Partial purification and characterization of an endothelial cell growth regulator from the bovine ovary*

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Summary. An inhibitor of endothelial cell thymidine incorporation *in vitro* was partly purified from cow ovaries using ammonium sulphate (AS) precipitation. Supernatant fluid from the 100 000 g pellet of freshly homogenized ovaries was subjected to stepwise AS precipitation. Precipitates were collected sequentially at 40%, 60%, 80% and 95% saturation, and then each was dissolved, dialysed (M_r 8000 cutoff) and examined in tissue culture for effects on cellular thymidine incorporation by cow pulmonary artery endothelial cells (CPAE) and mouse fibroblasts (L929 and 3T3). The 80% AS precipitate (ppt.) inhibited the in-vitro uptake of [3 H]thymidine by CPAE and L929 cells, but not 3T3 cells. Heparin–Sephadex (HS) chromatography of the 80% AS ppt. revealed that the inhibitory activity on CPAE and L929 cells did not bind to HS; the inhibitory fraction was found in the HS column breakthrough (80% BT). The 80% BT fraction reduced CPAE [3 H]thymidine uptake as determined by autoradiography and increased cellular uptake of trypan blue. Serial fractions from Sephacryl S-200 exclusion chromatography of the 80% BT contained CPAE inhibitory activity in the M_r range 30 000–50 000. The inhibitory activity on endothelial cells and L929 fibroblasts and the non-reduced molecular weight range of that fraction are similar to those of tumour necrosis factor alpha (TNF α). The results indicate that the cow ovary contains a fraction that inhibits endothelial cell growth *in vitro* and may have important roles in follicular atresia and luteal regression.

Keywords: endothelial cell; fibroblast; growth factor; ovary; tumour necrosis factor

Introduction

Growth of the follicle and corpus luteum is dependent on the growth of the vascular network that nourishes these metabolically active tissues (see Greenwald & Terranova, 1988). Conversely, follicular atresia and luteal regression are accompanied by involution of the follicular and luteal vascular systems (Lobel & Levy, 1968; Hay *et al.*, 1976; Niswender *et al.*, 1976; O'Shea *et al.*, 1978). The repeated restructuring of the vascular system is mediated, at least in part, by angiogenic factors in the ovary (Jakob *et al.*, 1977; Gospodarowicz & Thakral, 1978; Makris *et al.*, 1984; Gospodarowicz *et al.*, 1985; Frederick *et al.*, 1985; Koos, 1986). Extracts of bovine and rabbit corpora lutea were found to be potent stimulators of [3 H]thymidine uptake by bovine aortic endothelial cells *in vitro* (Gospodarowicz & Thakral, 1978). Gospodarowicz *et al.* (1985) later showed that in the cow this angiogenic activity was fibroblast growth factor (FGF).

Several studies have also indicated the presence of angiogenic factors in ovarian follicles. Non-luteal ovarian tissue from pigs exhibited stimulatory effects on endothelial cell proliferation and

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migration *in vitro* as well as angiogenic activity *in vivo* on the chick chorioallantoic membrane (Makris *et al.*, 1984). An angiogenic response to pig follicular fluid (Frederick *et al.*, 1985) and rat granulosa cell conditioned media (Koos, 1986) has also been demonstrated. Ovarian angiogenic factors have been proposed to stimulate and direct the development of blood vessels to follicles and corpora lutea (Findlay, 1986). A growth regulator directing the regression of blood vessels in the ovary has yet to be described. However, tumour necrosis factor (TNF), known to reduce endothelial cell proliferation (Sato *et al.*, 1986; Schweigerer *et al.*, 1987) and migration (Mano-Hirano *et al.*, 1987), has been immunohistochemically demonstrated in cow and rat ovaries (Roby & Terranova, 1989), has been reported to be produced by lipopolysaccharide (LPS)-stimulated corpora lutea in the rabbit (Bagavandoss *et al.*, 1988) and has significant effects on ovarian steroidogenesis in the rat (Emoto & Baird, 1988; Roby & Terranova, 1988; Adashi *et al.*, 1989). It was the intent of this study to examine the cow ovary for the presence of factors that might alter endothelial cell growth *in vitro*.

Materials and Methods

Ovaries: source and extraction

Ovaries from beef cattle were obtained from the Department of Animal Science at the University of Missouri, Columbia, Missouri, or from The State of Kansas Meat Plant at Oskaloosa, Kansas. The ovaries were kept on ice for 4–6 h until snap frozen on solid CO₂ and were maintained at –80°C until subjected to extractions.

The ovaries were kept at 4°C throughout the extraction procedure. The ovaries were used irrespective of stage in the oestrous cycle and therefore contained large and small follicles and corpora lutea. Ovaries (~300 g) were cut into small pieces with a scalpel and then homogenized in 1.5 volumes (w/v) 0.01 M-Tris-HCl buffer, pH 7.0. The homogenate was centrifuged at 10 000 *g* for 30 min. The resulting pellet was discarded and the supernatant fluid was centrifuged at 100 000 *g* for 60 min. The supernatant fluid (~540 ml), from which 10 ml were saved as crude supernatant, was subjected to sequential precipitation by addition of ammonium sulphate to a final concentration of 40%, 60%, 80% and 95%. For precipitation, ammonium sulphate was slowly added to the supernatant with stirring until the desired final concentration was reached. The mixture was incubated for 10 min at 4°C with stirring and then centrifuged for 15 min at 10 000 *g*. The supernatant fluid was subjected to the next addition of ammonium sulphate. The precipitate was dissolved in the smallest volume of Tris buffer that resulted in a clarified fluid. Each dissolved precipitate, referred to as “% ppt.”, was dialysed (cutoff *M_r* 8000) against Tris buffer at 4°C overnight (dialysate:buffer 1:100). The buffer was changed once after 6 h of dialysis. Then, each dialysate was clarified by centrifugation (10 000 *g*, 15 min) to remove and discard any precipitate that may have formed during dialysis. Each dialysate was examined for effects on cellular incorporation of [³H]thymidine *in vitro*.

Cell culture

Endothelial cells. Cow pulmonary artery endothelial cells (CPAE, supplied by Una Ryan, University of Miami, Miami, Florida) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Sigma, St Louis, MO, USA), 50 U penicillin/ml and 50 µg streptomycin/ml (Irvine Scientific, Santa Ana, CA, USA) and Ryan red (Ryan & Maxwell, 1986) at a concentration of 50%.

Fibroblast cells. BALB/c mouse embryo fibroblasts (3T3 cells) and the transformed mouse fibroblast (L929) (CCL 163 and CCL 1, respectively, American Type Culture Collection, Rockville, MD, USA) were maintained in DMEM supplemented with 10% FCS and antibiotics.

Thymidine incorporation assay. The classical in-vitro assay to detect the presence of TNFα in fluids is the L929 cell lytic assay (Carswell *et al.*, 1975; Ruff & Gifford, 1980; Aggarwal *et al.*, 1985). The inhibitory activity of TNFα on L929 cells has been quantitated using cellular uptake of [³H]thymidine, uptake of trypan blue, exclusion of crystal violet, or the release of ⁵¹Cr (Ruff & Gifford, 1980; Aggarwal *et al.*, 1985).

The media from confluent 25 cm² flasks of 3T3, CPAE and L929 cells were removed and the cells were washed once with Hanks Balanced Salt Solution (HBSS, calcium and magnesium free; Gibco Laboratories) and then trypsinized with 0.25% trypsin in 5 ml HBSS. The cells were incubated with the trypsin for 10 min and then with 1 ml FCS. The cells, in suspension, were centrifuged at 100 *g* for 10 min. The supernatant was removed and the cells were diluted to the desired density with DMEM supplemented with 10% FCS. For assay, cells were seeded in 96-well flat bottomed trays (Fisher, St Louis, MO, USA) at a density of either 20 000 L929 cells/200 µl/well or 10 000 3T3 and CPAE cells/200 µl/well. After an overnight incubation allowing for cellular attachment, the media were removed and the cells were washed twice with 300 µl DMEM without serum. Fresh DMEM (20 µl) without serum and the 50 µl sample to be

tested were added. All samples were diluted to the desired concentration with DMEM and tested in triplicate. After incubation for 48 h, 1 μCi [^3H]thymidine (ICN Radiochemicals, Irvine, CA, USA; sp. act. 59 Ci/mmol) in 10 μl DMEM was added to each well for 4 (L929) or 24 h (CPAE or 3T3). After the incubation with [^3H]thymidine, media were removed, the cells were washed once with HBSS and then loosened from the culture surface by incubation for 15 min with 200 μl 0.25% trypsin in HBSS. The cells were then harvested onto glass-fibre filter paper with an automated cell harvester (No. 7019, Skatron Inc., Sterling, VA, USA). Radioactivity in the nuclear fraction of the cells was determined by liquid scintillation.

Chromatography

All column chromatographic procedures were performed at 4°C. Heparin–Sephacryl CL-6B (HS) (Pharmacia, Piscataway, NJ, USA) was swollen for 1 h in 40 ml Tris buffer per 1 g HS and then washed with 5 changes of buffer. Excess buffer was removed from the HS and then the HS was incubated with the 80% AS ppt. for 1 h with gentle shaking. The HS was poured into a 10 ml column (1.5 cm $\times$ 6 cm) and the breakthrough (BT) was collected. The protein bound to the HS (BO) was eluted from the column at 15 ml/h with a 2 M-NaCl–Tris buffer step (approximately 2 column volumes collected as a single fraction). Fractions were diluted and assayed in triplicate in the [^3H]thymidine incorporation assays.

The breakthrough of the 80% AS ppt. (80% BT) which was generated from the heparin–Sephacryl column was applied to a Sephacryl S-200 column (100 ml packed volume, 1.5 cm $\times$ 110 cm; Sigma) equilibrated with 0.01 M-phosphate buffer saline (PBS) buffer. The Sephacryl column was precalibrated with the following standards: bovine albumin (M_r 66 000), carbonic anhydrase (M_r 29 000), cytochrome C (M_r 12 400), and aprotinin (M_r 6500) (Sigma). Buffer was pumped through the column at 15 ml/h, 2.5-ml fractions were collected and aliquants were tested in cell culture for effects on [^3H]thymidine incorporation into CPAE cells.

Autoradiography

For autoradiography, 20 000 CPAE cells were seeded in 400 μl DMEM containing 10% FCS in 4-chamber plastic slides (Thomas Scientific, Swedesboro, NJ, USA). After overnight incubation for cellular attachment, the cells were washed twice, 400 μl each, with serum-free DMEM. Then 400 μl DMEM without serum were added, followed by the addition of 100 μl diluted sample. After incubation for 48 h, 1 μCi [^3H]thymidine in 10 μl DMEM was added. After 24 h the media were removed and the cells were fixed in methanol twice for periods of 5 min each (Klagsburn *et al.*, 1977). After 4 washes with tap water, cold 5% trichloroacetic acid (TCA) was added twice for periods of 10 min each, followed by 4 washes with water. The chamber apparatuses were removed from the slides and the slides were dipped in NTB2 nuclear track emulsion (Kodak, Rochester, NY, USA) and exposed in the dark for 3 days. The emulsion-coated slides were developed in Dektol and then fixed. The percentage of labelled cells was determined by counting the labelled and unlabelled cells in 10 microscope fields, with each field containing more than 10 cells. The percentage of labelled cells (no. labelled/total no.) $\times$ 100 was determined for each field and the mean $\pm$ s.e.m. for 10 fields was calculated.

Trypan blue exclusion

CPAE cells were washed and plated as described above for autoradiography. At 18 h after the addition of 80% BT, the media were removed and 400 μl trypan blue were added. The numbers of cells stained with trypan blue (dead cells) and excluding trypan blue were counted.

Tumour necrosis factor

Human recombinant tumour necrosis factor- α (TNF) lot L9009AX, was supplied by Genentech Inc., South San Francisco, CA, USA. The specific activity of TNF determined by in-vitro LM fibroblast cell lysis was 4.03×10^7 U/mg and endotoxin present in the preparation was ≤ 0.06 EU/mg as determined by the *Limulus* amoebocyte lysate (LAL) assay. These determinations were made by Genentech Inc. and provided on the data sheet that accompanied the TNF α .

Protein determination

Protein determinations were made using the Bio-Rad Protein Assay of Bradford (1976) (Bio-Rad Laboratories, Richmond, CA, USA).

Statistical analysis

A total of 12 different batches of ovaries were extracted for these experiments. Data are presented as the mean $\pm$ s.e.m. of determinations from 3 replicate cultures within each treatment group and are derived from a single experiment. Observations were confirmed in 3–8 independent experiments. Student's *t* test was used to detect differences

between means in some experiments. When appropriate, data were analysed by 1-way or 2-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. Values were considered significant when $P \leq 0.05$.

Results

Ammonium sulphate extraction

CPAE cells (Fig. 1a). At high protein concentration, [3 H]thymidine uptake was significantly less ($P < 0.001$) with the 80% AS ppt. when compared with 0% FCS controls. At the lowest protein concentrations of the 80% AS ppt., the uptake of [3 H]thymidine was significantly greater ($P < 0.05$) than 0% FCS. The 40% AS ppt. increased the uptake of [3 H]thymidine incorporation compared to 0% FCS. [3 H]thymidine incorporation by CPAE cells was stimulated at the high protein concentrations with the 60% and 95% AS ppts. Higher protein concentrations of the 40 and 95% AS ppts could not be tested due to their low solubility.

3T3 cells (Fig. 1b). No inhibitory effect on the uptake of [3 H]thymidine by 3T3 cells was observed with any AS ppt. (40%, 60%, 80% and 95%). Stimulation of [3 H]thymidine uptake was observed with at least one concentration of the AS ppts tested.

Fig. 1. Effects of the 40–95% ammonium sulphate (AS) precipitates on [3 H]thymidine incorporation by cow pulmonary artery endothelial (CPAE) cells (a) and fibroblast (3T3) cells (b). The basal level of [3 H]thymidine uptake in response to media alone is indicated by the open circles. Values are the mean $\pm$ s.e.m. of 3 replicate cultures derived from a single experiment and are representative of 3 independent experiments. [3 H]thymidine incorporation in response to various AS precipitates was considered statistically different from that in response to 0% FCS (control) when the P value was ≤ 0.05 as determined by Student's t test. AS ppt. versus 0% FCS: * $P < 0.05$.

Heparin–Sephacrose chromatography

CPAE cells (Fig. 2). The portion of the 80% ppt. that exhibited inhibitory effects ($P < 0.001$) on CPAE cell uptake of [3 H]thymidine did not bind the HS but was contained in the breakthrough (80% BT) (Fig. 2). Dilution of the 80% BT to ~ 2 μ g/well stimulated ($P < 0.05$) [3 H]thymidine uptake by the CPAE cells. That portion of the 80% fraction bound to the HS (80% BO) which was eluted by a 2 M salt step did not alter [3 H]thymidine incorporation compared to 0% FCS.

3T3 cells (Fig. 3a). At the protein concentrations tested, the 80% AS ppt., 80% BT or 80% BO did not inhibit the uptake of [3 H]thymidine by 3T3 cells. The 80% AS ppt. and the 80% BT stimulated thymidine uptake (Fig. 3a) by 3T3 cells.

Fig. 2. Effects of 80% AS ppt., 80% heparin–Sephacrose column breakthrough (80% BT), and 80% heparin–Sephacrose column bound (80% BO) on [^3H]thymidine incorporation by CPAE cells. The values are the mean $\pm$ s.e.m. of 3 replicate cultures derived from a single experiment and are representative of 6 independent experiments. The basal level of [^3H]thymidine uptake in response to medium alone is indicated by the open circle. AS ppt. *versus* 0% FCS: * $P < 0.05$.

Fig. 3. Effects of 80% AS ppt., 80% heparin–Sephacrose column breakthrough (80% BT), and 80% heparin–Sephacrose column bound (80% BO) on [^3H]thymidine incorporation by (a) 3T3 cells and (b) L929 cells. Values are the mean $\pm$ s.e.m. of 3 replicate cultures derived from a single experiment and are representative of 4 independent experiments. The basal level of [^3H]thymidine uptake in response to medium alone is indicated by the open circles. AS ppt. *versus* 0% FCS: * $P < 0.05$.

Fig. 4. The effect of various amounts of 80% BT and human recombinant TNF α on [3 H]thymidine incorporation by CPAE cells. Values are the mean $\pm$ s.e.m. of 3 replicate cultures derived from a single experiment and are representative of 3 independent experiments.

Fig. 5. The effect of 80% BT on CPAE cell trypan blue uptake and [3 H]thymidine incorporation. After 18 h of incubation with the 80% BT, CPAE cells were stained with trypan blue, and the percentage stained (no. cells stained/total no. cells $\times$ 100) was determined. In a parallel study after 18 h of incubation with 80% BT, CPAE cells were pulsed for 4 h with [3 H]thymidine to determine thymidine incorporation. Control levels of trypan blue uptake and [3 H]thymidine incorporation are indicated by the open symbols.

L929 cells (Fig. 3b). Inhibitory effects on L929 cell [3 H]thymidine incorporation were apparent in the 80% AS ppt. and 80% BT fractions. Compared to controls, there was approximately a 60% reduction in [3 H]thymidine uptake by the L929 cells at the highest protein concentration of 80% AS ppt. and 80% BT tested. The 80% BO fraction, at the highest protein concentration tested, exhibited stimulatory effects ($P < 0.05$) on L929 [3 H]thymidine uptake. With a reduction in protein concentration the 80% BO fraction did not alter [3 H]thymidine incorporation compared to controls.

Effects of various amounts of 80% BT and TNF

The 80% BT and TNF α , each reduced the uptake of [3 H]thymidine by CPAE cells in a dose-dependent manner (Fig. 4). CPAE cells appeared to be more sensitive to TNF: 1 ng TNF reduced

Fig. 6. The percentage labelled CPAE cells (no. labelled/total no. $\times$ 100) as determined by autoradiography after incubation with crude (Cr), 80% AS ppt., 80% BT, or 0, 0.2, 1.0, 2.0% FCS. The percentage indicated is the mean $\pm$ s.e.m. of 10 microscopic fields. Total protein added/500 μ l was: crude, 76.5 μ g; 80% AS ppt. 163 μ g; 80% BT, 6.7 μ g. * P < 0.05, 0% FCS versus treatment, Student's t test.

Fig. 7. [3 H]thymidine incorporation by CPAE cells after incubation with the 80% BT fraction plus or minus various amounts of fetal calf serum (FCS). 80% BT versus serum control, * P < 0.05 (Student's t test).

thymidine incorporation to 13% of control values while 40 μ g were needed to give the same reduction for the 80% BT fraction.

Effects of 80% BT on CPAE cell trypan blue uptake and [3 H]thymidine incorporation

Increasing concentrations of 80% BT resulted in a dose-dependent increase in trypan blue uptake by CPAE cells (Fig. 5). The highest dose of 80% BT resulted in 80.6% of the CPAE cells staining with trypan blue. The lowest dose of 80% BT resulted in trypan blue uptake not different from control (13% and 13.8%, 80% BT and control, respectively). In a parallel study, [3 H]thymidine incorporation by CPAE cells was reduced in a dose-dependent manner with increasing concentration of 80% BT. The highest dose of 80% BT resulted in 10% [3 H]thymidine incorporation

Fig. 8. The effect of Sephadryl S-200 chromatography of the 80% BT on CPAE cell thymidine incorporation. The 80% BT was applied to a Sephadryl S-200 column. The horizontal line indicates levels of [^3H]thymidine incorporation in response to media alone. An inhibitory region of activity corresponds to fractions 39–42, a molecular weight range of 30 000–50 000. The absorbance of each fraction at 280 nm is indicated. Values are the mean $\pm$ s.e.m. of 3 replicate cultures derived from a single experiment and are representative of 3 independent experiments. An inhibitory region was considered when at least two consecutive fractions exhibited a statistically significant reduction in [^3H]thymidine incorporation compared with the control of media alone. * $P < 0.05$ effect of treatment compared with control.

when compared with control. The lowest dose of 80% BT resulted in 120% of the [^3H]thymidine incorporation compared to control.

Effects of 80% AS fractions

Incubation of CPAE cells with the 80% AS fraction (163 μg) resulted in 5.4% cell labelling with [^3H]thymidine as assessed by autoradiography (no. of labelled cells/total no. of cells/10 fields, in triplicate wells) (Fig. 6). The 80% BT fraction (6.7 μg) resulted in 3.9% cell labelling. The control conditions of incubation with media alone resulted in 42% CPAE cell labelling and incubation with 2% FCS resulted in 79% cell labelling with [^3H]thymidine.

Effects of fetal calf serum and 80% BT

The 80% BT fraction significantly ($P < 0.025$) reduced incorporation of [^3H]thymidine by CPAE cells compared with controls (Fig. 7). The inhibitory effect of the 80% BT was still apparent in the presence of 0.2% FCS. The addition of 1% or 2% FCS to the 80% fraction prevented the reduction in thymidine incorporation by CPAE cells.

Sephacryl S-200 chromatography of the 80% BT

After separation by molecular sieve chromatography, the molecular weight range for the fractions of the 80% BT that inhibited [3 H]thymidine uptake by CPAE cells corresponded to fractions 39–42 (M_r 30 000–50 000) (Fig. 8).

Discussion

The cow ovary contained a fraction (80% ammonium sulphate precipitate) that inhibited the incorporation of [3 H]thymidine by CPAE and L929 cells yet exhibited a stimulatory effect on 3T3 cell thymidine uptake at high and not low protein concentrations. This factor, partly purified from the bovine ovary (80% BT), resembles the actions of tumour necrosis factor- α (TNF α) that have been previously reported. The in-vitro bioactivity of TNF α revealed inhibition of [3 H]thymidine incorporation by endothelial (Sato *et al.*, 1986; Schweigerer *et al.*, 1987) and by L929 cells (Carswell *et al.*, 1975; Ruff & Gifford, 1980; Aggarwal *et al.*, 1985), and stimulation of [3 H]thymidine incorporation by 3T3 cells (Sugarman *et al.*, 1985; Vilcek *et al.*, 1986, 1987). The molecular weight range (30 00–50 000) of the 80% BT thymidine-incorporation inhibitory activity as determined by Sephacryl S-200 filtration is also similar to TNF α (Fisch & Gifford, 1983). TNF α has a molecular weight of 17 000 under reducing conditions (Pennica *et al.*, 1984; Aggarwal *et al.*, 1985) and 45 000 under non-reducing conditions (Aggarwal *et al.*, 1985; Old, 1985; Smith & Baglioni, 1987). Fisch & Gifford (1983) have shown that native, non-reduced TNF α elutes from Sephacryl S-200 at a molecular weight of about 48 000.

The extraction procedures utilized were a modification of those used by Gospodarowicz *et al.* (1985), Folkman *et al.* (1984) and Beach *et al.* (1983) for the extraction of endothelial cell growth regulators. TNF α has also been extracted from rabbit serum (Matthews, 1978; Matthews & Watkins, 1978) and *E. coli* cell lysates (Bringman & Aggarwal, 1987) with ammonium sulphate precipitation. A similarity therefore exists between methods for TNF extraction and methods used for the extraction of the 80% inhibitory activity. TNF α has also been localized immunocytochemically to the follicular and luteal compartments of the cow ovary (Roby & Terranova, 1989). It is possible that the 80% inhibitory activity is TNF or a TNF-like fraction. Western blot analysis and neutralization of bioactivity to TNF α are required but sufficient quantities of these specific antisera are at present unavailable.

The 80% AS ppt. and 80% BT fractions resulted in inhibition of [3 H]thymidine incorporation by CPAE and L929 cells only at the highest protein concentrations tested (Figs 1a, 2, 3b). The reason that different protein concentrations were tested among precipitates was due to the different solubilities. The precipitates were dissolved in the smallest volume of buffer to clarify the solution and thus each exhibited a different protein concentration. The thymidine incorporation assays were performed using the maximum protein concentration possible. Upon dilution of the 80% BT, [3 H]thymidine incorporation then increased beyond controls and this response was similar to the effect of TNF α on tumour cells (Goeddel *et al.*, 1986). The inhibitory effects of the 80% AS ppt. and 80% BT fractions were not entirely due to an excessively high concentration of protein. Various fractions exhibiting similar total protein concentrations did not inhibit thymidine uptake (see Fig. 1, 60% AS ppt.; Fig. 2, 80% BO).

Comparison of the 80% AS ppt. and TNF inhibition of [3 H]thymidine incorporation by CPAE cells (Fig. 4) indicates that both exhibit similar effects, although the TNF α was more potent. This observation is not unexpected and might be explained by the relative purity of each sample. TNF is a pure recombinant protein while the 80% ppt. is a protein fraction consisting of several compounds of different molecular weight (see Fig. 8).

Thymidine incorporation into the nuclear fraction was used as a method to assess cellular proliferation. The reduction in [3 H]thymidine incorporation observed after 80% fraction treatment may have been due to a decreased number of viable cells. Because cellular incorporation of thymidine does not always correlate with DNA replication and cell division, the effects of the 80% AS

ppt and the 80% BT fractions on cellular thymidine incorporation and viability were further examined by topical autoradiography and trypan blue exclusion. The 80% AS ppt. and 80% BT fractions reduced the percentage of CPAE cells that incorporated [^3H]thymidine as determined by autoradiography. Trypan blue uptake studies indicated an increase in percentage of CPAE cells incorporating the dye following 80% fraction treatment. Because exclusion of trypan blue by cells indicates cellular viability, these results demonstrate decreased cellular viability after treatment with the 80% fraction which correlates well with decreased [^3H]thymidine incorporation (autoradiography and cell harvest method). Collectively these results indicate that the 80% ppt. and 80% BT killed the CPAE cells. Future experiments to further confirm the effect of the 80% fraction on reducing [^3H]thymidine incorporation should include assessment of cellular DNA. The effect of the 80% fraction on reducing thymidine incorporation was not overcome by the addition of a small amount (0.1%) of serum to the culture; however, addition of a greater amount of serum (1–2%) resulted in loss of the inhibitory activity. Growth factors present in serum, when added in low concentrations (0.1%), were unable to overcome the most potent inhibitory effect of the 80% BT; however, the inhibitory effect of the 80% BT was overcome by the addition of greater amounts of growth factors (serum). Growth factors in the serum may overcome the effect of the 80% BT, but it is also possible that binding proteins in the serum may have inactivated the inhibitory compounds. *In vivo*, plasma may negate the effect of the inhibitory fraction and act as a point of physiological regulation.

What are the possible roles of this inhibitory activity of the 80% fraction in ovarian function? The inhibitory effect on endothelial cell proliferation might account for the marked difference observed in the vascularization of healthy and atretic follicles (Hay *et al.*, 1976). In the ewe, it has been shown that the inner capillary wreath of atretic follicles was about one-third as vascular and contained 85% fewer erythrocytes than did non-atretic follicles (Hay *et al.*, 1976). The level of capillary blood flow within the theca of follicles in advanced stages of atresia was significantly less than that in non-atretic follicles (Findlay & Carson, 1980; Carson *et al.*, 1986). Although alterations in blood flow were not detected in early atresia, another study has shown degenerative changes in the inner capillary network of the theca of primary atretic sheep follicles (O'Shea *et al.*, 1978). The inhibitory effects of the 80% fraction on the endothelial cells may mediate these vascular changes. The 80% inhibitory fraction may therefore participate in atresia by altering the follicular vasculature and the integrity of the membrana granulosa which could induce a complement reaction and cause lysis of the granulosa cells as suggested by Farookhi (1981). The ability of the 80% fraction to stimulate normal fibroblast (3T3) thymidine incorporation might also indicate another possible role for this fraction in atresia. During follicular atresia the thecal layer of the follicle increases in thickness due to cellular hypertrophy, cellular proliferation, and infiltration of fibroblasts ultimately forming the interstitial gland tissue (see Guraya, 1985). The activity of the 80% fraction may therefore have a physiological role in atresia by stimulating enlargement of the theca and formation of the interstitial gland tissues.

Endothelial cell growth factors extracted from the ovary have been implicated in the development of an extensive vasculature in corpora lutea (Gospodarowicz & Thakral, 1978; Goodman & Rone, 1985; Gospodarowicz *et al.*, 1985; Koos, 1986). It also seems possible that an inhibitor of endothelial cell growth could also function to limit growth and/or disrupt the organization of the vasculature during luteal regression. An early change during luteal regression is that the percentage volume of the corpus luteum occupied by vascular elements decreases linearly with time (Niswender *et al.*, 1976). Changes in endothelial cells with luteal regression include nuclear pycnosis and cellular disintegration (Lobel & Levy, 1968) which are similar to the actions of $\text{TNF}\alpha$ on endothelial cells (Sato *et al.*, 1986; Mano-Hirano *et al.*, 1987; Movat, 1987; Sato *et al.*, 1987). The results of the present study indicate that the bovine ovary contains an inhibitor of endothelial cells that exhibits biologically active characteristics similar to $\text{TNF}\alpha$ and it may have a role in follicular atresia and luteal regression.

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