Sertoli cells. Alkaline phosphatase activity assayed spectrophotometrically was found to be a useful biochemical marker for peritubular cell function and was utilized to determine the responsiveness of primary and subcultured cells to regulatory agents. Testosterone stimulated alkaline phosphatase activity associated with primary cultures of peritubular cells, thus supporting the observation that peritubular cells provide a site of androgen action in the testis. Retinol increased alkaline phosphatase activity in subcultured peritubular cells. Alkaline phosphatase activity increased in response to dibutyryl cyclic adenosine monophosphate (AMP) in both primary and subcultured peritubular cell cultures. Observations indicate that the ability of androgens and retinoids to regulate testicular function may be mediated, in part, through their effects on peritubular cells. This provides additional support for the proposal that the mesenchymal-epithelial cell interactions between peritubular cells and Sertoli cells are important for the maintenance and control of testicular function. Results imply that the endocrine regulation of tissue function may be mediated in part through alterations in mesenchymal-epithelial cell interactions.

INTRODUCTION

The process of spermatogenesis occurs within the seminiferous tubule, which is composed of germ cells, Sertoli cells, and peritubular myoid cells. Sertoli cells are of the epithelial cell type; they form the tubule and provide structural and nutritional support for the developing germinal cells. Peritubular cells are a mesenchymal cell type; they surround the Sertoli cells and form the exterior wall of the seminiferous tubule. Both peritubular cells and Sertoli cells cooperate in the production and formation of a complex extracellular matrix, which is the basement membrane for the tubule (Skin-

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ner et al., 1985). Therefore, one important peritubular cell function is to provide structural support for the seminiferous tubule and help maintain the proper cytoarchitecture of the epithelium (Hadley et al., 1985).

Sertoli cells create the unique microenvironment required for germinal cell development. Regulation of Sertoli cell function and differentiation, therefore, will indirectly affect the process of spermatogenesis. A complex array of regulatory agents influence Sertoli cell function and differentiation, including hormones (Skinner and Griswold, 1982; Mather et al., 1983) and locally produced paracrine factors (Skinner, 1987). Peritubular cells synthesize and secrete a paracrine factor termed P-Mod-S, which can modulate Sertoli cell function and differentiation (Skinner and Fritz, 1985a; Skinner and Fritz, 1986). The actions of P-Mod-S on Sertoli cell function are more profound than any individual regulatory agent previously described, including follicle-stimulating hormone (FSH) (Skinner et al., 1988). These observations indicate that peritubular cells are involved in the control and maintenance of Sertoli cell function and, therefore, may indirectly influence the process of spermatogenesis.

Androgen production by Leydig cells is required for the maintenance of spermatogenesis and testicular function. Within the seminiferous tubule, both Sertoli cells and peritubular cells contain androgen receptors (Verhoeven, 1979) and require androgens for their development (Bressler and Ross, 1972). Peritubular cells also respond to androgens by an apparent increase in the production of the paracrine factor P-Mod-S (Skinner and Fritz, 1985a,b). These observations have led to the proposed cellular interaction that Leydig cells produce androgens that act on peritubular cells to increase P-Mod-S production, which subsequently acts on Sertoli cells to influence functions involved in the control of germinal cell development (Skinner and Fritz, 1985a; Skinner, 1987). Peritubular cells, therefore, provide a potentially important mode of androgen action in the testis that may be essential for the maintenance of spermatogenesis.

Information currently available indicates that peritubular myoid cells play a critical role in the maintenance of the structural integrity of the seminiferous tubule as well as in the regulation of Sertoli cell function and development. The current study was designed to characterize peritubular cells using histochemical and biochemical techniques. This information is needed to understand more thoroughly the importance of the peritubular cell in the testis, as well as to develop biochemical markers for cellular functions.

MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (Dorrington et al., 1975) with a modified procedure previously described (Tung et al., 1984). The cells were plated at approximately 5×10^5 cells per well. Peritubular cells were obtained from the collagenase digestion supernatant of the testicular cell preparation after tubule fragments had gravity sedimented as previously described (Skinner et al., 1988). The cells were collected by low-speed centrifugation, resuspended, and plated at approximately $0.5–1.0 \times 10^5$ cells per well. Cells were maintained at 32°C in a 5% CO_2 atmosphere in Ham's F-12 medium with no additives, and the medium was changed every 48 h. Unless otherwise noted, primary cultures of peritubular cells were plated with 10% newborn calf serum (Hazelton, PA) for the first 2 days to promote cell attachment, and serum-free medium with no additives was used for the remainder of the culture period. Subcultured peritubular cells were obtained by trypsinization of confluent primary cultures of peritubular cells that had been grown in 10% serum. The cells were collected by centrifugation and plated in serum for the first 2 days of culture as outlined above. Co-culture of peritubular cells and Sertoli cells was maintained in the absence of serum. In some experiments, the cells were cultured with one or more regulatory agents (FSH, Pituitary Agency, Baltimore, MD, 100 ng/ml; insulin, 5 μg/ml; retinol, 0.35 μM; testosterone, 1 μM; dibutyryl cyclic AMP [dcAMP], 0.1 mM) and were treated as described in the Results section. Unless stated otherwise, treatments were initiated at the time of plating and maintained until the times stated in the Results.

Histology and Histochemistry

Cells were plated in 1-ml multi-well dishes over plastic coverslips (Thermanox, 15 mm round; Lux, Vangard, Neptune, NY). Optimal cell counts were obtained when the Sertoli cells were plated at approximately 1×10^5 cells/well. Peritubular cells were plated at approximately $0.5–1.0 \times 10^5$ cells per plate.

For hematoxylin and eosin staining, cells were fixed with 4% buffered formalin. The rinsed cells were then stained with Gill hematoxylin (Fisher, Atlanta, GA), rinsed with tap water, and counterstained with acidic eosin. The stained cells were dehydrated through graded ethanol washes, cleared with xylene, and mounted onto glass slides with Permount (Fisher, Atlanta, GA).

Desmin histochemistry was performed by using an immunoperoxidase technique. Briefly, cells were fixed with cold methanol, then incubated for 30 min in 0.3% H_2O_2 in methanol. The cells were incubated for 30 min in phosphate-buffered saline (PBS) with 1% horse serum (heat-inactivated) (PBS-HS) and then for 1 h with mouse monoclonal anti-desmin (1:100 in PBS-HS; Amersham, Arlington Heights, IL). Incubations (30–60 min) with biotinylated horse anti-mouse immunoglobulin G (IgG) (1:250) in PBS and Vectastain ABC Regent (Vector, Burlingame, CA) were followed by an incubation with the peroxidase substrate (0.5 mg/ml diaminobenzidine tetrahydrochloride prepared in 50 mM tris(hydroxymethyl)aminomethane [Tris] buffer, pH 7.6, with 0.03% H_2O_2) until reaction product was visualized. The rinsed cells were then counterstained with Gill hematoxylin and rinsed with tap water. The cells were rinsed extensively with PBS between incubations, except that after the incubation with the anti-desmin antibody PBS-HS was used instead of PBS. The cells were dehydrated, cleared, and mounted as outlined above. Desmin-positive cells are stained brown. This immunoperoxidase technique was used to test peritubular cells and Sertoli cells for the presence of a variety of cytoskeletal proteins and for fibronectin. The procedure was the same as for desmin, except that the incubation with the first antibody was varied in accordance with the protein being tested. The antiserum used for the first incubation was mouse anti-vimetin (Labsystems, Chicago, IL), mouse anti-cytokeratin (Labsystems), mouse anti-actin (Amersham), or rabbit anti-fibronectin (Calbiochem, LaJolla, CA). Biotinylated horse anti-mouse IgG (for vimentin, cytokeratin, and actin) or biotinylated horse anti-rabbit IgG (for fibronectin) was used as the secondary antibody; the cells were then incubated with Vectastain Reagent and peroxidase substrate as outlined above.

Endogenous alkaline phosphatase activity was visualized according to the procedure of Chapin (Chapin et al., 1987). Cells were fixed in 4% buffered formalin, rinsed, and immediately incubated 15–30 min with the alkaline phosphatase stain. The stain was prepared by dissolving 10 mg of the substrate (naphthol AS-biphosphoric acid) with 40 μl dimethyl sulfoxide and diluting the solution with 5 ml distilled water. The pH of the solution was adjusted by adding 5 ml AMP buffer (25 mM 2-amino-2-methyl-1-propanol containing 1.25 mM $MgCl_2$, pH 8.9). Immediately before the incubation, 10 mg of the dye (fast-blue RR salt) was added to the substrate solution; the mixture was vortexed extensively, and the precipitant was filtered with 0.2-μm nylon filter. After visualization of the reaction product, the cells were rinsed with AMP buffer and mounted on a glass slide with aquamount (Gur, Bath, England). Alkaline phosphatase-positive cells are stained blue.

The peritubular cell preparations were tested for the presence of low-density lipoprotein (LDL) receptors, a physiologic marker for endothelial cells and macrophages (Goldstein et al., 1979; Pitas et al., 1981). Cells were grown on glass coverslips; after 5 days of culture, the rhodamine-conjugated acetylated lipoprotein (Biomedical Technologies, Inc., Stoughton, MA), was added to the medium (1:20), and the cells were cultured for an additional 4 h. At the end of the incubation, the cells were rinsed, fixed with 4% buffered formalin, mounted on a slide with PBS-glycerol (1:3), and observed with a fluorescence microscope.

Sertoli cells and peritubular cells were counted at a magnification of 30×. Cells in a ruled mm^2 area were counted in five different areas of a slide. In many cases, two or more slides were counted/cell preparation. An average of 300 cells/slide for the peritubular cell preparation and 600 cells/slide for the Sertoli cell preparation were counted. From these data the percentage of stained cells/slide was calculated.

Alkaline Phosphatase Assay

At the end of the culture period, the medium was removed, buffer (145 mM sodium chloride, 10 mM sodium phosphate, pH 7.5; Sigma Chemical Co., St. Louis, MO) was added to the wells, and the cells were sonicated. An aliquot (100 μl) of the cell sonicate was immediately added to 25 μl of the phosphatase substrate (p-nitrophenyl phosphate, 1 mg/ml) prepared in diethanolamine buffer (DB) (5 mM $MgCl_2$, 10% diethanolamine, pH 9.8; Sigma). The assay volume was adjusted to 0.5 ml with DB buffer and incubated for 1 h at room temperature. The color reaction was terminated

by the addition of guanidine (final concentration, 1 M; Sigma). The reaction product was then quantified with an absorbance at 405 nm.

Creatine Phosphokinase Assay

Rinsed cells were sonicated in 10 mM Tris buffer, pH 7.5. An aliquot of the sonicate (100 µl) was incubated for 10 min at 37°C with 125 µl phosphocreatine solution (3 mg/ml, phosphocreatine prepared in Trizma buffer with magnesium, pH 7.5, from Sigma). The mixture was then incubated with 50 µl adenosine 5′-diphosphate (ADP)-glutathione solution (16 mM prepared in distilled water) for 30 at 37°C. The reaction was stopped by the addition of 50 µl of 1.2 mM p-hydroxymercuribenzoate, and the color reagents were added (250 µl of α-napthol, 20 mg/ml in 1.5 M NaOH and 250 µl of diacetyl, 0.05% in water). The assay volume was adjusted to 2 ml with water, and the color was developed for 20 min at 37°C. The samples were spun to remove any precipitate and the reaction product was quantified with an absorbance at 520 nm (all reagents were obtained from Sigma).

DNA and Protein Assays

DNA was measured fluorometrically with ethidium bromide (Karsten and Wollenberger, 1977) as previously described (Skinner et al., 1988). An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide, 100 units/ml heparin in ethidium bromide buffer [EBB: 20 mM sodium chloride, 5 mM ethylene diamine tetraacetate, 10 mM Tris, pH 7.8; Sigma]) and diluted 1:2 with EBB 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 (Bradford, 1976).

Gel Electrophoresis and Fluorography

Sertoli cell and peritubular cell cultures were maintained for 48 h starting on Day 5 of culture in glycine-free, cysteine-free, and methionine-free media containing 5 µCi/ml [^{35}S]methionine, [^{35}S]cysteine, and 5 µCi/ml [^{3}H]glycine. The medium was collected, centrifuged, and used for analysis. The amount of radiolabeled proteins analyzed was obtained from the same number of cells measured as µg DNA. Radiolabeled proteins were electrophorectically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (Laemmli, 1970) buffer system. The gels were fluorographed with diphenyloxazole in acetic acid as previously described (Skinner and Griswold, 1983a).

RESULTS

Secreted Proteins

A qualitative analysis of cellular function was investigated by examining radiolabeled secreted proteins from primary and subcultures of peritubular cells as well as primary cultures of Sertoli cells (Fig. 1). Proteins secreted by peritubular cells in primary culture were similar to those obtained for subcultured peritubular cells. Although minor differences were noted in the apparent abundance of individual proteins, the same proteins generally were present. Fibronectin was identified previously as a marker protein for peritubular cells (Tung et al., 1984) and a protein that co-migrates with fibronectin was identified in conditioned medium from both subcultured and primary cultures of peritubular cells. Fibronectin was not detected in the medium from cultured Sertoli cells. The proteins secreted by Sertoli cells were different from those obtained for cultured peritubular cells. Proteins that co-migrate with several previously identified Sertoli cell secreted proteins, including a 130,000 ceruloplasmin (Skinner and Griswold, 1983b) and 70,000 testicular transferrin (Skinner and Griswold, 1980), were not detected in peritubular cell-conditioned medium. Treatment of Sertoli cells with serum or peritubular cells with hormones did not significantly alter the secreted protein profiles shown in Figure 1 (data not shown). Data indicate that Sertoli cells and peritubular cells synthesize and secrete different proteins and suggest that there is minimal contamination of the peritubular cell preparation with Sertoli cells.

Morphology and Histochemistry

The morphology of cultured peritubular cells and Sertoli cells is shown in Figure 2. Sertoli cells plated

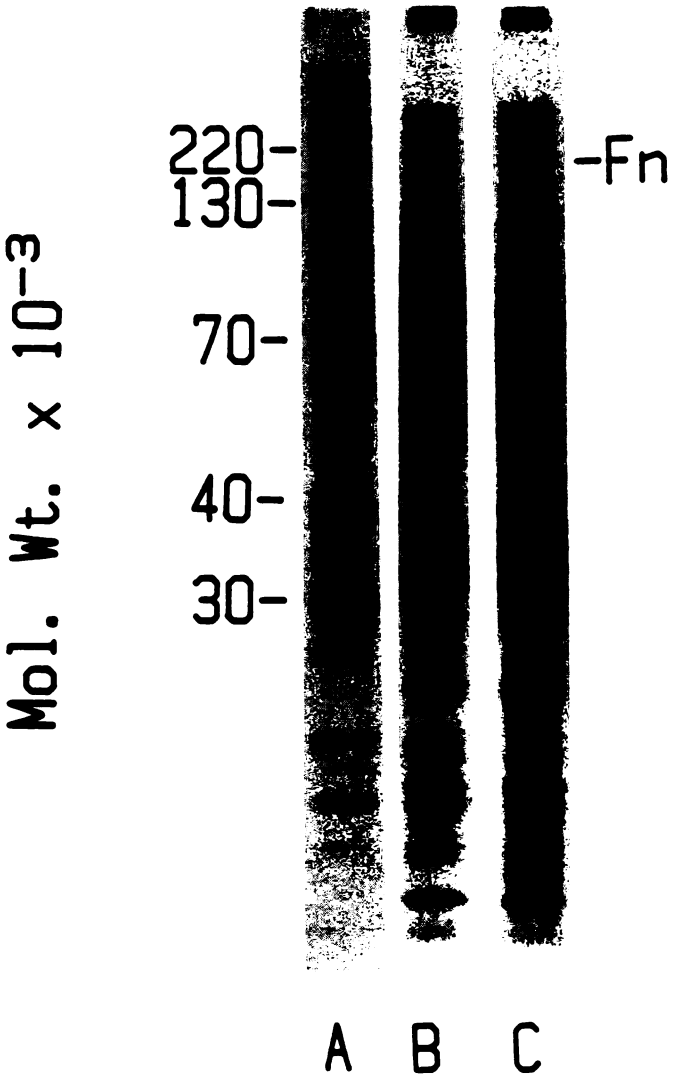

FIG. 1. Fluorograph of radiolabeled secreted proteins from peritubular cells and Sertoli cells. Primary (*B*) and subcultured (*C*) peritubular cells were cultured in the presence of 10% newborn calf serum for 5 days. Sertoli cells (*A*) were plated in the presence of a combination of regulatory agents FIRT (FSH, insulin, retinol, and testosterone). All cells were radiolabeled for 48 h with [^{3}H]glycine, [^{35}S]cysteine, and [^{35}S]methionine in glycine-free, cysteine-free, and methionine-free medium containing no additives, treatments, or serum. The migration of fibronectin (*FN*) is identified. The fluorograph is representative of at least four different experiments.

and cultured in the absence of serum and regulatory agents displayed a morphology that is characteristic for epithelial cells. After 5 days in culture, the aggregates of cells present at plating had spread and few germ cells were present (Fig. 2F). Sertoli cell morphology changed when the cells were plated in the presence of a combination of regulatory agents (FSH, insulin, retinol, and testosterone) (Fig. 2G). Under these conditions the cytoplasm formed long slender processes, the nucleus rounded-up, and the cells were more reticular in appearance. Peritubular cells cultured in serum for 5 days were fibroblast-like and uniform in appearance (Fig. 2E). When serum was not present, the plating efficiency was reduced, the cells became rounded, and the growth rate was retarded.

Desmin is a cytoskeletal protein that is usually found in muscle cells. Previous studies have suggested that this cytoskeletal protein may be present in peritubular cells (Virtanen et al., 1986). The Sertoli and peritubular cell preparations were tested immunocytochemically to determine if the desmin protein could be detected (Fig. 2A and 2B). Approximately 40% of the cells from the peritubular cell preparation were stained desmin-positive, whereas 2% of the cells were stained for this protein in the Sertoli cell preparations (Table 1). The immunocytochemical technique was used to test a variety of other cellular proteins as potential markers for peritubular cells or Sertoli cells. All of the cells from both the Sertoli and peritubular cell preparations stained positive when tested for vimentin, fibronectin, and α-actin, but neither Sertoli cells nor peritubular cells stained when tested for cytokeratin. Since cell-specific staining could be obtained only with desmin, studies were performed to establish that the desmin-positive cells were peritubular in origin. Isolated seminiferous tubules were tested immunocytochemically for desmin, embedded in paraffin, and then sectioned. Desmin staining was restricted to the periphery of the tubules,

TABLE 1. Quantification of desmin and alkaline phosphatase staining in Sertoli cells and peritubular cells cultured for 5 days, fixed, and analyzed for the presence of desmin or alkaline phosphatase activity.*

Cell type	Stain	% Staining	No. tested
Peritubular cell	desmin	39 ± 3	8
Sertoli cell	desmin	2 ± 1	7
Peritubular cell	alkaline phosphatase	69 ± 8	7
Sertoli cell	alkaline phosphatase	1 ± 1	7

*Data are the mean ± SEM of % staining cells/slide, with the number of cell preparations examined noted.

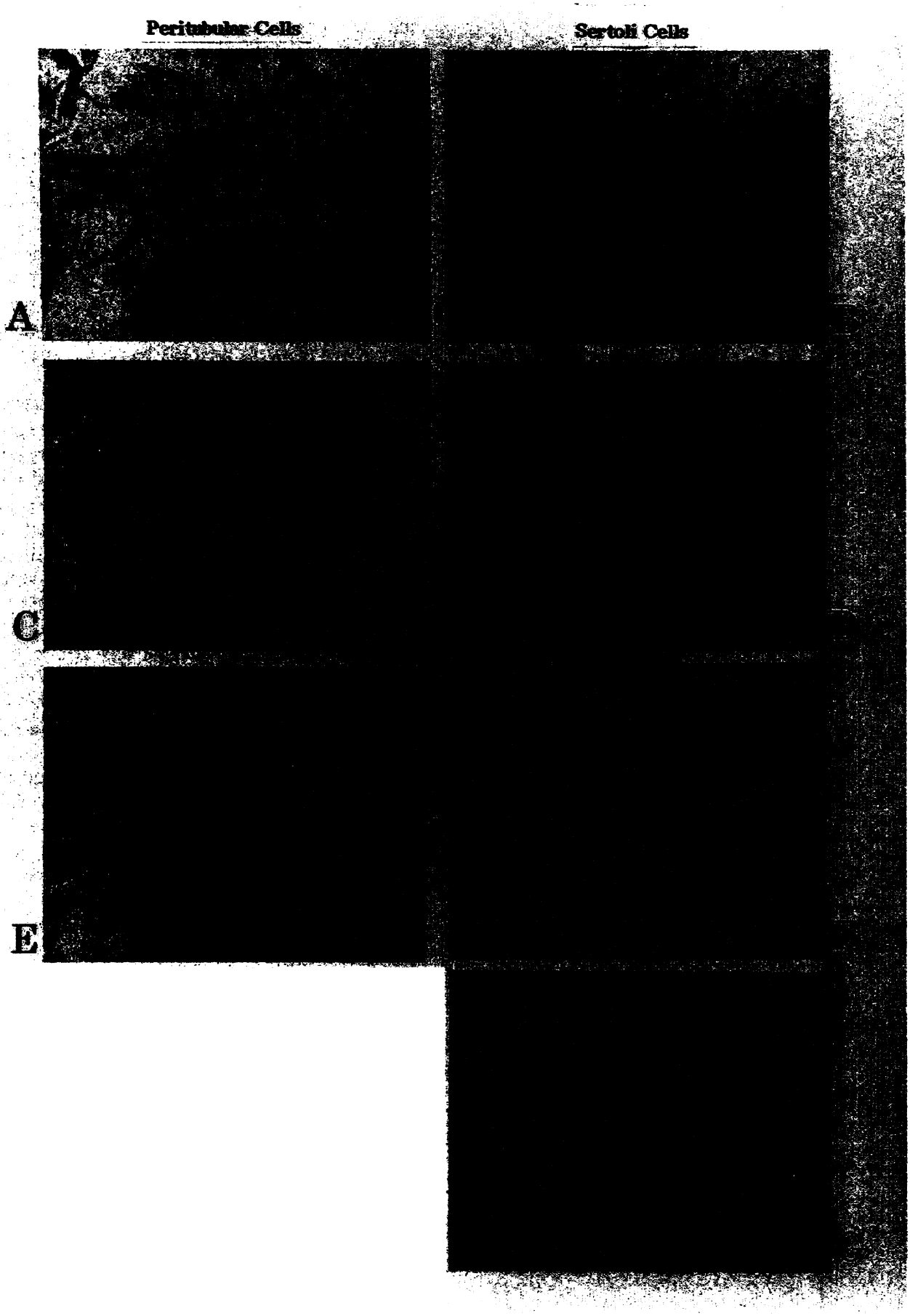
Peritubular Cells
Sertoli Cells
A
C
E

FIG. 2. Histochemistry of cultured peritubular cells and Sertoli cells. *Peritubular cells (A, C, E)* and *Sertoli cells (B, D, F,* and *G)* were tested immunocytochemically for the presence of desmin *(A* and *B)* and alkaline phosphatase activity *(C* and *D)* and stained with hematoxalin and eosin *(E, F,* and *G).* Sertoli cells were either cultured in the absence of regulatory agents *(B, D, F)* or in the presence of a combination of regulatory agents (follicle-stimulating hormone, insulin, retinol, and testosterone) *(G)* for 5 days. The cells were processed for histochemistry as outlined in *Materials and Methods.* The photomicrographs of the histochemically stained cells are representative of at least ten different cell preparations. (×170.)

which corresponds to the location of peritubular cells in situ. No reaction product was visualized when the anti-desmin antibody was omitted as a control (Fig. 3). Contamination of the peritubular cell preparation by endothelial cells and macrophages was also examined. Surface receptors for LDLs are used as a reliable marker for these cells (Goldstein et al., 1979; Pitas et al., 1981). A rhodamine-conjugated LDL probe identified receptors on the surface of bovine and rat endothelial cells as a positive control, but not on cells from the testicular peritubular cell preparation (data not shown). Combined data imply that the cells in the peritubular cell preparation are not endothelial cells or macrophages and that the desmin protein can not be identified in Sertoli cells. The desmin-positive cells appear to be peritubular cells, which implies that this histochemical technique will be useful for identifying peritubular cells in culture.

The Sertoli cell and peritubular cell preparations were also evaluated for alkaline phosphatase activity Fig. 2C and 2D). Approximately 70% of the cells from the peritubular cell preparation were stained blue, whereas 1% of the cells from the Sertoli cell preparation exhibited significant alkaline phosphatase activity (Table 1). The data indicate that the alkaline phosphatase histochemical technique can identify peritubular cells in culture. Preliminary data indicate that desmin-stained cells also had staining for alkaline phosphatase activity (data not shown).

Studies were performed to determine if these histochemical techniques could be used to quantify peritubular cells in a mixed cell population. For these experiments, Sertoli cells were plated at a constant density, and the peritubular cell density varied. The number of peritubular cells plated was determined by using a coulter counter. After a 5-day culture period, the preparations were stained and the percentage of stained cells was determined. With the desmin stain, the percentage of stained cells increased as the percentage of peritubular cells present in the culture increased (Fig. 4). Since a maximum of 42% of the cells in the peritubular cell preparation stain for desmin, the values observed with the cell mixtures were correspondingly lower. Adjusted values did reflect the percentage of peritubular cells present in the culture. With the alkaline phosphatase stain, neither the percentage of cells staining nor the adjusted values could reflect the percentage of peritubular cells present (Fig. 5).

Biochemical Characterization

Creatine phosphokinase is a marker protein for contractile cells (Shainberg et al., 1971). The rationale for

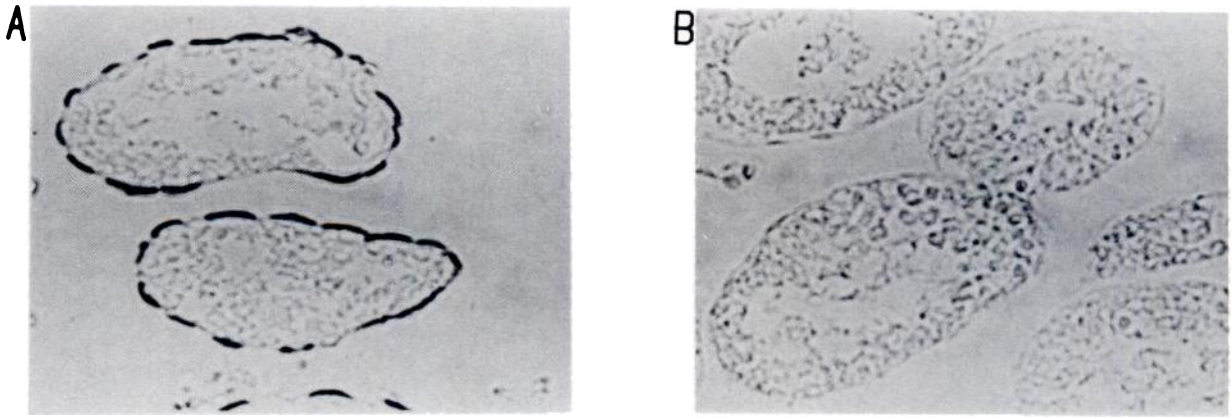

FIG. 3.Localization of desmin-staining cells in the seminiferous tubule. Isolated seminiferous tubules were fixed and tested for the presence of desmin as described in *Materials and Methods.* In *Panel A,* the tubules were incubated with the anti-desmin antibody; in *Panel B,* the primary antibody was omitted as a control. (×250.)

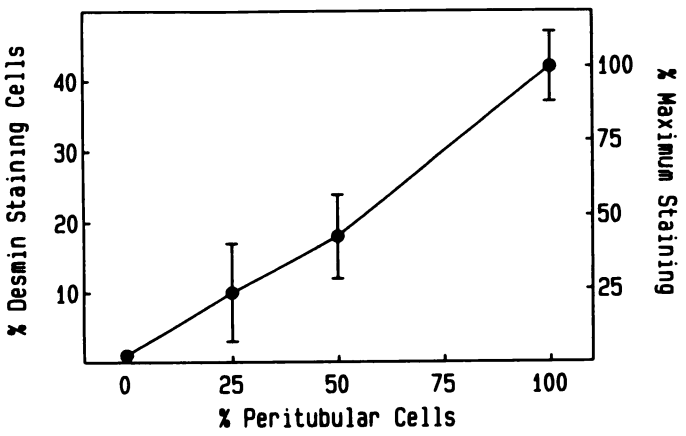

FIG. 4. Desmin staining in a co-culture of *peritubular cells* and *Sertoli cells*. Sertoli cells were plated at constant density whereas peritubular cells were plated at varying cell densities. After a 5-day culture period, the cells were stained and the percentage of positively stained cells are determined. The *left axis* illustrates the percentage of cells staining when 0, 25, 50, or 100% of the plated cells are peritubular cells. The *right axis* adjusts these values with 42% set as 100%, since this is the maximum staining percentage possible for desmin. The data are the mean ± SEM of cell counts from at least three different experiments.

its use is that a substrate for the enzyme, phosphocreatine, is a major energy source for muscle contraction. Peritubular cells are a myoid-like cell that may be involved in tubule contraction. A spectrophotometric assay for creatine phosphokinase is utilized to determine if the enzyme could be used as a marker for peritubular cells in culture. Significant levels of creatine phosphokinase were detected in cultured peritubular cells but not in cultured Sertoli cells (Table 2). The level of the enzyme increased as peritubular cell number increased (Fig. 6), but creatine phosphokinase levels were not altered when peritubular cells were treated with regulatory agents such as insulin, retinol, testosterone or dcAMP (data not shown). The data imply that cultured peritubular cells retain their differentiated state as myoid cells and suggest that creatine phosphokinase may be a useful biochemical marker for peritubular cells.

TABLE 2. Creatine phosphokinase activity in peritubular cells and Sertoli cells.*

Cell type	Absorbance 405 nm/μ DNA	% Peritubular cell activity
Peritubular cells†		
Primary culture	0.77 ± 0.01	100
Subculture	1.15 ± 0.17	149
Sertoli cells§	0.02 ± 0.01	3

*Data are mean ± SEM of triplicate wells from at least 2 experiments.

†Peritubular cells were plated with 10% newborn calf serum for 24 or 48 h and then maintained in 1% serum until Day 5 of culture.

§Sertoli cells were plated and then maintained without serum for the 5-day culture period.

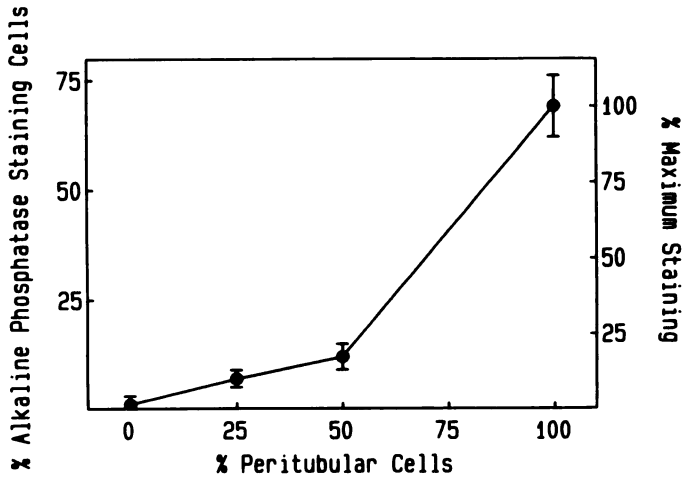

FIG. 5. Alkaline phosphatase staining in a co-culture of *peritubular cells* and *Sertoli cells*. Sertoli cells were plated at a constant density whereas peritubular cells were plated at varying cell densities. After a 5-day culture period, the cells were stained and the percentage of stained cells was determined. The *left axis* illustrates the percentage of cells staining when 0, 25, 50 or 100% of the cells plated are peritubular cells. The *right axis* adjusts these values with 70% set as 100%, since this is the maximum staining percentage possible for alkaline phosphatase. The data are the mean ± SEM of cell counts from at least three different experiments.

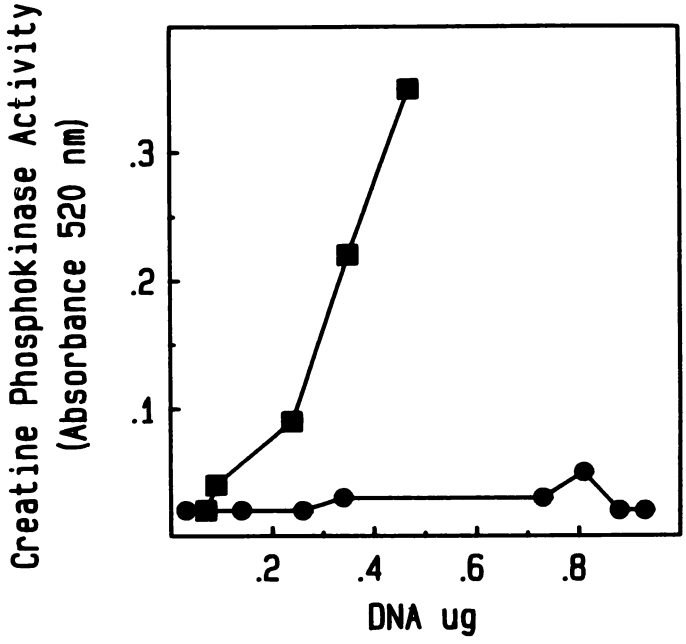

FIG. 6. *Creatine phosphokinase activity* with increasing numbers of peritubular cells and Sertoli cells. Untreated primary cultures of peritubular cells (■) or Sertoli cells (●) were cultured for 5 days. The cells were sonicated and aliquots were analyzed for DNA content and creatine phosphokinase activity. Data are the mean from triplicate wells of at least two experiments.

The possibility that alkaline phosphatase activity might be used as a biochemical marker of peritubular cell function was examined. Disrupted peritubular cells were incubated with the phosphatase substrate and the reaction product was measured spectrophotometrically. Preliminary studies indicated that component(s) present in serum interfered with the expression of alkaline phosphatase activity in peritubular cells. For this reason, peritubular cells were plated with 10% serum to promote cell attachment and growth, but after the initial 48 h of culture the cells were maintained in serum-free medium for the remainder of the culture period. The data presented in Figure 7 demonstrate that alkaline phosphatase activity increased as the number of assayed peritubular cells increased. Maximal activity occurred when an aliquot equivalent to 50 μg peritubular cell DNA was used. Very low levels of alkaline phosphatase activity were detected in Sertoli cells, and the levels showed no increase when cells were stimulated with regulatory agents. Alkaline phosphatase activity was routinely assayed in aliquots equivalent to 10–30 μg DNA.

A crude determination of the cellular localization of the alkaline phosphatase activity was made. Scraped cells were placed in phosphatase assay buffer then centrifuged at 13,000 × *g* to obtain a crude cytosol supernatant and cellular membrane pellet fraction. The supernatant and pellet were individually sonicated and assayed for the presence of alkaline phosphatase. The percentage of total activity present in the crude cellular membrane fraction was 77 ± 1% and 81 ± 1% for primary and subcultured peritubular cells, respectively. Therefore, the majority of the alkaline phosphatase activity was present in the crude cellular membrane fraction.

The possibility that alkaline phosphatase activity may change with culture duration was evaluated. Alkaline phosphatase activity was quantified in peritubular cells at several different time points throughout a 10-day culture period. Alkaline phosphatase activity was relatively stable throughout the culture period for untreated primary cultures of peritubular cells. Continuous treatment of cells with dcAMP, starting at the time of plating, stimulated alkaline phosphatase activity on Days 5–7 of culture, which subsequently declined by Day 10 (Fig. 8). In untreated subcultured peritubular cells, the alkaline phosphatase activity was relatively stable from Day 2 to Day 7 of culture, with higher levels on Day 10. Continuous treatment of subcultured peritubular cells with dcAMP stimulated alkaline phosphatase activity on Days 5 through 7 of culture (Fig. 9). In subsequent experiments, the cultures were terminated on Day 5 or Day 7 of culture (Fig. 9). The values

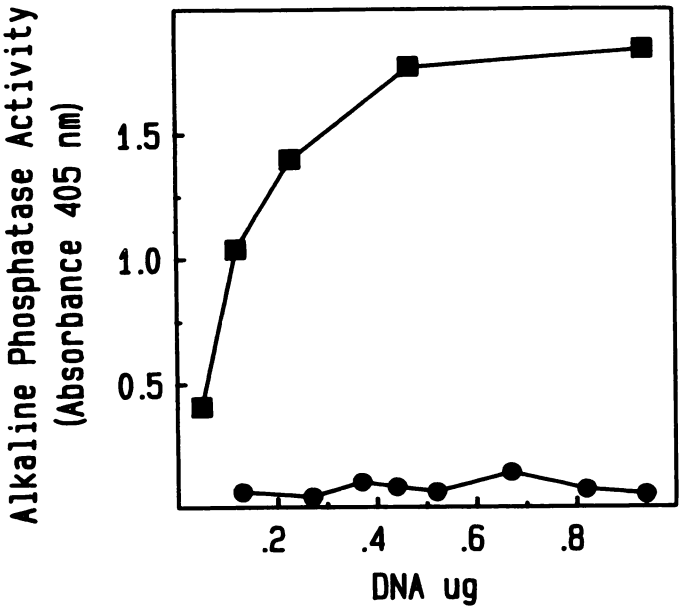

FIG. 7. *Alkaline phosphatase activity* with increasing numbers of peritubular cells and Sertoli cells. Primary cultures of peritubular cells (■) or Sertoli cells (●) were cultured for 5 days. Cells were sonicated and aliquots were analyzed for DNA content and alkaline phosphatase activity. Data presented is the mean of duplicate wells from a minimum of three different experiments.

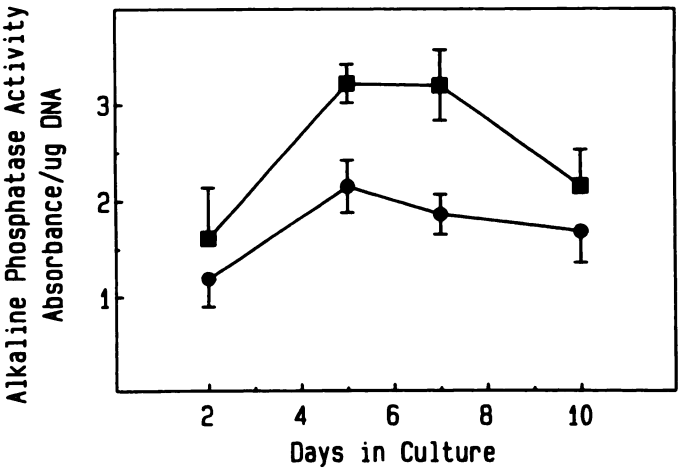

FIG. 8. Effect of culture duration on *alkaline phosphatase activity* in primary cultures of peritubular cells. At the indicated *time points*, the cultures were terminated, and the cells were sonicated and assayed for DNA content and alkaline phosphatase activity. Data, expressed as *Absorbance 405 nm/μg DNA*, are from control (●) and dibutyryl cyclic adenosine 3′,5′-monophosphate (■) treated cultures and are the mean ± SEM of duplicate wells from three different experiments.

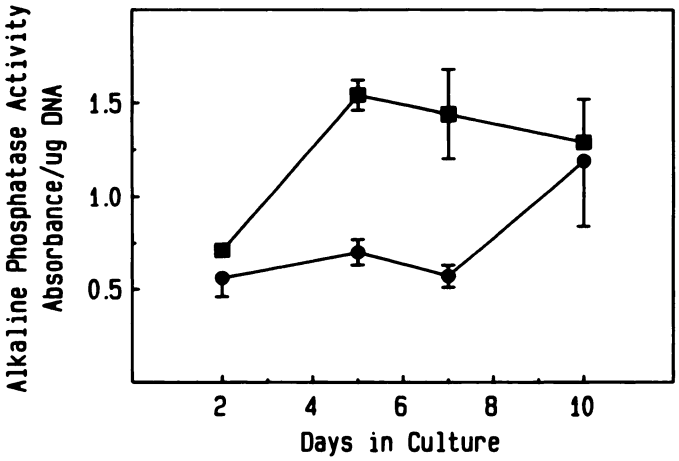

FIG. 9. Effect of culture duration on *alkaline phosphatase activity* in subcultured peritubular cells. At the indicated *time point*, the cultures were terminated, and the cells were sonicated and assayed for DNA content and alkaline phosphatase activity. Data, expressed as *Absorbance 405 nm/μg DNA*, are from control (●) and dibutyryl cyclic adenosine 3′,5′-monophosphate (■) treated cultures and are the mean ± SEM of duplicate wells from three different experiments.

obtained for subcultured peritubular cells were 30–50% lower than those obtained with primary cultures of peritubular cells. The intensity of the histochemical stain for alkaline phosphatase was also lower when subcultured peritubular cells were compared with primary cultures of peritubular cells. This observation implies that the reduced levels of alkaline phosphatase activity noted in subcultured peritubular cells was the result of a decrease in the specific activity of the enzyme and not a change in the composition of the peritubular cell preparation with subculture.

The response of peritubular cells to treatment with a variety of regulatory agents was tested by the alkaline phosphatase assay. The cells were treated for 5 or 7 days and data are expressed as fold increase over control. Testosterone treatment increased alkaline phosphatase activity in primary cultures of peritubular cells (Fig. 10). This stimulation was modest (30% over control) but reproducible and statistically significant. No increase in alkaline phosphatase activity as noted when subcultured peritubular cells were treated with testosterone (Fig. 11). Treatment with retinol stimulated alkaline phosphatase activity in subcultured peritubular cells but had no effect on the alkaline phosphatase activity associated with primary cultures of peritubular cells. Insulin did not increase alkaline phosphatase activity for either primary or subcultured cells and no synergism among regulatory agents (insulin, retinol, and testosterone) was demonstrated (data not shown). Treatment of cells with dcAMP resulted in the highest stimulation of alkaline phosphatase activity in both primary and subcultured peritubular cells. No alkaline phosphatase activity was detected in the stocks of regulatory agents tested, in unconditioned medium, or in

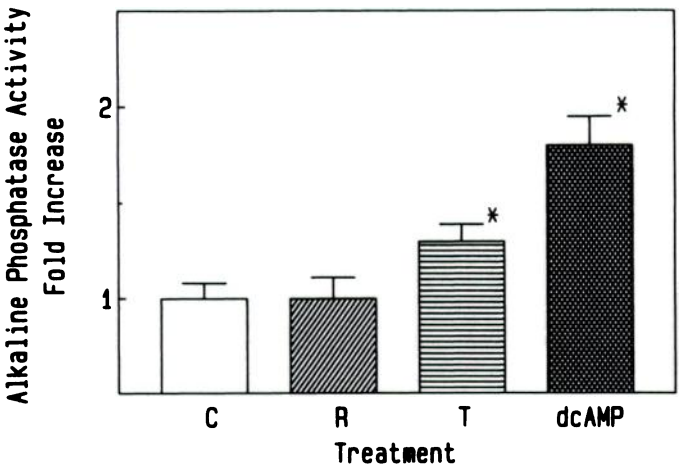

FIG. 10. Effect of regulatory agents on *alkaline phosphatase activity* in primary cultures of peritubular cells. Peritubular cells were plated and cultured for 5 or 7 days in the absence (*C*) or presence of regulatory agents: *R*, retinol; *T*, testosterone; and *dcAMP*, dibutyryl cyclic adenosine 3′,5′-monophosphate. Data are presented as fold increase over control values and are the mean ± SEM of duplicate wells from three different experiments. *Marked* treatments (*) are significantly different from control values, with $p<0.05$ using Student's *t*-test.

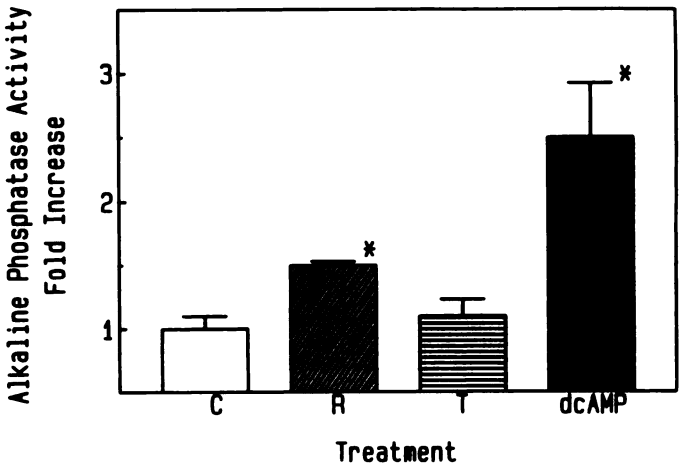

FIG. 11. Effect of regulatory agents on *alkaline phosphatase activity* in subcultured peritubular cells. Peritubular cells were subcultured and treated for 5 or 7 days in the absence (*C*) or presence of regulatory agent: *R*, retinol; *T*, testosterone; and *dcAMP*, dibutyryl cyclic adenosine 3′,5′-monophosphate. Data are presented as fold increase over control values and are the mean ± SEM of duplicate wells from three experiments. *Marked treatments* (*) are significantly different from control values, with $p<0.05$ using Student's *t*-test.

Sertoli cell- and peritubular cell-concentrated conditioned medium.

DISCUSSION

Fluorographs of radiolabeled secreted proteins provide qualitative information about cell function. Proteins secreted by subcultured and primary cultures of peritubular cells were similar, with only minor differences in the abundance of specific proteins. These observations suggest that there is no major alteration in cellular functions when peritubular cells are subcultured. The only peritubular cell-secreted proteins functionally identified are fibronectin (Tung et al., 1984), proteoglycans (Skinner and Fritz, 1985c), a plasminogen-activator inhibitor (Hettle et al., 1988), and the paracrine factor, P-Mod-S (Skinner et al., 1988). Further identification of secreted proteins will help elucidate specific functions of the peritubular cell. Sertoli cell-secreted proteins were found to be different from proteins secreted by peritubular cells. This analysis indicated that Sertoli cell contamination was nondetectable in peritubular cell preparations.

Histochemical techniques were established to identify peritubular cells cytochemically. A variety of cytoskeletal proteins were tested, and only desmin was found to be specific for peritubular cells. Desmin has previously been shown to be a marker for testicular peritubular cells (Virtanen et al., 1986). The percentage of cells in a peritubular cell preparation that stains for desmin is approximately 40% of the total cell number. The nonreactive cells in the peritubular cell preparation may be undifferentiated peritubular cells, nondifferentiated fibroblasts, or endothelial cells. Contamination of the peritubular cell preparation by endothelial cells was ruled out in these studies by determining the absence of detectable levels of LDL receptor. It is speculated that the unstained cells in the peritubular cell preparations may be undifferentiated peritubular cells. This hypothesis is based on the high percentage of cells in the peritubular cell preparations that displayed alkaline phosphatase activity. The alkaline phosphatase stain is an additional cytochemical marker for peritubular cells, as previously determined (Chapin et al., 1987). Approximately 70% of the population stained for alkaline phosphatase. Since desmin and alkaline phosphatase appear to counterstain the same cells, the majority of cells present appeared to be peritubular cells at different stages of differentiation.

Both histochemical procedures were in agreement that the percentage of staining cells in Sertoli cell preparations was low (1–2%), and the characteristic morphology of the stained cells suggests that they were peritubular cells. The advantage of these histochemical techniques is that they can provide a rapid and objective method for identifying peritubular cells in a Sertoli cell preparation. The quantification of peritubular cells in a Sertoli cell preparation was not equally dependable with these histochemical techniques. The alkaline phosphatase histochemical procedure stained a greater percentage of the cells in the peritubular cell preparation, but the number of cells stained in a mixed cell population did not accurately reflect the number of peritubular cells present. When similar analyses were performed using immunocytochemistry, the desmin stain provided a better estimate of peritubular cell contamination in Sertoli cell preparations. The creatine phosphokinase enzyme appears to be an additional useful marker for detecting peritubular cells. Further research is required to determine if the creatine phosphokinase assay will be a useful biochemical method for quantifying peritubular cells. Combined observations indicate that both desmin and alkaline phosphatase provide useful histochemical markers for the investigation of peritubular cell function and differentiation. The desmin stain may also be useful to identify and quantify peritubular cells in different testis cell preparation.

The presence of the alkaline phosphatase activity in peritubular cells was utilized to develop a biochemical assay to monitor peritubular cell function and regulation. Previously, the only method available to evaluate peritubular cell function was a bioassay for P-Mod-S activity (Skinner and Fritz, 1986). The current study demonstrates that alkaline phosphatase activity can be readily quantified in cultured peritubular cells, but negligible activity was associated with cultured Sertoli cells. The only agent previously known to regulate peritubular cell function and differentiation was testosterone (Bressler and Ross, 1972; Skinner and Fritz, 1985a). Androgen treatment stimulated alkaline phosphatase activity in primary cultures of peritubular cells. The stimulatory effect of testosterone decreased with duration of primary culture and was nondetectable in subcultures of peritubular cells. This decrease may reflect a decline in androgen receptor level, which is a phenomenon that has been documented for other steroid-responsive cells. The ability of androgens to stimulate alkaline phosphatase activity in peritubular cells

provides direct evidence for the previous proposal that androgens regulate peritubular cell differentiation and function (Bressler and Ross, 1972; Skinner and Fritz, 1985a). This observation supports the previous hypothesis that androgen effects on testicular function are in part mediated through actions on peritubular cells.

Retinoids have previously been shown to be required for the maintenance of spermatogenesis (Wohlback and Howe, 1925; Thompson et al., 1964) and retinol is known to directly influence Sertoli cell function (Karl and Griswold, 1980; Skinner and Griswold, 1982; Hugly and Griswold, 1987). A significant increase in alkaline phosphatase activity was observed in subcultured peritubular cells treated with retinol, but primary cultures of peritubular cells did not respond to the retinol treatment. It is speculated that the lower levels of alkaline phosphatase activity obtained in subcultured peritubular cells may represent a decline in the differentiated state of the peritubular cell and that retinol helps maintain peritubular cell differentiation. The effect of retinoids on the differentiation of a number of different cell types has previously been described. The present study is the first to indicate that retinol may directly influence peritubular cells function and differentiation. This observation implies that the effects of retinoids on spermatogenesis and testicular function may be mediated in part through actions on peritubular cells.

The highest level of stimulation in alkaline phosphatase activity was obtained when peritubular cells were treated with dcAMP. The pharmacologic effect of dcAMP implies that factors other than testosterone and retinol may be involved in peritubular cell regulation. Insulin treatment had no effect on the alkaline phosphatase activity in peritubular cells and no synergism among regulatory agents was demonstrated. Combined observations provide a better understanding of the endocrine regulation of peritubular cell function and expand the current information available on the mode of androgen and retinoid actions on testicular function.

Mesenchymal-epithelial cell interactions are postulated to be important for a variety of cellular function. It has been speculated that during embryogenesis mesenchymal cells produce inducer substances which may direct the differentiation of the adjacent epithelium (review, Cunha et al., 1983). The interaction between peritubular cells and Sertoli cells is an example of a mesenchymal-epithelial cell interaction. The production by peritubular cells of a paracrine factor, P-Mod-S, which modulates Sertoli cell function and differentiation, provides biochemical evidence for the hypothesis involving a mesenchymal inducer substance. The current study extends this observation and indicates that the mesenchymal cell (peritubular cells) may participate in the endocrine regulation of a tissue. Data indicate that testosterone and retinoids can act on peritubular cells, which may indirectly through a paracrine-mediated interaction influence Sertoli cell function. Further investigation into peritubular cell function and hormonal regulation is required to develop a better understanding of this important cell type in the testis, as well as provide insight into general mesenchymal-epithelial cell interactions. It is speculated that the endocrine regulation of tissue function and development may be mediated in part through a modulation of mesenchymal-epithelial cell interactions.

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