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# RETE TESTIS FLUID CONTAINS A GROWTH FACTOR FOR CULTURED FIBROBLASTS

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A new polypeptide mitogen has been detected at high specific activity in the rete testis fluid of rams (oRTF). The factor, which stimulates DNA synthesis in quiescent Swiss 3T3 cells, has a molecular weight of 45,000 as assessed by gel filtration through Ultrogel AcA 34. The factor is heat stable but is inactivated by proteolytic enzymes and by β-mercaptoethanol. The growth-promoting activity in oRTF does not bind to concanavalin A.

## INTRODUCTION

The Sertoli cells of the seminiferous epithelium, together with their secreted products, are considered to play an important role in the control of spermatogenesis (1). In addition, Sertoli cells are unusual in that primary cultures of the cells will attach, spread and survive in medium without added serum or macromolecular growth factors (2,3). For these reasons we considered it possible that Sertoli cells might themselves produce and secrete cell growth-promoting activity. We have investigated this question through an assay previously used to detect serum growth factors; namely the stimulation of DNA synthesis in quiescent cultures of mouse 3T3 fibroblasts (4,5). Although Sertoli cells from 20-day old rats can be maintained for long periods (weeks) in primary culture, the cells do not proliferate and cannot be readily passed. Consequently, large-scale cultures for harvesting conditioned medium are time-consuming and costly. We have therefore chosen to use ovine rete restis fluid (oRTF), which can be collected in relatively large volumes, as a source of Sertoli cell secreted

ABBREVIATIONS: oRTF; ovine rete testis fluid. PBS; phosphate-buffered saline. EGF; epidermal growth factor. IGF1, IGF2; insulin-like growth factors 1 and 2. PDGF; platelet-derived growth factor.

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proteins (6,7). We have found that this fluid contains a potent polypeptide growth factor which stimulates $^{3}$H-thymidine incorporation into quiescent fibroblasts. The factor appears to be different from other well-characterized growth factors.

## METHODS

Collection and concentration of rete testis fluid. Testicular semen was collected from conscious Clun Forest rams during the breeding season by cannulating the rete testis of each animal under pentobarbitone-halothane anaesthesia as described previously (8). The fluid was kept at 4°C during 24 h collection periods and then the spermatozoa were removed by centrifugation. The supernatant fluid (oRTF) was stored frozen at -20°C. For use in assay or for biochemical separation, the oRTF was thawed and concentrated ten-fold by pressure ultrafiltration at 4°C using Amicon equipment. Initially UM2 membranes were used but recent experiments have shown that a faster concentration can be made using PM10 membranes with no loss of cell growth-promoting activity. The final protein concentration of the ultrafiltration retentate was 6-8 mg/ml.

Cell cultures. Stock cultures of Swiss mouse 3T3 cells were maintained on 9 cm plastic culture dishes (Nunc) and passaged every 3 days in Dulbecco-Vogt modified Eagle's medium (DME) containing 10% newborn calf serum. Dishes were incubated at 37°C in a humidified atmosphere of 10% $CO_2$/90% air. For use in the $^{3}$H-thymidine incorporation assay, 5 x $10^{4}$ cells, in 2 ml DME + 10% calf serum, were seeded in 3.5 cm dishes (Nunc) which were incubated for at least 6 days. These cultures were "quiescent" as judged by a low level of $^{3}$H-thymidine incorporation. After a continuous 30 h exposure to $^{3}$H-thymidine, less than 1% labelling of cell nuclei is detected by autoradiography (5).

Measurement of $^{3}$H-thymidine incorporation. Aliquots of test agents were added directly to the depleted growth medium of quiescent cells. Twenty µl of $^{3}$H-thymidine were added per dish to give a final concentration of 1 µCi/ml (1 x $10^{-6}$M) After incubation for 36 h at 37°C, the medium was removed from the dishes and the cells were washed twice with cold PBS. The cells were extracted for 30 min with 1-2 ml of cold 5% trichloracetic acid, rinsed with ethanol, and air dried. The cells were dissolved in 1 ml of 0.1M NaOH and 0.5 ml of this solution were mixed with 10 ml of acidified scintillation fluid for $^{3}$H counting.

## RESULTS

To date, 6 ten-fold concentrates have been prepared from rete testis fluid collected from three different rams and all preparations have demonstrated similar levels of cell growth-promoting activity. The dose-response for the stimulation of DNA synthesis in quiescent 3T3 cells by one preparation is shown in Fig. 1 (curve a). For comparison, the dose-response curve to newborn calf serum is included in Fig. 1 (curve b). Both oRTF and calf serum stimulate $^{3}$H-thymidine incorporation to a similar maximal level. However the specific activity (activity/unit protein) of oRTF is 15 times that of calf serum with half-maximal stimulation achieved at 150 and 2700 µg protein/ml respectively.

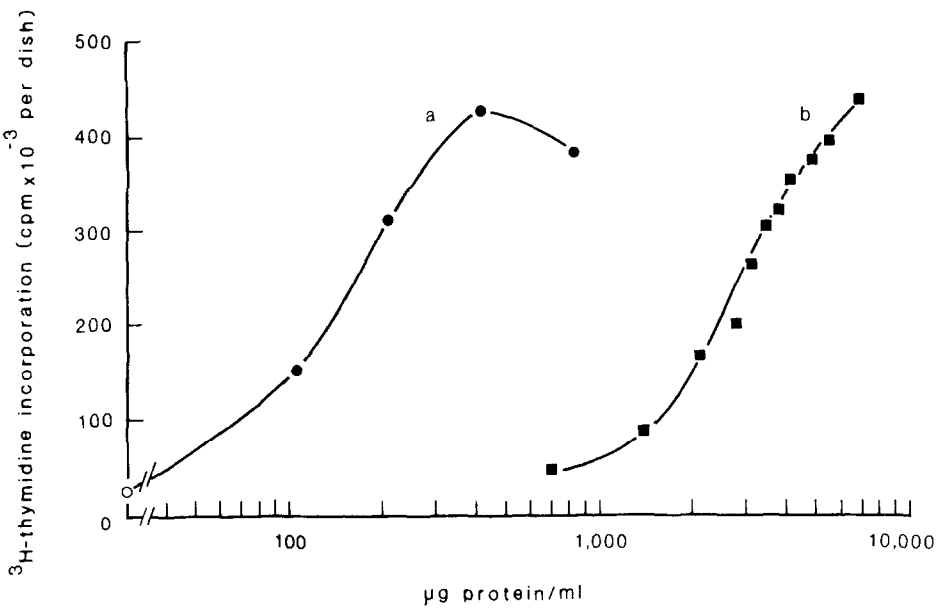


Fig. 1. Stimulation of $^3$H-thymidine incorporation in quiescent 3T3 cells as a function of the concentration of oRTF (●—●) or calf serum (■ — ■). The incorporation of $^3$H-thymidine was measured as described under METHODS. The values show the means of duplicate determinations which did not vary by more than 5%.

The slight inhibitory effect of higher concentrations of oRTF on $^3$H-thymidine incorporation has been observed with all preparations of oRTF tested.

oRTF was subjected to gel filtration on Sephadex G-50 equilibrated and eluted with phosphate-buffered saline (pH 7.4). All the stimulatory activity eluted in the void volume indicating that the active material has a molecular weight greater than 30,000 (results not shown). When oRTF was chromatographed on Ultrogel AcA 34 under the same conditions (PBS, pH 7.4) the elution profile shown in Fig. 2 was obtained. Minor peaks of mitogenic activity were detected at positions corresponding to molecular weights of 350,000 and 75,000. A major peak of activity eluted at a position corresponding to a molecular weight of approximately 45,000. Fractions 28-31 (Fig. 2) were pooled and concentrated 10 times by ultrafiltration. The dose-response to this material is shown in the insert to Fig. 2.

It has been reported that some two-thirds of the proteins secreted by rat Sertoli cells in culture are glycoproteins which bind to concanavalin A (9). We have found that approximately one-third of the protein in oRTF will bind to

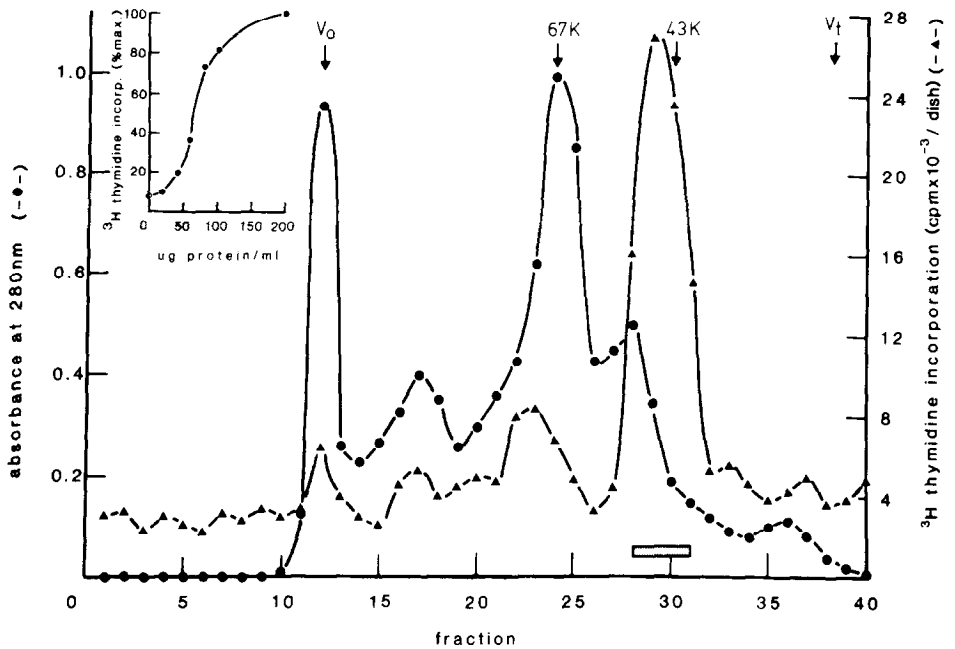


Fig. 2. Concentrated oRTF (4.5 ml) was applied to a 2.5 x 70 cm column of Ultrogel ACA 34 previously equilibrated with PBS (pH = 7.4). The column was eluted at 4°C with PBS at a flow rate of 20 ml/hr. Fractions (10 ml) were collected, the absorbance at 280 nm was measured (●-●), and aliquots (200 µl) were tested for ability to stimulate $^3$H-thymidine incorporation in quiescent 3T3 cells (▲-▲). The void volume (Vo) and the total volume (Vt) of the column were determined with blue dextran and potassium dichromate respectively. The elution positions of bovine serum albumin (67,000) and ovalbumin (43,000) are also marked. The results show the mean of duplicate determinations of $^3$H-thymidine incorporation. In this experiment, addition of 200 µl of concentrated oRTF or 200 µl of calf serum produced incorporation values of 74 x $10^3$ and 86 x $10^3$ cpm per dish respectively. Fractions 28-31 were pooled and reconcentrated four-fold by ultrafiltration (PM10 membrane). The dose-response of this material in the 3T3 $^3$H-thymidine incorporation assay was measured (insert).

a column of concanavalin A-Sepharose. However none of the growth-promoting activity bound to the lectin (Table 1) suggesting that the active material is not a glycoprotein.

The growth factor in oRTF appears to be a polypeptide that contains a disulfide linkage. Treatment of oRTF with trypsin/chymotrypsin resulted in a complete loss of stimulatory activity whereas treatment of oRTF with previously heat-inactivated enzymes did not cause any loss of activity (Table 11). However, the growth promoting activity in oRTF is heat stable (100°C for 20 min). Incubation of oRTF for 24 h at 4°C in the presence of β-mercaptoethanol (2%) resulted in a loss of 97% biological activity. Similar incubations with urea (6M) or guanidine HCl (4M) caused only 16% and 30% losses of activity

TABLE I

Concanavalin A-Sepharose fractionation of oRTF: stimulatory activity of bound and unbound fractions

| Additions | $^{3}$H-thymidine incorporation (cpm x $10^{-3}$/dish) |
|---|---|
| control, 100 µl PBS | 4.3 |
| oRTF, unfractionated, 100 µl | 192.8 |
| oRTF, conA bound, 100 µl | 12.5 |
| oRTF, conA non-bound, 100 µl | 123.3 |

A column containing 2 ml of concanavalin A-Sepharose was equilibrated with 0.1M ammonium acetate buffer (pH = 6.0) which contained 1M NaCl, $10^{-3}$M $CaCl_2$, and $10^{-3}$M $MgCl_2$. A 0.5 ml sample of concentrated oRTF was applied to the column. The unbound proteins were washed from the column with acetate buffer and collected as a single fraction. When the protein concentration of the eluate had returned to zero, the bound glycoproteins were eluted from the column with acetate buffer containing 0.1M α-methyl-D-mannoside. Both collected samples (unbound and bound proteins) were dialysed against 0.02M ammonium formate (pH = 6) at 4°C, lyophylized, reconstituted in 0.5 ml of PBS and tested in the 3T3 cell $^{3}$H-thymidine incorporation assay. The values show the mean of two determinations of $^{3}$H-thymidine incorporation which did not vary by more than 5%.

respectively (Table II). Similar results (not shown) were obtained with severa different samples of oRTF.

## DISCUSSION

Our results show that oRTF contains, at high specific activity, a polypeptide growth factor. The source of this factor remains to be determined. We suspect that the factor may be produced locally, probably by Sertoli cells, since it is unlikely that a polypeptide of this molecular weight (45,000) could cross the blood-testis barrier (10,11). In support of this conclusion is the recent report that homogenates of Sertoli cells isolated from the seminiferous epithelium of prepuberal mice contain a polypeptide mitogen (12). In addition, we have observed (results not shown) that conditioned medium harvested from rat Sertoli cells in culture also contains growth-promoting activity for mouse 3T3 cells. It is well established that Sertoli cells secrete a variety of polypeptides including androgen binding protein (13,14) and transferrin (15). Our results, together with those of Feig et al. (12) suggest that these cells, *in vivo* and *in vitro*, secrete a polypeptide growth factor.

TABLE 11

Susceptibility of the growth-promoting activity in oRTF to denaturing conditions, reduction, heat and proteolytic enzymes

| Expt. | Additions | $^{3}$H-thymidine incorporation (cpm x $10^{-3}$/dish) |
|---|---|---|
| I | control, PBS | 10.4 |
| | oRTF, PBS, 4°C, 24 h | 246.2 |
| | oRTF, 6M urea, 4°C, 24 h | 207.0 |
| | oRTF, 4M guanidine.HCl, 4°C, 24 h | 175.8 |
| | oRTF, 2% β-mercaptoethanol, 4°C, 24 h | 18.3 |
| | oRTF, control | 232.2 |
| | 10% calf serum | 370.6 |
| II | control, PBS | 9.4 |
| | oRTF + PBS | 87.2 |
| | oRTF + T/C in PBS | 5.6 |
| | oRTF + heat-inactivated T/C in PBS | 105.1 |
| | 10% calf serum | 93.4 |

Samples of concentrated oRTF were treated with dissociative or reducing agents for 24 h at 4°C as indicated. The samples were subsequently dialysed against PBS for 48 h at 4°C prior to analysis for growth-promoting activity. In a separate experiment, concentrated oRTF was treated with an equal volume of 1) PBS, 2) PBS containing trypsin and chymotrypsin (T/C), 3) PBS containing heat-inactivated T/C. Trypsin and chymotrypsin were present at a final concentration of 1 mg/ml. The samples were incubated at 37°C for 4 h and then heated at 100°C for 20 min prior to use in the 3T3 $^{3}$H-thymidine incorporation assay. The values show the mean of two determinations of $^{3}$H-thymidine incorporation which did not vary by more than 5%.

The molecular weight of the oRTF-derived growth factor is somewhat higher than the molecular weight of other polypeptides (e.g. EGF, FGF, PDGF, IGF1 and IGF2) with mitogenic activity for 3T3 cells (16,17). Since gel chromatography was carried out under non-dissociating conditions it is possible that the determined molecular weight represents a complex of a growth factor and a binding protein. However, the chromatographic elution profiles of oRTF on Sephadex G-50 or Ultrogel AcA34 showed no evidence of significant stimulatory activity in fractions containing lower molecular weight material. Thus, if the factor is bound to a carrier protein, the association appears to be very strong. The relationship between the growth factor in oRTF and the growth-promoting activity present in homogenates of mouse Sertoli cells (12) remains to be determined. The mouse-derived material is of a lower molecular weight, is inactivated by heating to 100°C, but is not inactivated by sulfhydryl-bond reduction. In contrast the oRTF-derived factor is heat stable, but is

inactivated after reduction by β-mercaptoethanol. The two factors are clearly not identical. We have recently found (results not shown) that homogenates of ram testis contains growth-promoting activity which is not inactivated by reduction with β-mercaptoethanol or dithiothreitol. This result suggests that a similar growth factor may be present in the testes both of mouse and sheep but the growth factor may be chemically modified in the course of secretion into the seminiferous tubules and rete testis.

Feig et al. (12) have suggested that a Sertoli cell-derived growth factor could play a role in the control of spermatogenesis, perhaps by regulating the proliferation of spermatogonia or by synchronizing DNA synthesis in preleptotene spermatocytes. These speculations remain exciting possibilities. In addition, our finding of growth-promoting activity in rete testis fluid suggests that Sertoli cell-derived factor is transported out of the testis and raises the possibility that its primary function may be to regulate cell division in the first part of the epididymis, where the mitotic rate is normally high (18,19).

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