

Antiproliferative Actions of Ovine Uterine Serpin

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PROBLEM: Ovine uterine serpin (OvUS) is a member of the serine proteinase inhibitor superfamily and is the major protein produced by luminal and glandular epithelium of the sheep endometrium during mid to late pregnancy. The protein does not have prototypical proteinase inhibitory activity but can inhibit a wide variety of lymphocyte functions such as mitogen-induced proliferation and natural killer cell cytotoxicity.

METHOD OF STUDY: The antiproliferative actions of OvUS were studied.

RESULTS: It was demonstrated that, in addition to inhibiting lymphocyte proliferation, OvUS inhibits growth of two tumor cell lines (D17 and PC-3). The protein also interrupts development of pre-implantation embryos. Inhibition of cell proliferation is not universal, however, as OvUS did not inhibit growth of two non-tumorigenic cell lines (MDBK and BEND). The mechanism of action of inhibitory effects of OvUS is not known although experiments with inhibitors of protein kinase A indicate that the protein does not inhibit lymphocyte proliferation through this pathway. Moreover, the protein does not induce apoptosis.

CONCLUSIONS: The finding that OvUS has antiproliferative activity is demonstrative of the wide range of functions exerted by members of the serpin superfamily. The antiproliferative property of OvUS may reflect the role of the protein during pregnancy and may be exploitable for design of new antiproliferative drugs.

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INTRODUCTION

The serpins are a superfamily of proteins of about 500 known members that fold into a conserved structure and inhibit serine proteinases through a unique suicide-like mechanism.^{1,2} Several members of this family have functions distinct from proteinase inhibition. Among these functions are regulation of blood pressure (angiotensinogen), inhibition of angiogenesis (pigment epithelium-derived factor), hormone binding (corticosteroid binding globulin, thyroxine binding globulin), regulation of neuron survival and differentiation (pigment epithelium-derived factor), and interactions with chromatin (MENT).^{1,2} Two serpins have been found to interfere with cell growth. One is maspin, which is a member of the ovalbumin clade of serpins expressed in mammary epithelial cells and which facilitates induction of apoptosis in carcinoma

cells.^{3,4} The second is ovine uterine serpin (OvUS), which is a member of the α_1 -antitrypsin clade expressed in uterine epithelial cells under the influence of progesterone⁵ and which can inhibit proliferation of $\alpha\beta$ T lymphocytes.^{6–9}

Genes related to OvUS exist in cow, goat, and pig^{1,10,11} but only the protein produced in the sheep uterus has been tested for lymphocyte-inhibitory activity. Although OvUS is a weak inhibitor of pepsin,¹² it does not appear to exist in the stressed conformation characteristic of inhibitory serpins¹³ and it may be an inactive proteinase inhibitor. OvUS can inhibit lymphocyte proliferation induced by mixed lymphocyte reactions, phytohemagglutinin (PHA), concanavalin A, and *Candida albicans*^{6–9} and also inhibit natural killer cell activity.^{14,15} It has been hypothesized that the major role of OvUS is to inhibit lymphocyte function during pregnancy and thereby

facilitate survival of the antigenically-foreign conceptus in the uterus.¹⁶ Progesterone administration delays allograft and xenograft rejection in the uterus^{17,18} and OvUS may mediate this effect of progesterone. OvUS also binds IgM, IgA and activin^{19,20} but the physiological significance of binding is not clear.

It is not known whether the antiproliferative activity of OvUS is specific for lymphocytes or whether the protein exhibits broad specificity as an antiproliferative molecule. The latter possibility may be the case because OvUS can bind to the surface of non-lymphoid cells²¹ and crude uterine fluid from pregnant ewes inhibits proliferation of MDBK, FG10L, and J774 cell lines.²² Inhibitory actions of OvUS on lymphocyte proliferation are somewhat selective, however, as the protein did not inhibit lymphocyte proliferation induced by pokeweed mitogen⁷ and did not inhibit activation of $\gamma\delta$ T cells.⁹

The present study was carried out to test whether OvUS can inhibit proliferation of a range of normal and tumor cells as well as pre-implantation embryos. A second objective was to determine whether the antiproliferative actions of OvUS on lymphocyte proliferation involved protein kinase A (PKA) because signaling through this pathway can lead to inhibition of lymphocyte proliferation.²³ Finally, it was tested whether, as for the serpin maspin,^{3,4} OvUS induces apoptosis.

MATERIALS AND METHODS

Materials

Tissue Culture Medium-199 (TCM-199), Eagle's Minimum Essential Medium (MEM), MEM with D-valine, RPMI 1640, Ham's F12, Dulbecco's phosphate-buffered saline (DPBS), penicillin-streptomycin, red cell lysis buffer, and Rp-8-Cl-cAMPS were purchased from Sigma-Aldrich (St Louis, MO, USA). Horse serum was obtained from Hyclone (Logan, UT, USA), fetal bovine serum was from Intergen (Purchase, NY, USA) and Fico-Lite 1077 was from Atlanta Biologicals (Norcross, GA, USA). The D-17 (canine lung osteosarcoma), PC-3 (human prostate cancer), MDBK (bovine kidney epithelial cell), and BEND (bovine endometrial cell) cell lines were purchased from ATCC (Rockville, MD, USA). Centricon ultrafiltration devices were from Amicon (Beverly, MA, USA). Carboxymethyl Sepharose and Sephacryl S-200 were from Amersham Biosciences (Piscataway, NJ, USA) and [³H]thymidine (6.7 Ci/mmol) was from ICN (Irvine, CA, USA). The RQ1 RNase-free DNase was from Promega (Madison, WI, USA), *in situ* cell death detection kit [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)] was from

Roche (Indianapolis, IN, USA), ProLong[®] Antifade kit was purchased from Molecular Probes (Eugene, OR, USA) and RNase A was from Qiagen (Valencia, CA, USA). Supplies for production of embryos by *in vitro* fertilization were obtained as described elsewhere.²⁴ Other reagents were from either Sigma-Aldrich or Fisher.

Purification of Ovine Uterine Serpin

Ovine uterine serpin was purified from the uterine fluid accumulating in the ligated horn of unilaterally-pregnant ewes at day 140 of pregnancy using a combination of cation-exchange chromatography with carboxymethyl Sepharose and gel filtration chromatography with Sephacryl S-200 as described previously.¹⁴ Purity of protein as assessed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis was >90%. After purification, OvUS was dialyzed against DPBS and concentrated using Centricon ultrafiltration devices. Protein concentration was determined using the Bradford procedure²⁵ with bovine serum albumin as standard.

Antiproliferative Actions of OvUS

Peripheral blood lymphocytes. Blood was obtained by jugular venipuncture from non-pregnant Rambouillet ewes by collection into heparinized tubes. Mononuclear cells were purified from the buffy coat fraction of blood by density gradient centrifugation as described previously except that adherent cells were not removed.¹⁵ Cells were suspended in modified TCM-199 [TCM-199 containing 5% (v/v) horse serum, 200 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM supplemental glutamine, and 10⁻⁵ M β -mercaptoethanol] to a concentration of 1 \times 10⁶ cells/mL.

The lymphocyte proliferation assay was performed by addition of 1 \times 10⁵ lymphocytes in culture medium containing 4 μ g/mL PHA and various concentrations of OvUS (0.25, 0.5 and 1 mg/mL) or ovalbumin (negative control; 1 mg/mL) in a final volume of 190 μ L. The final volume of DPBS (the diluent for OvUS) was the same in each well (5–25 μ L). After 48 hr, 0.1 μ Ci [³H]thymidine in 10 μ L culture medium was added. Cells were harvested onto glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD, USA) device at 24 hr after thymidine addition. Filters were counted for radioactivity using scintillation spectrometry. The experiment was performed three times using lymphocytes from a separate ewe for each replicate.

Cell lines. The D17 and MDBK cell lines were cultured continuously in MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/mL penicillin and 2 mg/mL streptomycin. The PC-3

cells were grown in Ham's F12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/mL penicillin and 2 mg/mL streptomycin. The BEND cells were grown in 1:1 (v/v) mixture of Ham's F12 and the D-valine modification of Eagle's MEM supplemented with 200 U/L insulin, 10% (v/v) heat-inactivated fetal bovine serum, 10% horse serum, 200 U/mL penicillin and 2 mg/mL streptomycin. At confluence, cells were trypsinized, mixed with an equal amount of culture medium, centrifuged for 5 min at 110 g, resuspended in culture medium, counted and adjusted to 1×10^5 cells/mL in culture medium.

The proliferation assay involved culture of 1×10^4 cells in 100 μ L total volume in individual wells of 96-well culture plates. After 24 hr at 37°C and 5% (v/v) CO₂, various concentrations of OvUS (0.25, 0.5 and 1 mg/mL final concentration) and ovalbumin (0.5 and 1 mg/mL) were added. For the control wells, an equivalent volume of DPBS (5–25 μ L) was added instead of OvUS or ovalbumin. The final volume was adjusted with cell culture medium to 190 and 10 μ L (0.1 μ Ci/well) [³H]thymidine (in culture medium) was also added to each well. The cells were incubated at 37°C and 5% (v/v) CO₂ for 48 hr, harvested onto glass-fiber filters and tritium measured as described as above. For each assay, all treatments were performed in triplicate. The experiment was performed on 12, 8, 6, and 4 separate occasions for D17, PC-3, MDBK, and BEND cells respectively.

Effects of OvUS on Development of Pre-implantation Embryos

Pre-implantation bovine embryos were produced by *in vitro* fertilization.²⁴ After fertilization, presumptive zygotes were removed from fertilization wells, denuded of cumulus cells by vortexing in ~40 μ L of HEPES-TALP, washed two to three times in HEPES-TALP, and cultured in groups of 14–30 embryos at 38.5°C and 5% (v/v) CO₂ in humidified air in 50 μ L microdrops of modified KSOM-BE2 containing either 0.5 mg/mL OvUS, 1.0 mg/mL OvUS, 1.0 mg/mL ovalbumin, or an equivalent volume of DPBS as used for OvUS and ovalbumin treatments (20 μ L). Microdrops were overlaid with mineral oil and embryos cultured at 38.5°C and 5% (v/v) CO₂ in humidified air. Embryos were evaluated for cleavage at day 3 and for blastocyst development at day 8. The experiment was replicated four times with a total of 78–97 oocytes per treatment.

Effect of Inhibition of Protein Kinase A on Antiproliferative Activity of OvUS

Lymphocyte proliferation assays were carried out as described above. Aliquots of 100 μ L lymphocytes (purified from jugular blood as described earlier) containing 1×10^5 cells were placed into wells of a

flat-bottomed, 96-well cell culture plate in a total volume of 190 μ L containing 5 μ g/mL PHA, one of two proteins (1 mg/mL OvUS or 1 mg/mL ovalbumin), either 5 μ M Rp-8-Cl-cAMPS (a non-hydrolyzable cAMP analog which is a selective inhibitor of cAMP-dependent type-I PKA) or an equivalent volume of vehicle (DPBS), and with modified M-199 to bring the final volume to 190 μ L. After 48 hr of incubation at 37°C, 10 μ L (0.1 μ Ci/well) [³H]thymidine (in modified M-199) was added to each well. Cells were incubated at 37°C and 5% (v/v) CO₂ overnight, harvested using a cell harvester and tritium incorporation measured using a liquid scintillation counter. The experiment was performed five times using lymphocytes from a separate non-pregnant ewe for each replicate.

Induction of Apoptosis in D17 and PC-3 Cells

The D17 and PC-3 cells were cultured as described before. At confluence, cells were trypsinized for 3 and 10 min respectively, mixed with an equal amount of culture medium, centrifuged for 5 min at 110 g, resuspended in culture medium, counted and adjusted to 1×10^5 cells/mL in culture medium. Cells were then plated in 96-well plates to determine effect of OvUS on proliferation (conducted as described before) or apoptosis. To determine apoptosis, 1×10^4 cells in 100 μ L were cultured with vehicle, 1 mg/mL OvUS or 1 mg/mL ovalbumin. For the vehicle treatment, an equivalent volume of DPBS was added instead of OvUS or ovalbumin. The final volume in all wells was adjusted with culture medium to 200 μ L. Cells were cultured at 37°C and 5% (v/v) CO₂ for 24 hr and then harvested for the detection of apoptotic cells by TUNEL. All treatments were performed in triplicate and the experiment was performed on three (D17) or one (PC-3) separate occasions.

Cells were processed for TUNEL staining as follows. Cells were washed by centrifugation with 0.1 M sodium phosphate, pH 7.4 with 0.9% (w/v) NaCl (PBS) and containing 1 mg/mL polyvinyl pyrrolidone (PBS/PVP), and then resuspended in 200 μ L 4% (w/v) paraformaldehyde in 0.2 M sodium phosphate, pH 7.4 with 0.9% (w/v) NaCl for 1 hr at room temperature. Cells were washed again with PBS/PVP, resuspended in 200 μ L PBS/PVP, and 100 μ L cell suspension was transferred to a poly-L-lysine coated slide and allowed to dry for at least 24 hr at room temperature. For TUNEL staining, slides were washed twice in PBS/PVP (2 min each) and then incubated with permeabilization solution [0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate in PBS] for 1 hr at room temperature. Positive controls were incubated with RQ1 RNase-free DNase (50 U/mL) at 37°C for 1 hr. Slides were washed in PBS/PVP and then incubated with 50 μ L TUNEL reaction mixture (containing

fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by the manufacturer) for 1 hr at 37°C in the dark. Negative controls were incubated in the absence of the enzyme. After washing, slides were incubated with RNase A (50 µg/mL) for 1 hr at room temperature and then with 12.5 µg/mL propidium iodide for 30 min at room temperature. Cells were washed four times in PBS/PVP to remove excess propidium iodide and coverslips mounted with mounting medium containing Prolong Antifade® as recommended by the manufacturer. Slides were observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter (Carl Zeiss, Inc., Göttingen, Germany). Images were acquired using AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Carl Zeiss, Inc., Thorwood, NY, USA). Percent apoptotic cells were determined by counting the total number of TUNEL-labeled nuclei at 10 different sites on the slide.

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models Procedure of SAS (SAS System for Windows, Version 8.02; SAS Institute, Cary, NC, USA). For each analysis, replicate was considered a random effect and other main effects were considered fixed. Error terms were determined based on calculation of expected mean squares. In some analyses, the pdiff mean separation test of SAS was performed to distinguish differences between various levels of an effect. In addition, variance associated with treatment was portioned into individual degree-of-freedom comparisons using orthogonal contrasts to compare groups of treatments (for embryo data) or using orthogonal polynomial contrasts to determine whether the response to increasing concentrations of OvUS was linear, quadratic or cubic (for cell line data).

RESULTS

Inhibitory Activity of OvUS on Proliferation of Lymphocytes and Cell Lines

Ovine US inhibited PHA-induced proliferation of lymphocytes in a concentration-dependent manner best that was best described as a quadratic response (Fig. 1). In addition, OvUS selectively inhibited growth of several different cell lines as measured by incorporation of [³H]thymidine into DNA (Fig. 2). While OvUS did not inhibit proliferation of BEND and MDBK cells, both of which are not derived from tumors, there was dose-dependent inhibition of proliferation of both D17 and PC-3 tumor cell lines by

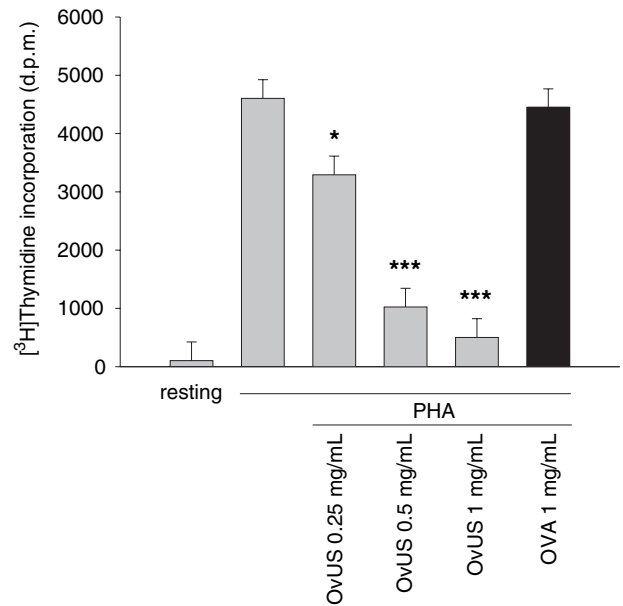


Fig. 1. Inhibition of phytohemagglutinin (PHA)-induced proliferation of peripheral blood lymphocytes by ovine uterine serpin (OvUS). Data represent least squares means ± S.E.M. There was a quadratic decrease in [³H]thymidine incorporation with increasing concentration of OvUS ($P < 0.003$). Means for PHA-stimulated cells that differ from the group treated with PHA alone are indicated by asterisks (* $P < 0.05$; *** $P < 0.001$).

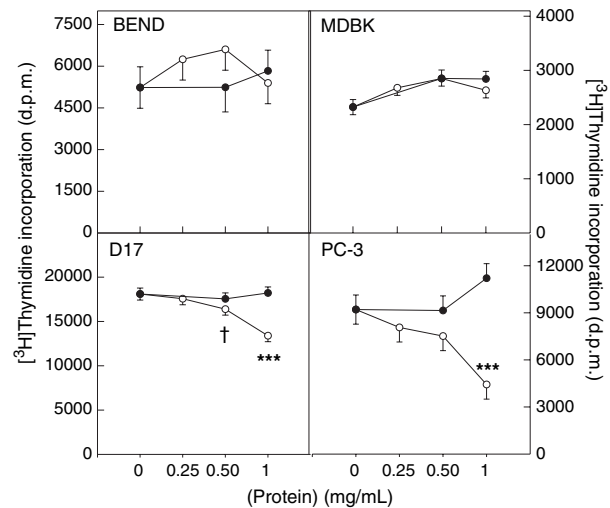


Fig. 2. Inhibition of proliferation of various cell lines by ovine uterine serpin (OvUS). Cells were cultured with various concentrations of OvUS (open circles) or ovalbumin (filled circles). Data represent least squares means ± S.E.M. OvUS did not effect proliferation of BEND and MDBK cells but caused a linear decrease in [³H]thymidine incorporation with increasing concentration of OvUS for D17 ($P < 0.001$) and PC-3 cells ($P < 0.001$). Means that differ from the group without added protein are indicated by symbols († $P = 0.08$; *** $P < 0.001$).

OvUS (linear effect of concentration, $P < 0.001$ for both D17 and PC-3 cells). In contrast, the control serpin, ovalbumin, did not inhibit growth of any of the cell lines tested. The percentage decrease in proliferation was less for D17 and PC-3 cells than for lymphocytes. At a concentration of 1 mg/mL OvUS, for example, incorporation of [3 H]thymidine into DNA was inhibited by 89% for lymphocytes (Fig. 1) versus 26% for D17 cells and 52% for PC-3 cells (Fig. 2).

Effect of OvUS on Development of Bovine Embryos

Addition of OvUS to bovine embryos produced by *in vitro* fertilization had no effect on the proportion of oocytes that cleaved but reduced ($P < 0.02$) the proportion of oocytes that became blastocysts at day 8 after fertilization (Fig. 3). In contrast, addition of 1 mg/mL ovalbumin did not affect the proportion of oocytes that cleaved or became blastocysts.

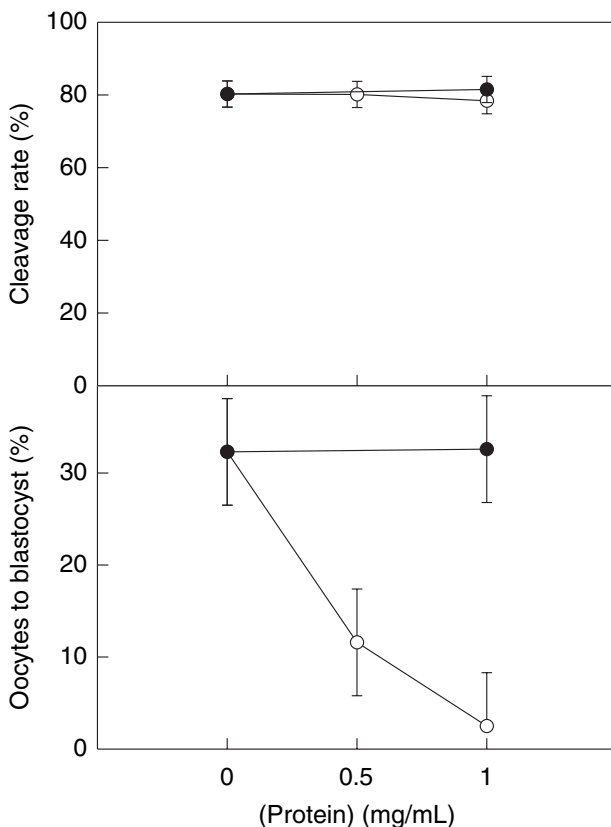


Fig. 3. Inhibition of bovine embryonic development by ovine uterine serpin (OvUS). Embryos produced by *in vitro* fertilization were cultured, beginning after the end of fertilization, with various concentrations of OvUS (open circles) or ovalbumin (closed circle). Data represent least squares means \pm S.E.M. As compared with untreated embryos and embryos cultured with ovalbumin, OvUS inhibited blastocyst formation ($P < 0.02$).

Effect of Inhibition of Protein Kinase A on Antiproliferative Activity of OvUS

An experiment was conducted to test whether the inhibitory effect of OvUS on lymphocyte proliferation was mediated by activation of PKA (Fig. 4). Proliferation, as measured by incorporation of [3 H]thymidine into DNA, was inhibited by OvUS ($P < 0.01$). Addition of the PKA inhibitor, Rp-8-Cl-cAMPS, did not affect the magnitude of proliferation in the absence of OvUS (i.e. in ovalbumin-treated control wells) and did not reverse the effect of OvUS on proliferation.

Apoptosis in D17 and PC-3 Cells

Data in Fig. 5 illustrate results of an experiment to test whether OvUS induces apoptosis in D17 and PC-3 cells. OvUS inhibited proliferation of both D17 and PC-3 cells (Fig. 5A). The proportion of cells that was TUNEL-positive in the absence of OvUS was very low (see Fig. 5B where percent TUNEL-positive was 1.1% for D17 cells and 0.5% for PC-3 cells). The proportion of cells that was TUNEL-positive was only slightly increased by OvUS (Fig. 5B; 2.2% for D17 cells treated with OvUS and 3.3% for PC-3 cells treated with OvUS; Fig. 5B). Thus, apoptotic cells were rare for all treatments (see Fig. 5C for examples of TUNEL staining).

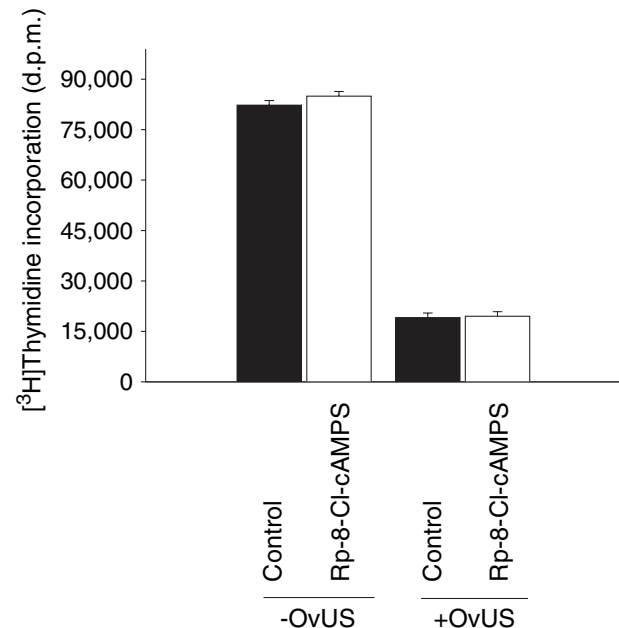


Fig. 4. Inhibition of protein kinase A by addition of Rp-8-Cl-cAMPS does not reverse anti-proliferative actions of ovine uterine serpin (OvUS) on phytohemagglutinin-induced proliferation of PBL. Data represent least squares means \pm S.E.M. Incorporation of [3 H]thymidine into cells was reduced by OvUS ($P < 0.01$) but was not affected by Rp-8-Cl-cAMPS or the interaction of OvUS and Rp-8-Cl-cAMPS.

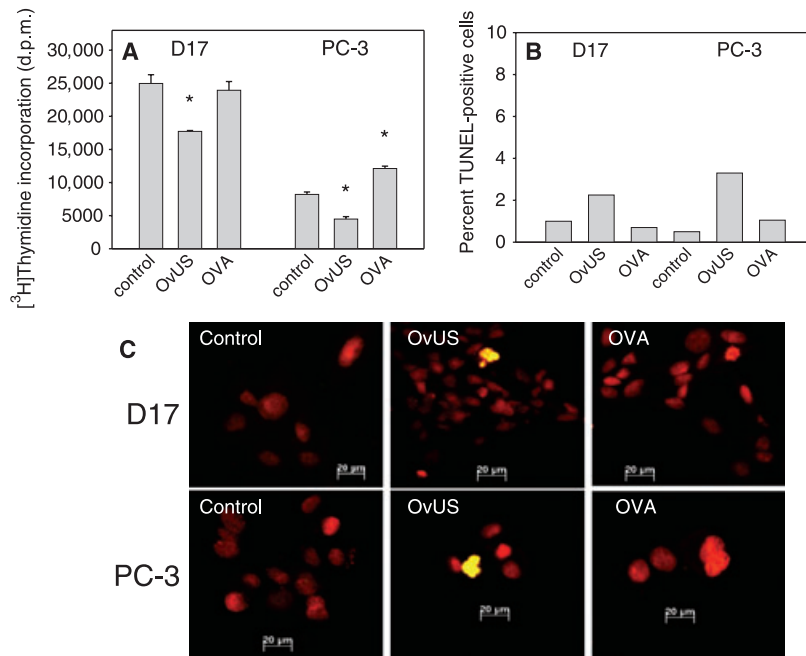


Fig. 5. Effect of ovine uterine serpin (OvUS) on induction of apoptosis in D17 and PC-3 cells as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) labeling. Data in **A** illustrate that addition of 1 mg/mL OvUS reduced incorporation of [³H]thymidine into D17 cells and PC-3 cells. Results for D17 cells are least squares means \pm S.E.M. and are based on three separate assays and with two separate batches of OvUS. Results for PC-3 cells are least squares means \pm S.E.M. and are based on one assay with two separate batches of OvUS. Asterisks represent means that differ from the control value at $P < 0.05$. Data in **B** represent the percent of cells that were TUNEL-positive after 24 hr culture. Data are from a representative assay. **C** shows representative patterns of TUNEL labeling for D17 cells (top row) and PC-3 cells (bottom row). Note that yellow cells are considered apoptotic.

DISCUSSION

While it has long been known that OvUS can inhibit lymphocyte proliferation,⁶⁻⁹ present results demonstrate that OvUS is a more broadly-active inhibitor of cellular proliferation. In particular, OvUS inhibited proliferation of two tumor cell lines (D17 and PC-3) as well as development of pre-implantation bovine embryos. In contrast, there was no growth inhibition for two cell lines not derived from tumors (MDBK and BEND). One possibility is that antiproliferative actions of OvUS are more likely to be exerted on cells with rapid or less-controlled growth.

The observation that OvUS inhibited growth of the two tumor-cell lines tested indicates the potential for use of OvUS, or molecules based on the active site of the OvUS molecule, as an anti-tumor treatment. The absolute magnitude of inhibition of D17 and PC-3 cells was less than for lymphocytes, suggesting that lymphocytes are more susceptible to inhibition than the former cells. Perhaps receptors or binding proteins through which OvUS interact to inhibit cell proliferation are present in greater abundance in lymphocytes. Alternatively, there are unique pathways for inhibition for lymphocytes not present in D17 or PC-3 cells (for example, inhibition of IL-2 receptor

expression,⁹) or intracellular mechanisms to counteract inhibitory actions of OvUS are less prominent in lymphocytes.

The inhibitory effects of OvUS on proliferation of tumor cell lines is reminiscent of the actions of another serpin, maspin, which can block growth of carcinomas through induction of apoptosis.^{3,4} The mechanism by which OvUS blocks cellular proliferation is not known. Given that PKA can be inhibitory to lymphocyte activation,²³ it was tested whether inhibition of PKA would block the inhibitory effects of OvUS on proliferation. The failure of Rp-8-Cl-cAMPS to reduce anti-proliferative effects of OvUS indicate that the mechanism for inhibition is independent of PKA activation. It remains possible, however, that OvUS inhibits lymphocyte activation through induction of cAMP synthesis involving downstream pathways independent of PKA.²⁶ The very small increase in apoptosis in D17 and PC-3 cells treated with OvUS indicates that, unlike maspin,^{3,4} OvUS does not inhibit cell growth by inducing apoptosis.

The lymphocyte-inhibitory actions of OvUS are believed to represent an important mechanism by which the allogeneic conceptus escapes graft rejection by the maternal immune system. Progesterone, which induces endometrial synthesis of OvUS,^{5,27} also leads to

a reduction in tissue rejection responses *in utero*.^{17,18} The concentrations of OvUS required to inhibit lymphocyte proliferation are physiologically relevant and, in fact, lower than the several milligram per milliliter concentrations of OvUS in uterine fluid of unilaterally-pregnant ewes.^{28,29} The physiological significance of the anti-proliferative actions of OvUS on non-lymphoid cells is not clear. As compared to several other species, trophoblast cells of the sheep placenta exhibit only limited invasiveness into the endometrium.^{30,31} It is conceivable that OvUS participates in limiting trophoblast invasion into the uterus by inhibiting trophoblast proliferation.

The inhibition of growth of the pre-implantation embryo seen in the present study is a further reflection of the anti-proliferative actions of OvUS. In a typical pregnancy, pre-implantation embryos would not come in contact with OvUS as the protein is first detected in pregnant ewes at day 16 of gestation²⁷ whereas the embryos used in this study represent stages of development characteristic of the first week after fertilization. However, treatments that advance the exposure of the endometrium to progesterone have been shown to reduce pregnancy rate in cattle,³² and it is possible that this effect of premature exposure to progesterone involves secretion of uterine serpin into the uterine lumen at a time when development is sensitive to disruption by OvUS.

In conclusion, present results indicate that OvUS is an antiproliferative molecule with actions on a variety of cell types. The finding that OvUS has antiproliferative activity extends the range of functions exerted by members of the serpin superfamily and may be relevant to the role of the protein during pregnancy.

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