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Identification of a non-mitogenic paracrine factor involved in mesenchymal-epithelial cell interactions between testicular peritubular cells and Sertoli cells

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Summary

Seminiferous peritubular cells have previously been shown to secrete a protein termed P-Mod-S which modulates the functions of Sertoli cells. The present study provides an initial characterization of P-Mod-S and examines the actions of P-Mod-S on Sertoli cells. Gel filtration chromatography demonstrates that P-Mod-S has an apparent molecular weight of 70 000 that could not be dissociated to a lower molecular weight form. A 40- to 90-fold purification of P-Mod-S was obtained with a predicted half maximal effective concentration for Sertoli cells of less than 10^{-9} M. Through an analysis of the actions of P-Mod-S on Sertoli cells it is demonstrated that P-Mod-S stimulates the Sertoli cell to a greater extent than any single hormone or vitamin known to influence the cell. P-Mod-S maximally stimulates testicular transferrin and androgen-binding protein production by Sertoli cells, but does not stimulate levels of plasminogen activator activity. P-Mod-S also appears to induce the synthesis of several proteins that are not detected in control non-treated Sertoli cell cultures. One such protein whose synthesis was stimulated by P-Mod-S treatment of Sertoli cells was a component having a molecular mass of 20 kDa. This 20 kDa Sertoli cell-secreted protein was specifically immunoprecipitated with an antibody against an epididymal lactalbumin-like protein. This implies that P-Mod-S can induce Sertoli cells to synthesize and secrete a lactalbumin-like protein. P-Mod-S was found not to contain mitogenic activity. Data presented indicate that testicular peritubular cells synthesize and secrete a 70 kDa non-mitogenic paracrine factor termed P-Mod-S which has a dramatic influence on Sertoli cell functions. Results are discussed with respect to modulation of epithelial (Sertoli) cell functions by components produced by mesenchymal (peritubular) cells.

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Abbreviations: P-Mod-S, protein produced by peritubular cells which modulates Sertoli cell functions; MEM, Eagle's modified minimal essential medium; NRK, rat kidney fibroblast cell line; IGF, insulin-like growth factor; EGF, epidermal growth factor; ABP, androgen-binding protein; PA, plasminogen activator; FIRT, follicle-stimulating hormone, insulin, retinol and testosterone.

Most organs have a functioning epithelial cell which is in close proximity or contact with a mesenchymal (i.e. stromal) cell. When placed in contact with each other, mesenchymal cells from one organ can direct the apparent embryonic morphogenesis and differentiation of epithelial cells from another organ (review, Cunha et al., 1983). From these observations investigators have postulated that mesenchymal cells produce inducer substances which influence the functions of adjacent epithelial cells (Grobstein, 1967; Kratochwil, 1969). The types of interactions that are possible between mesenchymal cells and epithelial cells are mediated by cell surface (e.g. extracellular matrix) components or through the secretion of soluble substances. In the present study, cellular interactions in the testis have been examined to investigate mesenchymal-epithelial cell interactions.

Within the seminiferous tubules, where spermatogenesis occurs, the 2 types of somatic cells are the epithelial-like Sertoli cell and the mesenchymal/stromal-like peritubular (myoid) cell. The Sertoli cell helps form the seminiferous tubule and provides the cytoarchitecture and microenvironment required for spermatogenesis. Peritubular cells surround the tubule on the basal surface of the Sertoli cells and between the 2 cell types is a basement membrane. The structural relationship between peritubular cells and Sertoli cells provides an example of mesenchymal-epithelial cell associations. Primary cultures of relatively homogeneous populations of Sertoli cells and peritubular cells can readily be prepared and maintained under defined conditions (Tung and Fritz, 1980; Tung et al., 1984a), thereby providing a useful model system for the investigation of some aspects of mesenchymal-epithelial cell interactions.

We have previously demonstrated that Sertoli cells and peritubular cells synthesize different ECM components (Tung et al., 1984b; Skinner et al., 1985a). Results imply that Sertoli cells and peritubular cells cooperate in the production and formation of the seminiferous tubule basal lamina (Tung and Fritz, 1984; Tung et al., 1984b; Skinner et al., 1985a). Expression of a given differentiated state for many cell types is dependent on the presence of an ECM (review, Hay, 1981). An additional cellular interaction which may have a regulatory role between 2 different cell types is that mediated by the secretion of soluble substances. Components involved in this type of interaction have been termed paracrine factors. Peritubular cell-Sertoli cell interactions were examined to determine whether paracrine factors are involved in mesenchymal-epithelial cell interactions.

Modulation of Sertoli cell functions has been assessed through analysis of the production of specific secreted proteins such as testicular transferrin (Skinner and Griswold, 1980), androgen-binding protein (ABP) (Fritz et al., 1976) and plasminogen activator (Lacroix et al., 1977). Previous observations have indicated that the presence of peritubular cells in coculture with Sertoli cells results in an increased production of ABP by Sertoli cells (Tung and Fritz, 1980; Hutson and Stocco, 1981). In a preliminary study we demonstrated that cultured peritubular cells secrete into the medium a protein(s) between 50 and 100 kDa which can stimulate the production of testicular transferrin and ABP by Sertoli cells (Skinner and Fritz, 1985a). This protein was termed P-Mod-S to indicate a protein(s) secreted by peritubular cells (P) which modulates (Mod) Sertoli cell (S) functions. P-Mod-S appears to be a paracrine factor involved in mesenchymal-epithelial cell interactions in the seminiferous tubule and provides evidence for the mesenchymal inducer substance previously postulated.

Analysis of embryonic development of mesenchymal and epithelial tissues has indicated that in steroid-responsive organs the mesenchymal cell may be the primary site of steroid action (Cunha et al., 1983). Normal spermatogenesis requires the presence of androgens and the seminiferous tubule is a target tissue for androgens (Fritz, 1978). Sertoli cell-enriched preparations respond to testosterone by increasing the production of androgen-binding protein (ABP) (Louis and Fritz, 1979). Recently, the procedure to isolate Sertoli cells has been improved and Sertoli cell preparations of less than 0.3% peritubular cell contamination can be obtained (Tung et al., 1984a). Testosterone treatment of these purified Sertoli cell preparations did result in a slight increase in ABP production (Skinner and Fritz, 1985b). However, the presence of peritubular cells in a coculture, 50% Sertoli and 50% peritubular cells, significantly augmented the testosterone response to a maximum (Skinner and

Fritz, 1985b). This study indicated that the presence of peritubular cells can enhance the apparent actions of testosterone on Sertoli cells. Previously it was found that peritubular cells contain a high percentage of the androgen receptors in the seminiferous tubule (Sar et al., 1975; Verhoeven, 1980). The presence of testosterone in primary cultures of peritubular cells was found to increase the production of P-Mod-S (Skinner and Fritz, 1985a). This combined information implies that androgens can act on peritubular cells to increase the production of P-Mod-S which can then act on Sertoli cells to modulate specific functions. Therefore, the development of a better understanding of peritubular cell-Sertoli cell interactions will provide insight into the actions of hormones on the seminiferous tubules.

Because the structural relationship between mesenchymal cells and epithelial cells is present in most organs, it is postulated that cellular interactions mediated by paracrine factors such as P-Mod-S may be a general biological phenomenon. This type of cellular interaction may play an integral role in the development, regulation and maintenance of the functions of many different organs. Several initial questions which arise in analyzing mesenchymal-epithelial cell interactions are (1) is more than one protein involved in the effects observed, (2) does the paracrine factor(s) contain mitogenic activity and is a known growth factor, and (3) what are the overall effects of the paracrine factor on the target cell, such as the induction of new functions. These questions are addressed in the present study through a preliminary characterization of P-Mod-S and a detailed analysis of the actions of P-Mod-S on Sertoli cells.

Materials and methods

Cell culture

Sertoli cells were isolated from the testis of 20-day-old Wistar rats by sequential enzymatic digestion (Dorrington et al., 1975) with a modified procedure previously described (Tung et al., 1984a; Skinner and Fritz, 1985a). Decapsulated testis fragments were digested first with trypsin (2.5 mg/ml) (Gibco), to remove Leydig cells, followed by a collagenase digestion (1 mg/ml Type I, Sigma) and then a hyaluronidase digestion (1 mg/ml, Sigma). Sertoli cells were then plated in 24-well (1 ml per well) Linbro plates at approximately 5×10^5 cells per well, corresponding to approximately 1.3 μg of DNA/cm^2. Cells were maintained at 32°C in a 5% CO_2 atmosphere in Eagle's minimal essential medium (MEM) (Gibco) supplemented as described (Tung et al., 1975). The degree of contamination of these Sertoli cell preparations by peritubular cells was $\leq 0.3\%$ (Tung et al., 1984a). Sertoli cultures were treated as described in the Results section 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 Sertoli cell culture was obtained for analysis and the cells harvested for a DNA assay. Sertoli cell cultures were treated as outlined in the Results section with FSH (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM) and testosterone (1 μM).

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (Skinner and Fritz, 1985a). Cells were plated in 150 mm-diameter tissue culture plates containing 25 ml of 10% calf serum in MEM and cultured for 5 days. Cells were then removed by brief treatment with trypsin (1.7 mg/ml), washed, and replated at 1/4 density in 150 mm dishes. These subcultured cells grew to confluence in 4 days in MEM containing 10% calf serum. Cells were then washed with serum-free medium, incubated 6 h in fresh serum-free medium, and washed again. These peritubular cells were then maintained in serum-free medium and conditioned medium collections were made at 48–72 h intervals for periods up to 2 weeks. Sertoli contaminants in subcultured peritubular cell cultures were not detectable as determined morphologically or biochemically by the absence of Sertoli cell-secreted proteins, testicular transferrin or androgen-binding protein, in the peritubular cell-conditioned medium.

Preparation of peritubular cell-conditioned medium

Peritubular cell serum-free conditioned medium was centrifuged ($10\,000 \times g$ for 30 min at 4°C) upon collection and stored at −20°C. Conditioned medium was concentrated and fractionated

with an Amicon ultrafiltration system. Ultrafiltration membrane types YM-2, YM-10, XM-50, XM-100 and XM-300 with molecular weight exclusion limits of 2000, 10 000, 50 000, 100 000 and 300 000 respectively, were used as indicated in the Results section to concentrate the medium approximately 200-fold. The concentrated samples were washed with 10 mM Tris-Cl, pH 7.5, and centrifuged at $15\,000 \times g$ for 30 min at 4°C, and the supernatants were stored at −20°C.

Gel filtration chromatography

Peritubular cell-secreted proteins were applied to a Sephacryl S-200 gel filtration column (0.7 × 100 cm) and eluted with 50 mM NaCl, 25 mM Tris-Cl, pH 7.5, at 4°C and collected in 1.2 ml fractions. Fractions were centrifuged at $13\,000 \times g$ for 30 min at 4°C and the supernatant stored at −20°C. Standardization of the gel filtration column for molecular weights was accomplished with the standards immunoglobulin (150 kDa), bovine serum albumin (68 kDa), ovalbumin (42 kDa) and chymotrypsinogen (25 kDa).

Amounts of [^{35}S]methionine incorporated into total radiolabeled proteins secreted by Sertoli cells were determined in cells which had been maintained for 4 days in Linbro wells (1 ml) in serum-free MEM in the absence or presence of hormones or peritubular cell-conditioned medium. On day 5 of culture, the medium was changed to 50% methionine-reduced MEM containing [^{35}S] methionine (10 μCi/ml). After a 24 h period of culture, the medium was collected and guanidine hydrochloride was added to a final concentration of 4 M. Samples were applied to a P-6 (BioRad) gel filtration column (1 × 10 cm) and eluted with 4 M guanidine hydrochloride. The total amount of radioactivity in the void volume, containing the radiolabeled proteins, was determined and expressed as dpm per μg of Sertoli cell DNA.

Quantitative assays for Sertoli products

Transferrin was analyzed with a radioimmunoassay utilizing a rabbit anti-rat transferrin antibody (Cappel Laboratories) and a goat anti-rabbit immunoglobulin (Sigma) second antibody precipitation with polyethylene glycol as previously described (Skinner and Griswold, 1982). ABP levels were determined with a radioimmunoassay using the antibody and standard supplied by the National Hormone and Pituitary Program (National Institute of Child Health and Human Development, Bethesda, MD). Our ABP assay consisted of 2 incubations for 24 h at 4°C using a double antibody precipitation with polyethylene glycol as previously described (Skinner and Fritz, 1985a, b). Plasminogen activator was analyzed with a plasminogen-dependent ^{125}I-labeled fibrin degradation assay (Lacroix et al., 1977). The DNA content of culture wells was determined by the procedure of Karsten and Wollenberger (1977) as previously described (Lacroix et al., 1977). All data for Sertoli cells were normalized per μg of cell DNA. Since the amount of DNA per confluent peritubular cell culture varied a maximum of 10%, the data were normalized per peritubular cell plate unless otherwise specified. Amount of protein was determined with the Hartree procedure (1972).

Quantitative assays for growth factors and mitogenic activity

To determine the presence of mitogenic activity, a rat kidney fibroblast cell line (NRK) was utilized. NRK cells were plated at 10% confluence in a 24-well Linbro plate in 1 ml/well of MEM containing 1% calf serum at 37°C in a 5% CO_2 atmosphere. Cells were maintained for 24 h and then the medium was replenished with MEM containing 0.1% calf serum. After an additional 24 h of culture the medium was replenished with 0.5 ml of MEM containing 0.1% calf serum and cultured for another 24 h at which time samples of interest were used to treat the cells. After 24 h of treatment the cells were radiolabeled with 1 μCi of [^{3}H]thymidine for 2 h. The medium was then removed and the cells washed twice with fresh medium. Cells were then incubated for 15 min in 0.5 ml of 0.25% trypsin followed by sonication for 10 s at high power with a microprobe (Branson Sonicator, Fisher). Aliquots were then applied to DE81 filters (Whatman) and rinsed with 50 mM phosphate, 150 mM NaCl pH 7.0 in a Gilson multifiltration apparatus. Filters were then counted in ACS (Amersham) scintillation fluid on a liquid scintillation counter. Other aliquots of the cells were used for a DNA determination with a previously described procedure (Karsten and Wollen-

berger, 1977). As a positive control 10 ng of epidermal growth factor (EGF) was added to treat the cells which generally resulted in a 5-fold increase in dpm [^{3}H]thymidine/μg DNA over control non-treated cells.

The presence of EGF in peritubular cell-conditioned medium was analyzed with an [^{125}I]EGF receptor displacement assay using NRK cells as previously described (Kudlow and Colbrin, 1984). This assay was graciously done by Dr. Jeffrey Kudlow, University of Toronto, who has established and validated the assay in his laboratory. The presence of insulin-like growth factor (IGF) was determined with an IGF radioimmunoassay as previously described (Armstrong et al., 1983), which did not differentiate between IGF I and IGF II. This assay was graciously performed by Dr. Morely Hollenberg, University of Calgary, who has established and validated the assay in his laboratory.

Electrophoresis, fluorography and immunoprecipitation

Sertoli cells were radiolabeled for 24 h on day 5 of culture in methionine-free MEM containing 5 μCi/ml [^{35}S]methionine. The medium was collected, centrifuged, and used for analysis. Radiolabeled proteins were electrophoretically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (1970) buffer system. The gels were fluorographed with diphenyloxazole in acetic acid as previously described (Skinner and Griswold, 1983a).

Immunoprecipitation of Sertoli cell-secreted radiolabeled proteins was accomplished with an antiserum to an epididymal lactalbumin-like protein (Klinefelter and Hamilton, 1984) generously provided by Dr. David Hamilton (Dept. Anatomy, University of Minnesota, Minneapolis). A previously described double antibody immunoprecipitation technique was utilized (Skinner and Griswold, 1983b).

Results

Initial characterization of P-Mod-S

The influence of peritubular cell-conditioned medium on transferrin production by Sertoli cells was utilized to detect the presence of P-Mod-S. Conditioned medium from cultured peritubular cells was obtained from a 72 h collection period on day 3 or day 15 of serum-free cell culture and subsequently used to treat Sertoli cells. As the percentage (v/v) of conditioned medium added to the Sertoli cell cultures increased the amount of transferrin produced by Sertoli cells increased (Fig. 1). Similar stimulation was observed for both collection periods with a slight decrease in medium collected on day 15. These results confirm previous observations with medium collected on day 3 of peritubular cell culture (Skinner and Fritz, 1985a) and indicate that the stimulatory activity detected is present for up to 2 weeks in serum-free cell culture of peritubular cells.

Conditioned medium was collected from subcultured peritubular cells for up to 2 weeks of culture with collections every 48–72 h. Medium

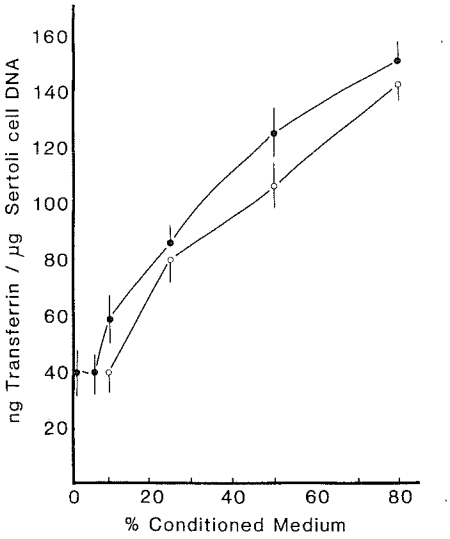

Fig. 1. Influence of peritubular cell-conditioned medium on transferrin production by Sertoli cells. Sertoli cells were cultured in serum-free medium in the presence of varying amounts of serum-free conditioned medium from peritubular cell cultures. Conditioned medium was added at the time of plating and replenished after 48 h when the medium was changed. A 72 h medium collection on day 5 of Sertoli cell culture was analyzed for levels of transferrin and expressed as ng transferrin/μg Sertoli cell DNA. Peritubular cell-conditioned medium from a 72 h medium collection on day 3 (●) or day 15 (○) of serum-free cell culture was used to treat Sertoli cells. Points represent the mean $\pm$SD ($n = 9$) for 3 separate experiments performed in triplicate.

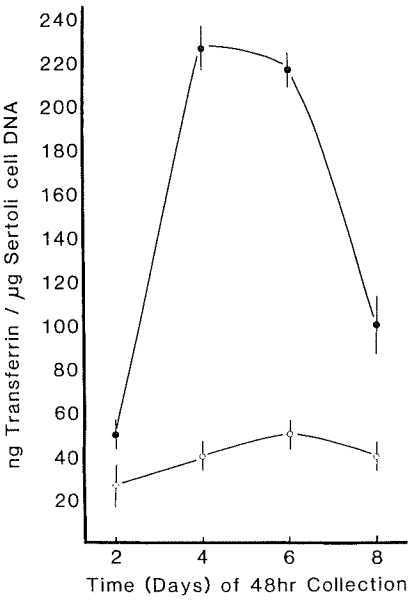

Fig. 2. Time course of the influence of P-Mod-S on transferrin production by Sertoli cells. Sertoli cells were cultured in the absence (○) or presence (●) of a P-Mod-S preparation, 1.5 μg/ml of a 200-fold concentrated 10–300 kDa peritubular cell-conditioned medium fraction, at the time of plating and replenished every 48 h when the medium was changed. Medium collected from Sertoli cell cultures was analyzed for transferrin levels expressed as ng transferrin/μg Sertoli cell DNA. Points represent the mean ± SD ($n = 9$) for 3 separate experiments performed in triplicate.

was concentrated 200-fold and fractionated into a greater than 10 000 and less than 300 000 molecular weight fraction by ultrafiltration. Small aliquots of this concentrated medium, 1.5 μg protein, were used to treat Sertoli cell cultures. Sertoli cells were treated at the time of plating and retreated every 48 h when the medium was collected and replenished. The amount of transferrin present in the Sertoli cell medium was determined for both treated and non-treated control cultures (Fig. 2). Control cultures had a negligible change in transferrin production with time in culture. Treatment with concentrated peritubular cell medium resulted in a small increase in transferrin production on day 2 of culture followed by a large increase reaching a maximum between days 4 and 6 of culture. This stimulation of transferrin production started to decline on day 8 of Sertoli cell culture. From these results it was determined that a 72 h medium collection between days 2 and 5 of Sertoli cell culture would be useful for the routine analysis of the effects of P-Mod-S on Sertoli cells.

Further characterization of P-Mod-S was accomplished with gel filtration chromatography on a Sepharose S-200 column (Fig. 3). The sample applied to the column was 1 ml of a concentrated 10–300 kDa fraction of peritubular cell-conditioned medium. An aliquot of each fraction collected was used to treat cultured Sertoli cells. The ability of the fractions to stimulate transferrin production by Sertoli cells demonstrated that P-Mod-S has an approximate molecular weight of 70 000 (Fig. 3). Elution buffers such as 1 M acetic acid, pH 4.5, or 2 M ammonium acetate, pH 4.5, did not dissociate or reduce the apparent molecular weight of P-Mod-S (data not shown). Previously it was reported that a 50–100 kDa fraction of peritubular cell medium stimulates transferrin and ABP production by Sertoli cells (Skinner and Fritz, 1985a). To determine whether the 70 kDa protein stimulates the production of both proteins, the ability of the fractions to stimulate ABP pro-

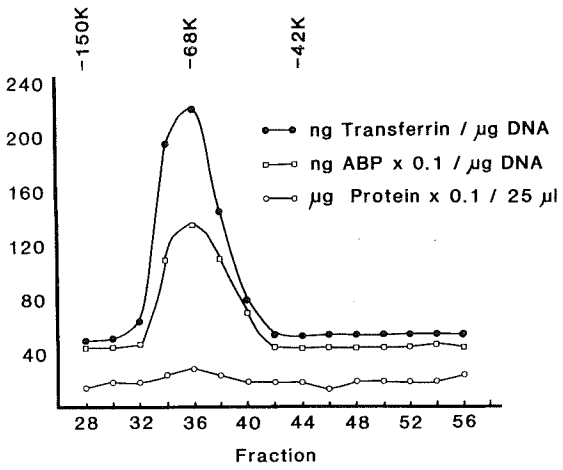

Fig. 3. Gel filtration chromatography of P-Mod-S. A Sephacryl S-200 gel filtration column (0.7 × 110 cm) was equilibrated and eluted with 50 mM NaCl, 25 mm Tris-Cl, pH 7.5 at 4°C. The sample applied to the column was 1 ml of a 200-fold concentrated 10–300 kDa peritubular cell-conditioned medium fraction. Aliquots (25 μl) of each collected fraction (1.2 ml) were used to treat Sertoli cell cultures to determine their influence on transferrin (○) production (ng transferrin/μg Sertoli cell DNA) and ABP (□) production (ng ABP × 10^{-1}/μg Sertoli cell DNA). Another 25 μl aliquot was analyzed for protein content expressed as μg protein × 10^{-1}/25 μl. Similar results were obtained for 3 separate experiments.

duction was also examined (Fig. 3). The same fractions which stimulated transferrin production also increased ABP production.

For the most active gel filtration fractions of peritubular cell-conditioned medium, the effective concentration of protein required for half-maximal stimulation of transferrin production was 50–100 ng/ml under conditions described (Fig. 3). The minimal effective concentration of this active fraction was 10–25 ng/ml while a maximum stimulation required 250–500 ng/ml. In contrast, the effective concentration of protein required from the 10–300 kDa fraction of peritubular cell-conditioned medium, 200-fold concentrated, was between 4000 and 4500 ng/ml for half-maximal stimulation. These results indicate a 40- to 90-fold purification of P-Mod-S by gel filtration chromatography.

Actions of P-Mod-S on Sertoli cells

The influence of P-Mod-S on the production of Sertoli cell-secreted proteins other than transferrin and ABP was analyzed. Peritubular cell-conditioned medium was fractionated and concentrated by ultrafiltration sequentially on different molecular weight exclusion membranes. Fractions of 2–10 kDa, 10–50 kDa, 50–100 kDa, 100–300 kDa and $\geq$ 300 kDa were concentrated 200-fold and used to treat cultured Sertoli cells. The influence of these fractions on plasminogen activator (PA) production by Sertoli cells is shown in Table 1. None of the peritubular cell medium fractions stimulated PA activity regardless of duration of treatment or time of collection (data not shown). However, fractions 50–100 kDa and $\geq$ 300 kDa significantly reduced PA production by Sertoli cells. Addition of a mixture of hormones (FSH, insulin and testosterone) and retinol to Sertoli cell cultures stimulated PA production which confirms previous observations of the enhancement of PA levels by FSH (Lacroix et al., 1977). Stimulation of PA levels with FSH is essentially the same as for a mixture of FSH, insulin, retinol and testosterone (data not shown).

The effects of P-Mod-S on the synthesis of radiolabeled proteins secreted by Sertoli cells were examined. The incorporation of [^{35}S]methionine into total proteins secreted by Sertoli cells into the medium under basal conditions was 7000 ± 500 dpm/μg DNA/24 h. Treatment of the cells with a mixture of hormones (FSH, insulin and testosterone) and retinol, previously shown to maximally stimulate Sertoli cells (Karl and Griswold, 1980; Skinner and Griswold, 1982), increased incorporation to $33\,000 \pm 1800$ dpm/μg DNA/24 h. Sertoli cells cultured in the presence of a P-Mod-S fraction, either the concentrated 50–100 kDa fraction or an active gel filtration fraction, stimulated incorporation of [^{35}S]methionine into secreted proteins to $29\,000 \pm 1100$ dpm/μg DNA/24 h. These results indicate that P-Mod-S can stimulate radiolabel incorporation into Sertoli cell-secreted proteins to the same extent as maximal hormonal stimulation.

TABLE 1

EFFECTS OF CONCENTRATED PERITUBULAR CELL-CONDITIONED MEDIUM FRACTIONS ON LEVELS OF PLASMINOGEN ACTIVATOR (PA) ACTIVITY PRODUCED BY SERTOLI CELLS

A 25 μl aliquot of the 200-fold concentrated fractions was used which contained protein concentrations of 2 μg for the 2–10 kDa; 4 μg for the 10–50 kDa; 1.5 μg for the 50–100 kDa; 1.7 μg for the 100–300 kDa and 3 μg for the $\geq$ 300 kDa fraction. FIRT represents treatment with FSH, insulin, retinol and testosterone. Cells were treated at the time of plating and retreated after 48 h of culture when the medium was replenished. A 72 h medium collection on day 5 of culture was used for determination of PA levels with a fibrin degradation assay. Values obtained were normalized per μg of Sertoli cell DNA and represent the average $\pm$ SD for 3 different experiments done in triplicate ($n = 9$). Different superscripts between different values indicate a statistically significant difference, $P < 0.005$ with a Student t-test.

Addition to Sertoli cell cultures	None	FIRT	Peritubular cell medium fraction				
			2–10 kDa	10–50 kDa	50–100 kDa	100–300 kDa	$\geq$ 300 kDa
Units PA μg DNA	59 ± 7^{a}	212 ± 22^{b}	56 ± 7^{a}	56 ± 7^{a}	33 ± 5^{c}	48 ± 10^{a}	32 ± 7^{c}

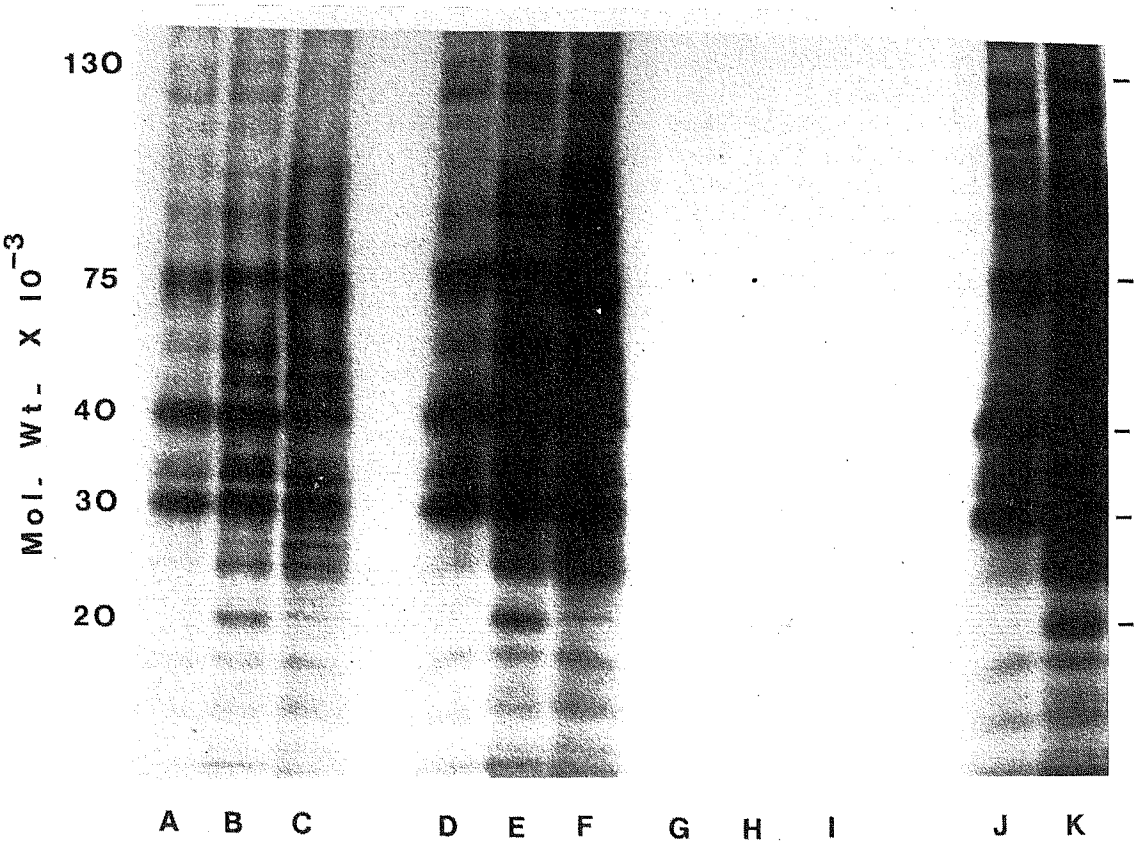

Fig. 4. Fluorograms of the electrophoretic profiles of Sertoli cell-secreted proteins. Radiolabeled secreted proteins were obtained from Sertoli cell control non-treated cultures (A, D, J), cultures treated with P-Mod-S (B, E, K), or Sertoli cell cultures treated with a mixture of FSH, insulin, retinol and testosterone (C, F). Also presented are immunoprecipitates obtained with antisera against epididymal lactalbumin of radiolabeled proteins secreted by control cultures (G) and by P-Mod-S-treated cultures (H). A control immunoprecipitate with non-immune rabbit serum of radiolabeled proteins secreted by P-Mod-S-treated cultures is shown in (I). Triplicate or duplicate lanes represent different experiments with different Sertoli cell preparations.

Radiolabeled Sertoli cell-secreted proteins from the above treatments were electrophoretically analyzed. Fluorograms of the electrophoretic profiles of radiolabeled proteins secreted by stimulated Sertoli cells indicate several bands not evident in secretions from control non-treated cells (Fig. 4). A radiolabeled protein of approximately 20 kDa was abundant in Sertoli cell preparations which had been cultured in the presence of a P-Mod-S fraction from peritubular cell-conditioned medium. A lactalbumin-like protein having a molecular mass of approximately 20 kDa is produced by epididymal cells (Klinefelter and Hamilton, 1984) and is also suspected to possibly be produced by Sertoli cells. Radiolabeled proteins secreted by Sertoli cells were immunoprecipitated with an antibody preparation against a lactalbumin-like protein, isolated from the epididymis (Klinefelter and Hamilton, 1984), and resulted in the immunoprecipitation of the 20 kDa band exclusively (Fig. 4). Similar results were obtained with an antibody to rat mammary gland lactalbumin (a gift of Dr. P. Quasba, NIH, Bethesda, MD), but these findings were not as reproducible (data not shown). These data indicate that the 20 kDa radiolabeled protein synthesized and secreted by Sertoli cells in response to P-Mod-S is immunologically similar to lactalbumin. Lesser amounts of

radioactivity were found in comparable immunoprecipitates of radiolabeled proteins secreted by Sertoli cells cultured in the presence of FSH, insulin, retinol and testosterone. However, no detectable immunoprecipitate was detected in Sertoli cells maintained under basal conditions (Fig. 4). These findings are indicated by the relative intensities of the 20 kDa bands in the fluorograms of the electrophoretic profiles from 3 separate experiments, two of which are overexposed to demonstrate this more clearly (Fig. 4). Proteins other than the 20 kDa lactalbumin-like protein that also demonstrate increased intensities in the fluorograms after stimulation of Sertoli cell preparations are 25–30 kDa and 45–70 kDa. The identity of these proteins is presently unknown.

Identification of mitogenic activity produced by peritubular cells

Peritubular cell-conditioned medium was examined to determine whether it contained growth factors, and whether P-Mod-S contained mitogenic activity. Peritubular cell-conditioned medium, concentrated 10–300 kDa fraction, was applied to a Sephacryl S-200 column and eluted. The influence of an aliquot of each fraction was also used to detect the stimulation of DNA synthesis of NRK cells (Fig. 5). Fractions which stimulated transferrin production by Sertoli cells did not contain mitogenic activity. However, peritubular cell-conditioned medium did contain growth factor(s) with apparent molecular weights of 50 000 and 25 000.

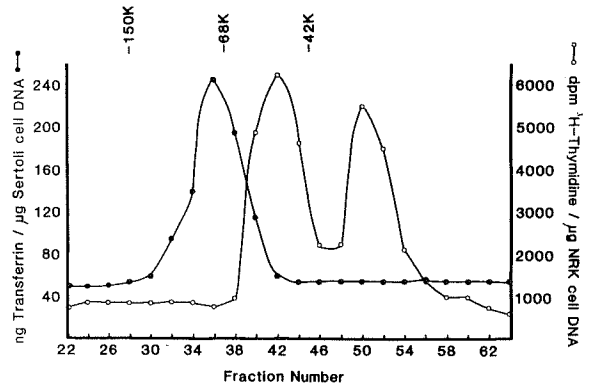

Fig. 5. Gel filtration chromatography of P-Mod-S and mitogenic activity. The peritubular cell-conditioned medium sample utilized and Sephacryl S-200 column conditions are outlined in the legend for Fig. 3. A 25 µl aliquot of each collected fraction was used to treat Sertoli cell cultures to determine an influence on transferrin production (●) expressed as ng transferrin/µg Sertoli cell DNA. Another aliquot was utilized to treat a NRK cell line to detect mitogenic activity through an analysis of [^{3}H]thymidine incorporation into NRK cell DNA (○) and is expressed as dpm [^{3}H]thymidine/µg NRK cell DNA. Similar results were obtained for 3 separate experiments.

The growth factor(s) detected had no apparent influence on the production of any Sertoli cell-secreted protein examined, nor on Sertoli cell proliferation in culture (data not shown). Preliminary experiments were initiated to determine if the growth factor(s) could be identified as EGF or IGF activity. The concentrated 10–300 kDa fraction of peritubular cell-conditioned medium did not displace ^{125}I-EGF from its receptor (data not shown). In contrast, the concentrated peritubular cell-conditioned medium fraction did contain IGF-like activity, as detected by the IGF radioimmunoassay (data not shown). These results indicate IGF-like activity secreted by peritubular cells could partly account for the mitogenic activity observed in Fig. 5.

Discussion

Conditioned medium from peritubular cell cultures stimulated Sertoli cells in a dose-dependent manner as previously reported. Peritubular cells are shown to produce this stimulatory activity, P-Mod-S, for a minimum of 2-weeks in serum-free cell culture. This observation implies that the activity is not an artifact of the early treatment of the cells with serum but is caused by a product produced by the cells. A previous observation which supports this assertion is that a non-relevant fibroblast cell line (3T3) treated in the same manner as peritubular cells did not contain stimulatory activity in its conditioned medium (Skinner and Fritz, 1985a). The amount of activity present after 2 weeks of serum-free culture was essentially the same as the first 3 days of culture. Therefore, collection of large quantities of conditioned medium is possible and this medium provides a source of P-Mod-S for subsequent purification.

A time course of the effect of P-Mod-S on Sertoli cells demonstrated a significant stimulation of transferrin production between days 4 and 6 of Sertoli cell culture (Fig. 2). This time course of

stimulation is similar to that observed with hormonal treatment of Sertoli cells (Skinner and Griswold, 1982). Therefore P-Mod-S, like hormones, actively stimulates transferrin production by Sertoli cells in culture. This is in contrast to the time course observed for androgen-binding protein (ABP) secretion in which there is a decline in production with time in culture (Skinner and Fritz, 1985b). After stimulation of Sertoli cells with hormones or P-Mod-S there is a reduction in the rate of decline of ABP production but stimulation above initial levels is not observed. The mechanism by which transferrin production can be stimulated while ABP production is declining is not known at present. Stimulation of transferrin production declined on day 8 of Sertoli cell culture (Fig. 2). This reduction is similar to that shown previously after hormonal stimulation. The decline after day 8 may be due to a decrease in the viability of the Sertoli cells with time in culture and/or an alteration in the differentiated state of the Sertoli cell with time in culture.

Initial characterization of P-Mod-S by gel filtration chromatography revealed an approximate molecular mass of 70 kDa (Fig. 3). Elution of P-Mod-S with high salt or acid did not dissociate the 70 kDa species to a lower molecular weight form. These results imply but do not prove that the 70 kDa species is probably not an aggregated form of a low molecular weight protein nor a small protein bound to a binding protein. The specific activity of P-Mod-S observed after gel filtration chromatography indicated that this would provide a useful initial step in the ultimate purification of P-Mod-S, which is presently in progress. The same fractions from the gel filtration column which stimulated transferrin production by Sertoli cells also stimulated ABP production (Fig. 3). This implies that a single protein species may be responsible for the observed activities of P-Mod-S. However, unequivocal demonstration of a single protein species will require purification of P-Mod-S.

Analysis of the actions of P-Mod-S on the production of known secretory proteins of Sertoli cells demonstrated that P-Mod-S stimulated transferrin and ABP production but not plasminogen activator (PA) secretion (Table 1). Peritubular cell medium fractions containing P-Mod-S apparently suppressed PA activity. This observation is in contrast to that observed after hormonal stimulation of Sertoli cells with FSH. These results imply that P-Mod-S may be selective in its actions on Sertoli cells by inhibiting or stimulating the production of specific protein products. The mechanism of such a selective action on the Sertoli cell would be complex. However, an alternate interpretation is that a protease inhibitor is produced by peritubular cells which inhibits PA. The presence of such an inhibitor would mask a stimulation by P-Mod-S simply by inhibiting the protease activity. Inhibition of PA by several molecular weight fractions of peritubular cell medium supports this interpretation (Table 1). A recent study provides experimental evidence that a protease inhibitor is produced by seminiferous peritubular cells which can inhibit PA activity (A. Hettle and I.B. Fritz, unpublished observation). Therefore, more highly purified preparations of P-Mod-S will be needed to determine whether PA production is stimulated by the actions of P-Mod-S on Sertoli cells.

The influence of P-Mod-S on total secreted proteins was determined through an analysis of radiolabel incorporation into secreted proteins. Both P-Mod-S and hormone treatments increased incorporation 4-fold over control levels. Electrophoretic analysis of these radiolabeled proteins secreted by Sertoli cells revealed a number of proteins in stimulated cultures that were not produced by non-treated control Sertoli cell cultures. A 20 kDa protein was a major product in medium after P-Mod-S treatment of Sertoli cells, but was present in only small amounts in hormone-stimulated cultures and absent in control cultures. It appears that P-Mod-S may play an important role in inducing the synthesis of this protein by Sertoli cells. To determine the possible identity of this 20 kDa protein, a comparison was made with lactalbumin, a 20 kDa protein produced by the mammary gland, that has previously been shown to be present in the reproductive tract (Hamilton, 1981). An antibody has been produced to a purified epididymal lactalbumin-like protein which was used for cytochemical localization studies (Klinefelter and Hamilton, 1984). Results from these studies indicated that epididymal epithelial cells appear to synthesize and secrete this lactalbumin-like protein which then localizes on the surface of

epididymal sperm. The function of a lactalbumin-like protein in the reproductive tract has been postulated to modulate the enzymatic activity of galactosyltransferase (Hamilton, 1980). The presence of galactosyltransferase and lactalbumin in the reproductive fluids from rat testis and epididymis leads to the speculation that lactalbumin may be produced by the Sertoli cell. With the use of the antibody to the epididymal lactalbumin-like protein, the 20 kDa protein secreted by Sertoli cells after P-Mod-S treatment was specifically immunoprecipitated (Fig. 4). This result provides direct evidence that Sertoli cells synthesize and secrete a lactalbumin-like protein immunologically similar to the epididymal lactalbumin-like protein. In addition, results indicate that this lactalbumin-like protein is primarily produced by Sertoli cells stimulated with P-Mod-S. The function of this Sertoli cell-secreted lactalbumin-like protein during spermatogenesis and/or in the reproductive tract is at present unknown. It is clear that the cellular interaction between peritubular cells and Sertoli cells mediated via P-Mod-S plays an integral role in regulating the secretion of the lactalbumin-like protein by Sertoli cells. This observation may therefore provide insight toward understanding the possible functions of the lactalbumin-like protein.

The dramatic influence of P-Mod-S on Sertoli cells was similar to that found for maximal hormonal stimulation. Both transferrin and ABP production are stimulated maximally with P-Mod-S (Skinner and Fritz, 1985a). A mixture of FSH, insulin and retinol is required to obtain a maximal stimulation which none of the hormones or vitamin alone can provide (Karl and Griswold, 1980; Skinner and Griswold, 1982). Therefore, P-Mod-S appears to have a greater influence on transferrin and ABP production by Sertoli cells than any single hormone or vitamin known to influence the cell. The same was found for radiolabel incorporation into total Sertoli cell-secreted proteins.

In an electrophoretic analysis of these radiolabeled proteins, the majority of proteins stimulated by hormones were also stimulated with P-Mod-S. Some proteins, for example the lactalbumin-like protein, were more prominent after P-Mod-S stimulation than after hormone stimulation. This indicates that P-Mod-S can induce or regulate Sertoli cell functions which normally are not influenced by hormones. The active gel filtration fraction of partially purified P-Mod-S required for a half-maximal effective concentration was 50–100 ng/ml and had an approximate molecular mass of 70 kDa. By calculation, an effective concentration of less than 10^{-9} M will be required for the actions of P-Mod-S on Sertoli cells. From these observations P-Mod-S clearly may play an important role in regulating Sertoli cell functions.

A major class of proteins involved in cell-cell interactions are growth factors. In many physiological systems growth factors are important in regulating the development and growth of specific cell types. Examples of growth factors involved in cellular communications are found in organs such as the mammary glands (Turkington, 1969), pituitary (Holley and Kiernan, 1968; Gospodarowicz et al., 1975) and brain (Gospodarowicz, 1974). A growth factor has also been implicated in mesenchymal-epithelial cell interactions in pancreatic tissue (Ronzio and Rutter, 1973). Therefore, it was of interest to determine whether P-Mod-S contained mitogenic activity and if it was a known growth factor. Fractions obtained from a gel filtration column containing P-Mod-S activity were found not to contain mitogenic activity (Fig. 5). These results imply that P-Mod-S is not a growth factor but a locally produced regulatory protein. This type of protein is analogous to a locally produced hormone, and can be termed a non-mitogenic paracrine factor. Unlike a growth factor which may act on many cell types, such a paracrine factor could bind a specific receptor on a single cell type to elicit a response. Whether such a factor would be more important during development or in regulation of differentiated cellular functions remains to be determined. However, the specificity of such a factor may allow a cellular interaction to occur that is specific to a given organ thus permitting induction of a function which is specific to a given cell type. Cellular interactions mediated by non-mitogenic paracrine factors potentially may provide a specificity which is not necessarily possible with growth factors. Whether P-Mod-S actions are specific to Sertoli cells remains to be determined.

Although P-Mod-S appears not to be a growth

factor, the peritubular cells did secrete a growth factor(s) with approximate molecular weights of 50 000 and 25 000. Unfractionated peritubular cell medium contained insulin growth factor-like (IGF) activity. Peritubular cells did not produce EGF, but the production of another growth factor in addition to IGF remains a possibility. Further characterization and identification of the growth factors produced by peritubular cells is in progress.

Analysis of mesenchymal-epithelial cell interactions has primarily been directed at an examination of the influence of different cell types on the embryonic development of a specific tissue (Grobstein, 1967; Kratochwil, 1969). From these studies it has been postulated that an inducer substance is produced by the mesenchymal cell which modulates the differentiation and functions of an adjacent epithelial cell (Cunha et al., 1983). Results from these types of studies have also implied that steroids may act on the mesenchymal cells, in a steroid-responsive organ, to regulate the production of the putative inducer substances (Cunha et al., 1983). The interaction between peritubular cells and Sertoli cells mediated by P-Mod-S provides evidence for the proposed mesenchymal-derived inducer substance. Androgens can act on primary cultures of peritubular cells to stimulate the production of P-Mod-S (Skinner and Fritz, 1985a). From these observations it is proposed that Leydig cells produce androgens which stimulate peritubular cells to produce P-Mod-S that modulates the differentiation and functions of Sertoli cells which in turn support spermatogenesis. This cellular interaction mediated by P-Mod-S supports the hypothesis that mesenchymal cells may play an integral role in the control and regulation of an epithelial cell's function. More detailed examination of the interaction between peritubular cells and Sertoli cells via P-Mod-S will be useful to understand more fully the development, maintenance and regulation of testicular functions.

The present study demonstrates that P-Mod-S is a 70 kDa non-mitogenic paracrine factor involved in mesenchymal-epithelial cell interactions between peritubular cells and Sertoli cells. P-Mod-S modulates Sertoli cell functions to a greater extent than any agent known to influence the cell. The structural relationship between mesenchymal cells and epithelial cells is present in many physiological systems. It is proposed that cellular interactions similar to those mediated via P-Mod-S may occur in many different organs. Whether the same or different proteins would be involved in the interactions in these other organs remains to be determined. The unique functions of each organ and the specificity of individual epithelial cell types suggest that different modulatory proteins may be produced by the different mesenchymal cell types.

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