Expression and Properties of Recombinant Ovine Uterine Serpin

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Ovine uterine serpin (OvUS) is produced in the uterus of sheep under the influence of progesterone. It weakly inhibits pepsin and reduces proliferation of lymphocytes, tumor cell lines, and preimplantation embryos. When purified from uterine fluid, the concentration required for its antiproliferative effect *in vitro* is ~0.25–1 mg/ml. Here we show that recombinant (r) OvUS is a more potent regulator of cell proliferation than native (n) OvUS purified from uterine fluid. To produce rOvUS, RNA was extracted from endometrium from a pregnant ewe and cDNA was amplified by reverse transcription–polymerase chain reaction using gene-specific primers. The purified OvUS cDNA was inserted into the ampicillin-resistant plasmid vector pcDNA3.1/V5-His-TOPO. The plasmid was introduced into the TOP10 *Escherichia coli* strain, purified, and used for transfection of Freestyle 293-F cells. Digestion of rOvUS with protein N-glycosidase F confirmed that rOvUS was N-glycosylated. Both rOvUS and nOvUS inhibited proliferation of phytohemagglutin-activated sheep lymphocytes and the P388D1 mouse lymphoma and PC-3 prostate cell lines. Inhibition was greater for rOvUS than for nOvUS, and concentrations as low as 15 μg/ml rOvUS were effective at reducing lymphocyte proliferation. Addition of rOvUS to fertilized bovine embryos reduced the cleavage rate and the percentage of embryos that became blastocysts. Native OvUS did not affect cleavage rate and had a smaller effect on development to the blastocyst stage. Experiments demonstrate that OvUS is a more potent inhibitor of cell proliferation and embryonic development than previously believed and add credence to the putative role for the protein in regulating cell proliferation. Exp Biol Med 231:1313–1322, 2006

Key words: serpin; uterus; lymphocytes; cell proliferation

The uterine serpins are a class of progesterone-induced proteins secreted by the epithelium of the uterine endometrium in sheep, cattle, pigs, and goats (1–4). Of these, the most well-characterized is ovine uterine serpin (OvUS). This protein is a weak inhibitor of pepsin activity (2, 5); can bind immunoglobulin A (IgA), IgM, and activin (6, 7); and can inhibit a variety of lymphocyte functions. Among its actions on lymphocytes are inhibition of mitogen-induced and mixed-lymphocyte–culture-induced proliferation (8–10), down-regulation of interleukin-2 receptor expression (11), and inhibition of natural killer cell activity *in vitro* (12, 13). *In vivo*, OvUS inhibits polyI•polyC-induced (i.e., natural killer cell–mediated) abortion in mice (12). Because of its actions in the immune system, it has been postulated that the role of OvUS during pregnancy is to inhibit maternal immune responses directed against the conceptus. Recently, it has been shown that the antiproliferative effects of OvUS are not limited to lymphoid cells but they also occur for two tumor cell lines and preimplantation embryos (14).

While OvUS exerts antiproliferative activity, it is not a potent inhibitor of cell proliferation because concentrations from ~250 to 1000 μg/ml are required to cause inhibition. Although these concentrations are well within the range of concentrations of OvUS in the uterus, the low potency of OvUS for inhibition raises the possibility that (i) the antiproliferative activity associated with OvUS is due to a trace contaminant present in preparations of the protein purified from uterine fluid; or (ii) much of the protein recovered from the uterus is biologically inactive. The second possibility is credited because OvUS is typically purified from uterine fluid of unilaterally pregnant ewes at Day 140 of pregnancy and it is possible that much of the protein in that fluid has undergone degradation or denaturation. Indeed, OvUS in uterine fluid exists in part in aggregates and as proteolytic fragments (15, 16).

In this paper, it is demonstrated that recombinant OvUS (rOvUS) produced in mammalian cells possesses antiproliferative activity more potent than previously described. This result confirms that inhibition of cell proliferation is one property of the protein and indicates the utility of the

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recombinant protein for further studies on the function of this progesterone-induced uterine serpin.

Materials and Methods

Collection of Uterine Fluid and Purification of Native OvUS. Ewes of Rambouillet genotype ($n = 4$) were made unilaterally pregnant as described elsewhere (17) by ligating one uterine horn. Ewes were slaughtered at Day 140 of pregnancy by captive bolt stunning and exsanguination. Crude uterine fluid was collected from the ligated uterine horn after slaughter by aspiration. Uterine fluid was clarified by centrifugation at 3600 *g* and stored at –20°C until purification of native OvUS (nOvUS) by a combination of cation-exchange chromatography and gel filtration chromatography as described previously (13).

RNA Extraction. Immediately following slaughter, interplacentomal endometrium (~2 g) was excised from randomly determined regions of the pregnant endometrium of a unilaterally pregnant ewe. Tissue was snap-frozen in cryogenic vials in liquid nitrogen and stored at –70°C until total RNA extraction was performed using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocols. RNA concentration was determined spectrophotometrically.

Cloning of OvUS cDNA. OvUS cDNA was amplified from total endometrial RNA by reverse transcription–polymerase chain reaction (RT-PCR) using gene-specific primers spanning the 5′ and 3′ whole coding sequence of a previously published sequence of *Ovis aries* uterine milk protein precursor A (GenBank accession number M21027). The forward primer was 5′-CACC ATG TCC CAC AGG AGA ATG-3′ and the reverse primer was 5′-CTC AAC TTG GGG GTT GAG GAC T-3′. The forward primer included a CACC sequence to ligate the insert in the vector and the first 18 bases of the signal sequence of the OvUS preprotein. The stop codon was omitted from the reverse primer to allow synthesis of a V5-His–tagged recombinant protein. The expected size of the PCR product was 1291 base pairs (bp).

The RT-PCR was performed using the SuperScript One Step RT-PCR kit with Platinum Taq (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To verify that PCR products were amplified from RNA only, the SuperScript reverse transcriptase was omitted from control reactions. The cDNA synthesis and predenaturation reactions were one cycle at 50°C for 30 mins and 94°C for 2 mins, respectively. The PCR amplification consisted of 35 cycles at 94°C for 30 secs, 46°C for 30 secs, and 72°C for 2 mins; and 1 cycle at 72°C for 5 mins for a final extension. A 2× reaction buffer (containing 0.4 m*M* each dNTP, and 2.4 m*M* $MgSO_4$) supplied in the kit was used in all PCR runs. The PCR products were separated by electrophoresis using a 0.8% (w/v) agarose gel in Tris-acetate buffer (40 m*M* Tris-acetate, pH 8.5 containing 2 m*M* EDTA) that included 0.5 μg/μl ethidium bromide (Sigma-Aldrich). Bands were visualized on a UV transluminator and photographed using either a Polaroid camera (Waltham, MA) or a Sony Mavica CD400 digital camera (Tokyo, Japan).

Sequencing of OvUS cDNA. Amplicons were gel-purified using the S.N.A.P. gel purification kit (Invitrogen,) according to the manufacturer's protocol. Briefly, PCR products were run on 0.8% (w/v) agarose gels containing 5 μg/μl ethidium bromide and bands corresponding to OvUS were excised, loaded into spin filters provided in the kit, and centrifuged at 14,000 *g* at 4°C for 10 mins. The filtrate was washed with DEPC water (Biotecx, Houston, TX) and concentrated by centrifugation twice at 5000 *g* for 20 mins using a Centricon YM30 (Millipore Corporation, Bedford, MA) filter apparatus. The concentration of the DNA was determined spectrophotometrically and purity was assessed by electrophoresis.

Sequencing of the gel-purified OvUS cDNA sample was performed at the University of Florida DNA Sequencing Core Laboratory using ABI Prism BigDye Terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA). The fluorescently labeled extension products were analyzed on an Applied Biosystems Model 373 Stretch DNA Sequencer or on a 3100 Genetic Analyzer (Perkin-Elmer). The primers for double-strand sequencing were the oligonucleotides used for PCR described above and two additional oligonucleotides (forward, 5′-ATG GTC TCA GGG TTC AGG-3′; reverse, 5′-TTT CAG CCC AAT CTC ACC-3′) synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Nucleotide sequences were aligned and assembled using programs in the Sequencher 3.0 software package (Gene Codes Corp., Ann Arbor, MI).

Cloning of OvUS cDNA. Gel-purified OvUS cDNA was ligated into the expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) and the resultant plasmid construct was introduced into TOP10 *Escherichia coli* strain (Invitrogen) by using the pcDNA3.1/V5-His-TOPO cloning kit (Invitrogen) according to the manufacturer's protocol. The positive colonies were selected by culturing *E. coli* on Luria broth (LB) agar containing 100 mg/ml ampicillin (Becton Dickinson, Spark, MD) overnight at 37°C. Positive colonies were cultured in 500 ml of LB with 10 mg/ml ampicillin overnight, and the plasmid was isolated and purified from 5 to 10 ml in an overnight culture using the Miniprep DNA Purification System (Qiagen, Valencia, CA). The purity of the plasmid was evaluated by agarose gel electrophoresis on 1% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. The presence and direction of the insert in the plasmid was confirmed by digestion of the plasmid construct with *Pst*I and *Xba*I restriction enzymes (Promega, Madison, WI), and analysis of digestion products on 0.8% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Also, the plasmid was sequenced using one of the forward primers used to amplify insert DNA and a reverse primer corresponding to an internal sequence (bovine growth hormone) in the vector.

Establishment of Cells Secreting rOvUS. Freestyle 293-F cells (Invitrogen) were transfected with the plasmid construct above by using Freestyle 293 cell transfection kit (Invitrogen) as described in the kit. Briefly, plasmid DNA (30 μg) and 293fectin reagent (60 μl) were mixed with 1 ml of OPTI-MEM-I medium (Invitrogen) in separate tubes, incubated at room temperature for 5 mins, combined together, and incubated at room temperature for 25 mins. The final DNA and 293fectin mixture was pipetted into a T75 cell culture flask containing Freestyle 293-F cells cultured overnight on a shaker in 28 ml of culture medium. Cultures continued for 3 days at 37°C and 5% (v/v) CO_2. The transfected cells (1 ml from the original flask) were placed into new flasks containing 20 ml of modified Freestyle 293 cell medium supplemented with 700 μg/ml geneticin (Invitrogen or RPI, Mount Prospect, IL) for selection and stabilization of transfected cells.

Transfected 293-F cells (hereafter called 293-OvUS cells) were stabilized by passaging and continuously culturing in selective media for at least 3 weeks. Conditioned medium was harvested every 5–7 days for purification of rOvUS. Presence of the OvUS DNA in the 293-OvUS cells was confirmed by RT-PCR using total RNA from 293-OvUS cells.

Purification of His-Tagged rOvUS from Conditioned Medium. Conditioned medium from 293-OvUS cells was clarified by centrifugation at 4000 *g* for 15 mins, and the supernatant was removed and concentrated using Centricon PL-80 or 20 concentration devices (molecular weight exclusion limit = 30,000; Millipore, Bedford, MA). Purification was achieved using HisTrap (Amersham Biosciences, Piscataway, NJ) or HisSelect (Sigma-Aldrich) nickel-chelating columns. The binding buffer was 20 m*M* sodium phosphate buffer pH 8.0, 0.3 *M* NaCl, 15 m*M* imidazole; and the elution buffer was 20 m*M* sodium phosphate buffer pH 8.0, 0.3 *M* NaCl, and with 300 or 500 m*M* imidazole. The concentrated medium was diluted with at least an equal volume of binding buffer and loaded onto the column using a fast protein liquid chromatography system (Pharmacia, Piscataway, NJ) or by using a syringe. The protein recovered after elution was buffer-exchanged with Dulbecco phosphate-buffered saline (DPBS) and concentrated using Centricon PL-20 filters. Concentration of rOvUS was determined using the Bradford assay (18) and purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4%–15% polyacrylamide Tris-HCl gradient gels (Bio-Rad, Richmond, CA) and staining by Coomassie blue G-250.

Expression and Purification of β-Galactosidase. The pcDNA3.1/V5-His-TOPO/lacZ plasmid containing the gene for β-galactosidase (Invitrogen) was used to express a His-tagged β-galactosidase as a control protein. Freestyle 293-F cells were transfected and selected as previously described for rOvUS. Transfected cells were harvested and the cell pellet was collected by centrifugation at 100 *g* (110 *g*) for 10 mins at room temperature. The pellet was resuspended in 20 m*M* sodium phosphate pH 8.0 containing 0.3 *M* NaCl and 35 m*M* imidazole, and a proteinase inhibitor cocktail diluted according to the manufacturer's recommendations (Sigma-Aldrich). Cells were lysed by two freeze-thaw cycles. The supernatant was collected by centrifugation and the recombinant His-tagged protein was purified as described for rOvUS.

Western Blotting. Aliquants (4.5–9 μg protein) of conditioned culture medium from 293-OvUS cells and purified rOvUS in elution buffer were subjected to SDS-PAGE using 4%–15% polyacrylamide, Tris-HCl gels (Bio-Rad, Richmond, CA) and were then subjected to Western blotting as described elsewhere (19). The primary antibody was an anti-OvUS monoclonal antibody (HL 218) (20) at a 1:25,000 or 1:32,000 dilution of hybridoma supernatant.

N-Glycosylation of rOvUS. Purified rOvUS and nOvUS were digested with PNGase F using the PNGase assay kit (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Briefly, 20 μg (8 μl) of rOvUS or nOvUS and 100 μg of RNase B (control protein) were mixed with 1 μl of 10% (v/v) β-mercaptoethanol and 5% (w/v) SDS and boiled for 10 mins. After allowing the samples to cool, 1 μl of 0.5 *M* sodium phosphate pH 7.5, 1 μl of 10% Nonidet 40, and 5 μl of 500,000 units/ml PNGase F were added to each sample and incubated at 37°C for 1 hr in a water bath. After dissociation by boiling in gel loading buffer containing 5% (v/v) β-mercaptoethanol, proteins were separated according to molecular weight using SDS-PAGE and 7.5% polyacrylamide, Tris-HCl gels (BioRad).

Lymphocyte Proliferation Assay. Blood was obtained by jugular venipuncture from nonpregnant Rambouillet or Rambouillet crossbred ewes by collection into heparinized tubes. Mononuclear cells were purified from the buffy coat fraction of blood by density gradient centrifugation as described previously (13) except that adherent cells were not removed. Cells were suspended in modified Tissue Culture Medium-199 (Tissue Culture Medium-199 [Sigma-Aldrich] containing 5% [v/v] heat-inactivated horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin, 2 m*M* supplemental glutamine, and 10^{-5} *M* β-mercaptoethanol) to a concentration of 1×10^6 cells/ml. The lymphocyte proliferation assay was performed by addition of 1×10^5 lymphocytes in 100 μl of culture medium containing 4 μg/ml phytohemagglutinin (PHA) and various concentrations of nOvUS and rOvUS (up to 1000 μg/ml) or ovalbumin (negative control) in a final volume of 200 μl. The final volume of DPBS (the diluent for OvUS and ovalbumin) was the same in each well (up to 25 μl). After 48–72 hrs, 0.1 μCi [^{3}H]thymidine (6.7 Ci/mmol; ICN, Irvine, CA) in 10 μl of culture medium was added. Cells were harvested onto glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD) device at 12 hrs after the addition of [^{3}H]thymidine. Filters were counted for radioactivity using scintillation spectrometry. Experiments were performed using lymphocytes from two to six sheep.

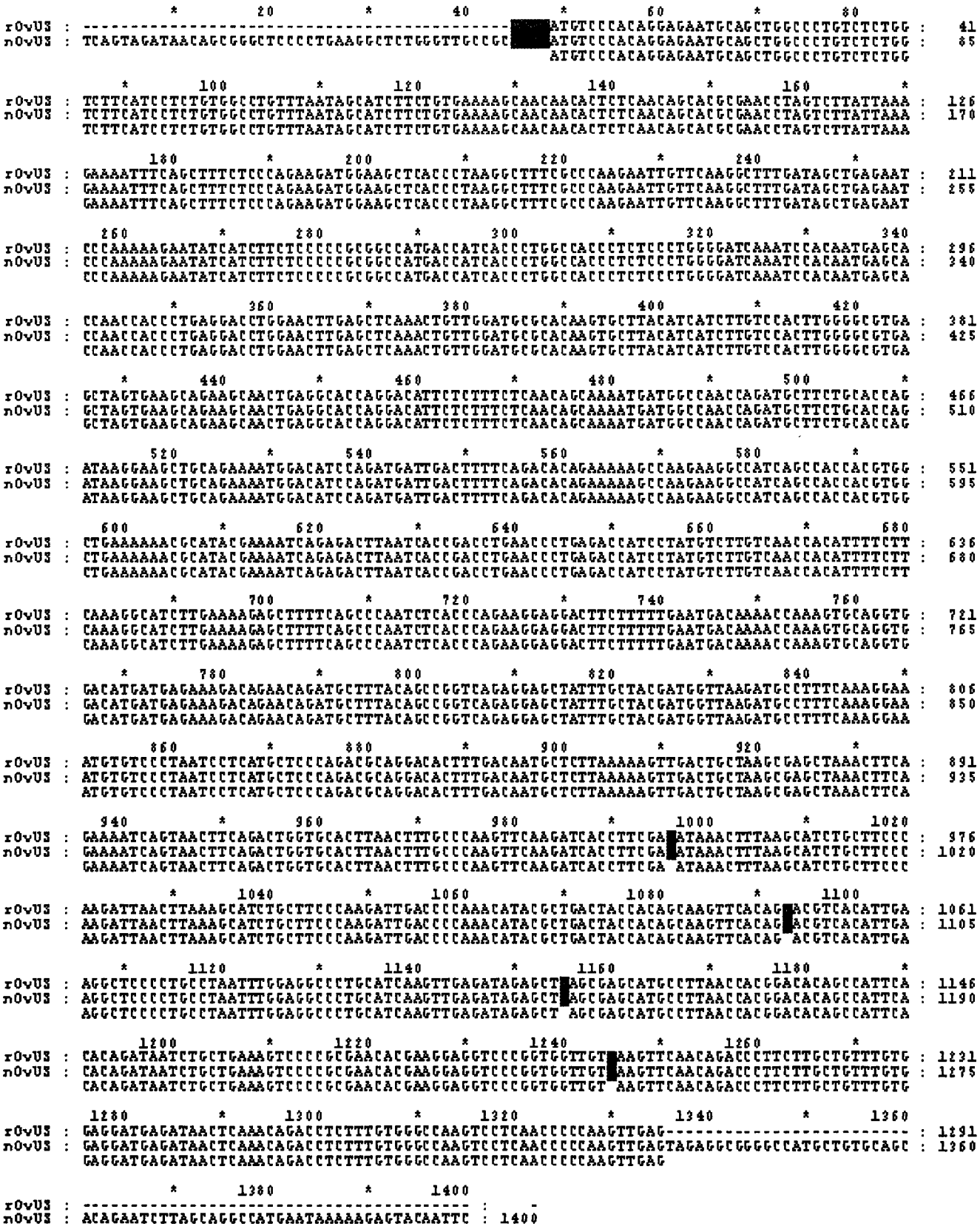

Figure 1. Alignment of the nucleotide sequence of the OvUS cDNA used for recombinant protein production (rOvUS; GenBank accession number AY679518) with a previously published OvUS nucleotide sequence (nOvUS; GenBank accession number M21027). Nucleotides that differ are shaded. Note that the sequence used for recombinant protein production also includes a 5′-CACC used for ligation into the expression vector.

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                *         20          *         40          *         60
rOvUS : MSHRRMQLALSLVFILCGLFNSIFCEKQQHSQQHANLVLLKKISAFSQKMEAHPKAFAQE :  60
nOvUS : MSHRRMQLALSLVFILCGLFNSIFCEKQQHSQQHANLVLLKKISAFSQKMEAHPKAFAQE :  60
        MSHRRMQLALSLVFILCGLFNSIFCEKQQHSQQHANLVLLKKISAFSQKMEAHPKAFAQE

                *         80          *         100         *         120
rOvUS : LFKALIAENPKKNIIFSPAAMTITLATLSLGIKSTMSTNHPEDLELELKLLDAHKCLHHL : 120
nOvUS : LFKALIAENPKKNIIFSPAAMTITLATLSLGIKSTMSTNHPEDLELELKLLDAHKCLHHL : 120
        LFKALIAENPKKNIIFSPAAMTITLATLSLGIKSTMSTNHPEDLELELKLLDAHKCLHHL

                *         140         *         160         *         180
rOvUS : VHLGRELVKQKQLRHQDILFLNSKMMANQMLLHQIRKLQKMDIQMIDFSDTEKAKKAISH : 180
nOvUS : VHLGRELVKQKQLRHQDILFLNSKMMANQMLLHQIRKLQKMDIQMIDFSDTEKAKKAISH : 180
        VHLGRELVKQKQLRHQDILFLNSKMMANQMLLHQIRKLQKMDIQMIDFSDTEKAKKAISH

                *         200         *         220         *         240
rOvUS : HVAEKTHTKIRDLITDLNPETILCLVNHIFFKGILKRAFQPNLTQKEDFFLNDKTKVQVD : 240
nOvUS : HVAEKTHTKIRDLITDLNPETILCLVNHIFFKGILKRAFQPNLTQKEDFFLNDKTKVQVD : 240
        HVAEKTHTKIRDLITDLNPETILCLVNHIFFKGILKRAFQPNLTQKEDFFLNDKTKVQVD

                *         260         *         280         *         300
rOvUS : MMRKTEQMLYSRSEELFATMVKMPFFKGNVSLILMLPDAGHFDNALKKLTAKRAKLQKISN : 300
nOvUS : MMRKTEQMLYSRSEELFATMVKMPFFKGNVSLILMLPDAGHFDNALKKLTAKRAKLQKISN : 300
        MMRKTEQMLYSRSEELFATMVKMPFFKGNVSLILMLPDAGHFDNALKKLTAKRAKLQKISN

                *         320         *         340         *         360
rOvUS : FRLVHLTLPKFKITF INFKHLLPKINLKHLLPKIDPKHTLTTTASSQ VTLKAPLPNLE : 360
nOvUS : FRLVHLTLPKFKITF INFKHLLPKINLKHLLPKIDPKHTLTTTASSQ VTLKAPLPNLE : 360
        FRLVHLTLPKFKITF INFKHLLPKINLKHLLPKIDPKHTLTTTASSQ VTLKAPLPNLE

                *         380         *         400         *         420
rOvUS : ALHQVEIELSEHALTTDTAIHTDNLLKVPANTKEVPVVVKFNRPFLLFVEDEITQTDLFV : 420
nOvUS : ALHQVEIELSEHALTTDTAIHTDNLLKVPANTKEVPVVVKFNRPFLLFVEDEITQTDLFV : 420
        ALHQVEIELSEHALTTDTAIHTDNLLKVPANTKEVPVVVKFNRPFLLFVEDEITQTDLFV


rOvUS : GQVLNPQVE : 429
nOvUS : GQVLNPQVE : 429
        GQVLNPQVE
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Figure 2. Alignment of the predicted amino acid sequence of the OvUS cDNA used for recombinant protein production (rOvUS; GenBank accession number AY679518) with the predicted amino acid sequence of a previously published OvUS nucleotide sequence (nOvUS; GenBank accession number M21027). Amino acids that differ are shaded.

Proliferation of P388D1 and PC-3 Cells. The P388D1 (mouse lymphoma) and PC-3 (human prostate cancer) cell lines were purchased from ATCC (Rockville, MD). The P388D1 cells were cultured continuously in Dulbecco modified eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated horse serum (Hyclone, Logan, UT), 200 U/ml penicillin, and 200 μg/ml streptomycin. The PC-3 cells were grown in DMEM/F12 medium (Gibco-Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 200 U/ml penicillin, and 200 μg/ml streptomycin.

The proliferation assay was performed in 96-well culture plates at 37°C and 5% (v/v) CO_2 with all treatments in triplicate. For P388D1 cells, 2×10^3 cells in 100 μl of culture medium were cultured for 6 hrs before the addition of various concentrations (0, 125, 250, 500, and 1000 μg/ml) of nOvUS, rOvUS, and ovalbumin (negative control). For PC-3 cells, 1×10^4 cells in 100 μl were cultured for 24 hrs before addition of 0, 62.5, 125, 250, 500, or 1000 μg/ml of nOvUS, rOvUS, or ovalbumin. For control wells, an equivalent volume of DPBS was added instead of proteins. The final volume was adjusted to 200 μl with culture medium. After 24 hrs of culture, 0.1 μCi of [^{3}H]thymidine in 10 μl of culture medium was added and cells were harvested onto glass-fiber filters using a cell harvester device at 24 hrs after thymidine addition. Filters were counted for radioactivity using scintillation spectrometry. Experiments were performed on three and five separate occasions for P388D1 and PC-3 cells, respectively. Another experiment with PC-3 cells was performed as described above except with recombinant β-galatcosidase at concentrations of 50, 100, and 200 μg/ml in nine replicates in one assay.

Development of Bovine Preimplantation Embryos. Preimplantation bovine embryos were produced by *in vitro* fertilization from slaughterhouse ovaries using previously described procedures (21). Approximately 8 hrs

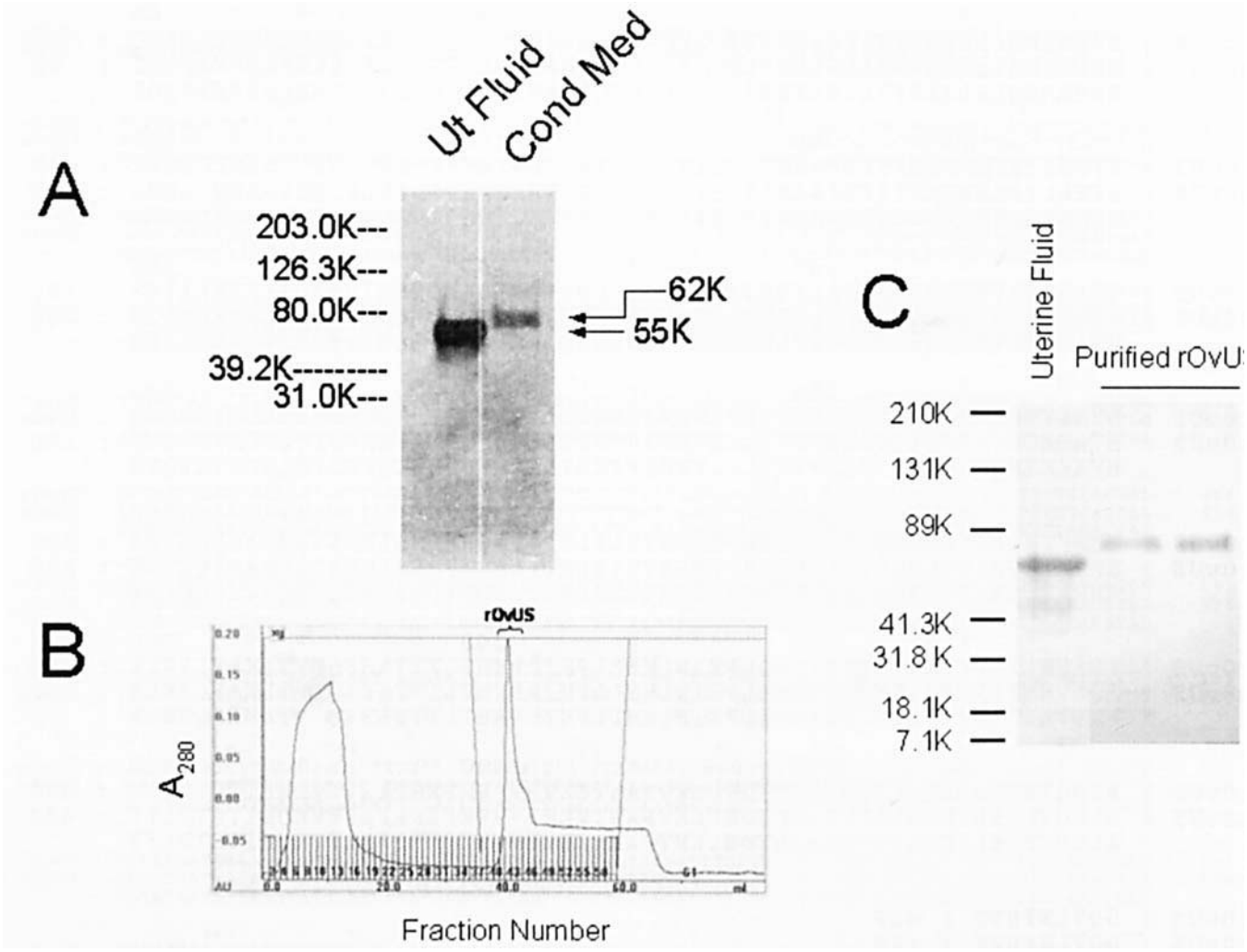

Figure 3. Characterization and purification of rOvUS. (A) Western blotting of uterine fluid (left panel) and conditioned medium from transfected Freestyle 293-F cells (right panel) reveals the presence of immunoreactive OvUS. Note that the estimated molecular weight of recombinant OvUS was higher (62,000) than the molecular weight of native OvUS (55,000) from uterine fluid. (B) A representative example of rOvUS purification from conditioned medium by fast protein liquid chromatography using a His-Trap column. The elution profile is shown in the top figure (brace indicates elution peak for rOvUS) and the purity of the resultant preparation as determined by SDS-PAGE and staining with Coomassie blue R-250 is shown in Panel C. (C) Example of purified rOvUS as resolved by SDS-PAGE using 4%–15% polyacrylamide SDS-PAGE.

after insemination, presumptive zygotes were denuded of cumulus cells by vortexing in 100 μl of 1000 U/ml hyaluronidase and then cultured in groups of 5–15 embryos at 38.5°C and 5% (v/v) CO_2 in humidified air in 25-μl microdrops of modified KSOM-BE2 containing either 0, 125, 250, 500, or 1000 μg/ml nOvUS, rOvUS, or ovalbumin. The zero treatment group contained an equivalent volume of DPBS as that used for treatments. Embryos were evaluated for cleavage on Day 3 and for blastocyst development on Day 8 postinsemination. The experiment was replicated three times with a total of 60–66 embryos per treatment.

Statistical Analysis. Data on [^{3}H]thymidine incorporation were analyzed by least-squares ANOVA using the General Linear Models Procedure of SAS (SAS for Windows, Release 8.02; SAS Institute Inc., Cary, NC). Ewe (for lymphocyte assays) or replicate (for assays of cell lines) was considered as a random effect and other main effects were considered fixed. Error terms were determined by calculating expected mean squares. In some analyses, the pdiff mean separation test of SAS was performed to determine treatments that differed from untreated cells. Categorical data collected on preimplantation embryos (percent cleavage and percent development) were analyzed by the CATMOD procedure of SAS. The model included effect of protein type (rOvUS vs. nOvUS), concentration (0,125, 250, 500, and 1000 μg/ml), protein type × concentration, and replicate. For analysis of the proportion of cleaved embryos becoming blastocysts, there were no observations for the 1000 μg/ml concentration of rOvUS (because no putative embryos cleaved). Therefore, this concentration was excluded from analysis.

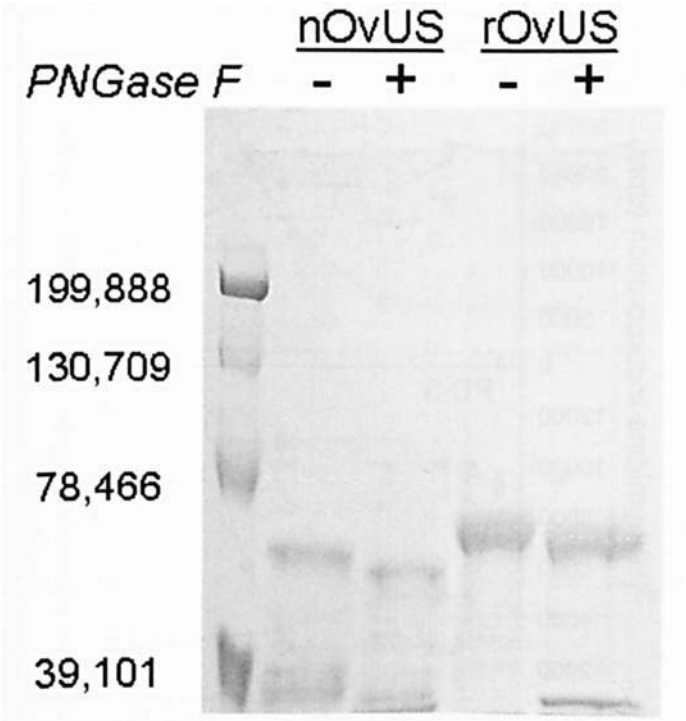

Figure 4. Digestion of native OvUS and recombinant OvUS by PNGase F. Molecular weight standards are in the first lane. Treatment with PNGase F reduced the apparent molecular weight of nOvUS from 55,000 to 50,300 (nOvUS – and +, respectively) and the molecular weight of rOvUS from 61,500 to 57,500 (rOvUS – and +, respectively). Lane 1 shows molecular weight standards with sizes indicated by the numbers to the left.

Results

Generation of cDNA Encoding the OvUS Preprotein. A 1290-bp cDNA encoding the OvUS preprotein was produced by subjecting total RNA isolated from endometrium of a Day 140 pregnant ewe to RT-PCR. The sequence of the cDNA for the OvUS preprotein (GenBank accession number AY679518) was nearly identical to a previously published cDNA sequence (GenBank accession number M21027). Of the four sequences that were different, three represented a G substitution in AY679518 for a C in M21027, whereas the fourth represented a G in AY679518 for a T in M21027 (Fig. 1). The predicted amino acid sequence is shown in Figure 2. The sequence is identical to the predicted amino acid sequence for M21027 except for amino acid 316 (glutamic acid in AY679518 vs. aspartic acid in M21027) and amino acid 349 (aspartic acid in AY679518 vs. histidine in M21027).

Establishment of a Transgenic 293 Cell Line Producing rOvUS. The cDNA encoding the OvUS preprotein was in-frame cloned into pcDNA3.1/V5-His-TOPO and the resultant plasmid, pcDNA3.1D/V5-His-TOPO-OvUS, was transfected into Freestyle 293-F cells. Production of rOvUS by colonies of transfected Freestyle 293-F cells was verified by Western blot analysis of the conditioned medium using a monoclonal antibody against OvUS. As predicted, based on the presence of the additional 45 amino acids on the N-terminus of the recombinant protein, the protein was slightly higher in molecular weight (~62,000) than nOvUS (~55,000) as determined by SDS-PAGE (Fig. 3A). There was also a small amount of protein at a molecular weight of ~45,000 in the nOvUS preparation that represents breakdown product. Histidine-tagged rOvUS was purified to apparent homogeneity from conditioned medium by using HisTrap or HisSelect columns (Fig. 3B, C).

Effect of PNGase F on Molecular Weight of Secreted rOvUS. The presence of glycosylation on rOvUS was determined by testing whether PNGase F caused a reduction in molecular weight of the protein. Treatment with PNGase F reduced the apparent molecular weight of nOvUS from 55,000 to 50,300 and the molecular weight of rOvUS from 61,500 to 57,500 (Fig. 4).

Inhibition of Mitogen-Induced Proliferation of Peripheral Blood Lymphocytes. In the first experiment, both nOvUS and rOvUS inhibited ($P < 0.001$) [^{3}H]thymidine incorporation by PHA-stimulated lymphocytes at concentrations of 500 and 1000 (g/ml) (Fig. 5A). In contrast, there was no inhibition caused by ovalbumin, a noninhibitory serpin used as a negative control protein. The degree of inhibition of [^{3}H]thymidine incorporation was greater for rOvUS than for nOvUS ($P < 0.02$). In a second experiment, effects of lower concentrations of rOvUS on lymphocyte proliferation were determined. Recombinant OvUS inhibited ($P < 0.05$) proliferation at 15.5, 31.0, and 62.0 μg/ml (Fig. 5B).

Antiproliferative Actions on P388D1 and PC-3 Cell Lines. Both nOvUS and rOvUS inhibited proliferation of P388D1 cells as measured by incorporation of [^{3}H]thymidine into DNA (Fig. 6A). All concentrations of rOvUS tested were inhibitory, with the lowest concentration being 125 μg/ml. For nOvUS, in contrast, there was inhibition only at 500 μg/ml ($P < 0.10$) and 1000 μg/ml ($P < 0.001$), and the magnitude of the reduction in proliferation at any concentration was greater for rOvUS than for OvUS. The control protein, ovalbumin, did not inhibit proliferation.

Similar results were obtained for PC-3 cells (Fig. 6B) except that in this case the only significant inhibition was for rOvUS. All concentrations tested, including a concentration as low as 62.5 μg/ml, reduced [^{3}H]thymidine incorporation ($P < 0.001$). While it was not significant, the highest concentration of nOvUS (1000 μg/ml) tended to reduce [^{3}H]thymidine incorporation. In another experiment, there was no inhibitory effect of recombinant β-galactosidase on proliferation of PC-3 cells. Incorporation of [^{3}H]thymidine in cells treated with 50, 100, and 200 μg/ml β-galactosidase was 114%, 110%, and 112% of values for cells cultured without β-galactosidase.

Cleavage and Embryonic Development. Native OvUS did not have any effect on the proportion of oocytes that cleaved following fertilization, but rOvUS caused a dose-dependent decrease in cleavage rate (Fig. 7A; nOvUS

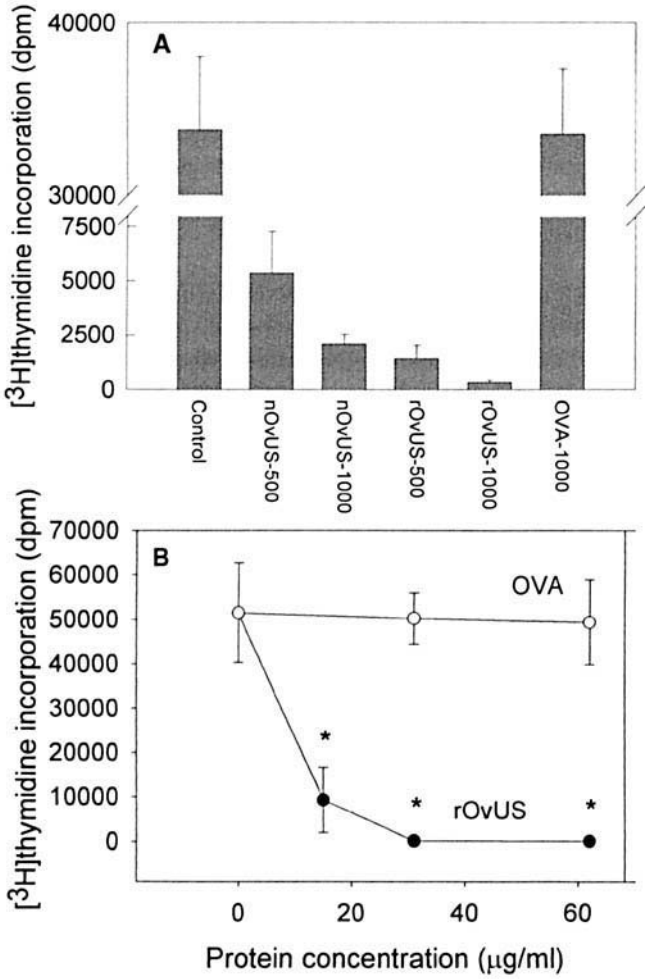

Figure 5. Inhibition of PHA-induced proliferation of lymphocytes. [^{3}H]thymidine was added at either 72 hrs (A) or 48 hrs (B) after addition of PHA (4 μg/ml) and 500 or 1000 μg/ml of OvUS purified from uterine fluid (nOvUS) or recombinant OvUS (rOvUS). A control protein, ovalbumin (OVA) was also tested. (A) Least-squares means ± SEM of results for [^{3}H]thymidine incorporation from six separate ewes. (B) Data for [^{3}H]thymidine incorporation from two separate ewes. Data were log-transformed before analysis and results are presented as untransformed means ± SEM of results. Means that differ from untreated cells are indicated by asterisks (*$P < 0.05$; ****$P < 0.001$). For (A), [^{3}H]thymidine incorporation was lower ($P < 0.02$) for cells treated with rOvUS as compared with OvUS.

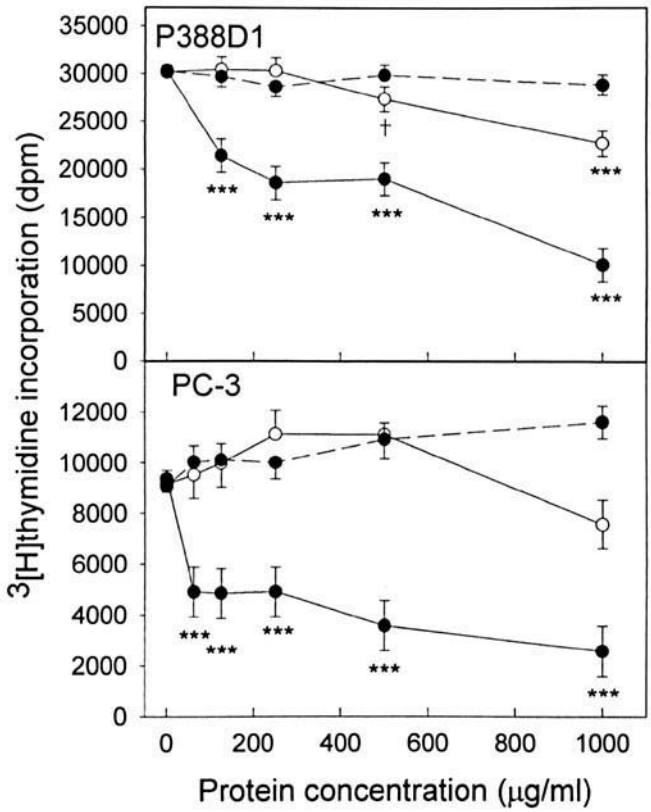

Figure 6. Inhibition of [^{3}H]thymidine incorporation of P388D1 cells and PC-3 cells by native (n) and recombinant (r) OvUS. Cells were cultured with various concentrations of nOvUS (open circles), rOvUS (filled circles and solid line) or ovalbumin (filled circles and dashed line). Data represent least square means ± SEM. Means that differ from untreated cells are indicated by symbols (†$P < 0.06$; ***$P < 0.001$).

vs. rOvUS, $P < 0.001$; protein type × concentration, $P < 0.01$). Inhibition of cleavage was nearly total at a concentration of 500 μg/ml (1.7% cleavage) and no oocytes cleaved when they were cultured with 1000 μg/ml rOvUS. The proportion of oocytes that became blastocyts was affected by both nOvUS and rOvUS with rOvUS being a more potent inhibitor than nOvUS (Fig. 7B; nOvUS vs. rOvUS, $P < 0.01$; protein concentration, $P < 0.001$). In large part, the decrease in the percentage of oocytes that became blastocysts caused by rOvUS reflects the reduction in cleavage rate. However, both nOvUS and rOvUS also reduced the potential for cleaved embryos to develop to the blastocyst stage because the proportion of cleaved embryos that became blastocysts was reduced by nOvUS and rOvUS (Fig. 7C). For concentrations from 0 to 500 μg/ml, the inhibition was greater for rOvUS than for nOvUS (protein type × concentration, $P < 0.001$).

Discussion

The antiproliferative actions of OvUS toward lymphocytes are well established (8–11). More recently, OvUS has been shown to also inhibit proliferation of various tumor cell lines and preimplantation embryos (14). Until the present paper, however, OvUS appeared to be only a weak inhibitor of cell proliferation because concentrations required to inhibit cell proliferation have generally ranged in the hundreds of micrograms per milliliter range. In the present work, it has been demonstrated that OvUS is a more potent inhibitor of cell proliferation than previously believed. Indeed, concentrations of the recombinant protein as low as 15 μg/ml inhibited lymphcoyte proliferation. This concentration is within the range of active concentrations for maspin, another serpin capable of regulating cell function (22–24). Given that concentrations of OvUS in uterine fluid are in the milligram per milliliter range (15), it is likely that OvUS inhibits cell proliferation *in vivo*.

The increased activity of rOvUS as compared with

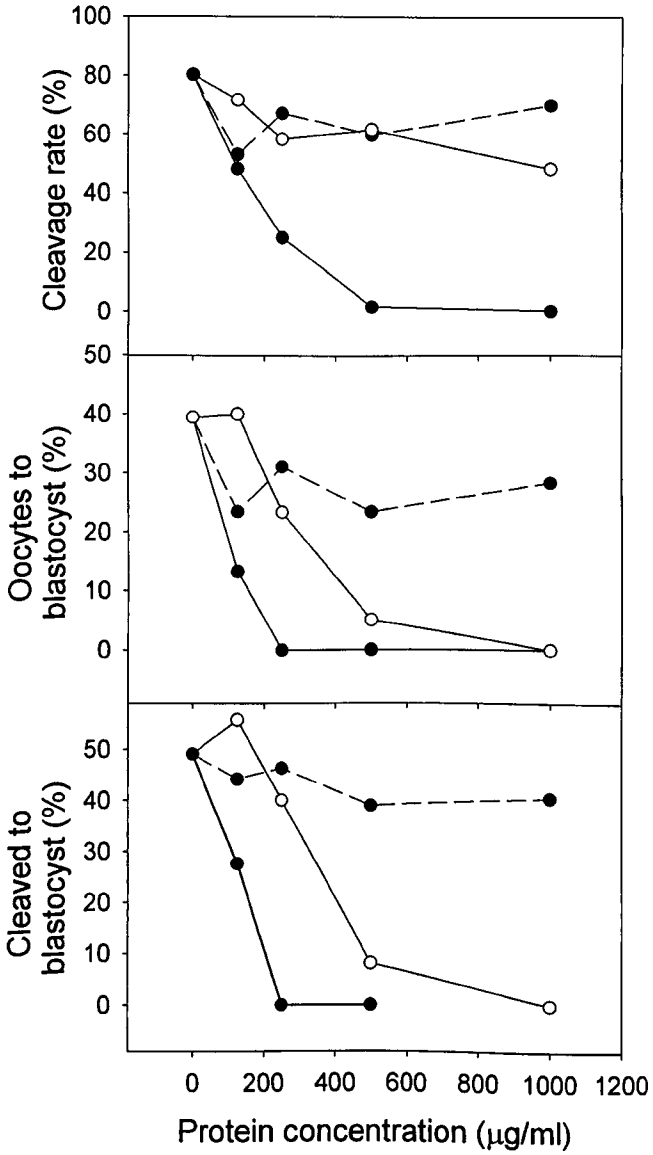

Figure 7. Inhibition of cleavage and embryonic development of bovine embryos caused by native (n) and recombinant (r) OvUS. Beginning after fertilization, bovine embryos were cultured with various concentrations of nOvUS (open circles), rOvUS (filled circles and solid line), or ovalbumin (filled circles and dashed line). Data represent the percentage of oocytes that cleaved (top panel), the percentage of oocytes that became blastocysts at Day 8 after insemination (middle panel), and the percentage of cleaved embryos that became blastocysts at Day 8 after insemination (bottom panel). When comparing responses to nOvUS and rOvUS, cleavage rate was affected by protein type ($P < 0.001$), protein concentration ($P < 0.001$), and protein type × concentration ($P < 0.01$). The percentage of oocytes that became blastocysts was affected by protein type ($P < 0.001$), protein concentration ($P < 0.01$), and protein type × concentration ($P < 0.09$). The percentage of cleaved embryos that became blastocysts was affected by protein type × concentration ($P < 0.001$).

OvUS purified from uterine fluid is probably a reflection of the degradation of the protein occurring *in utero*. Uterine fluid used for purification of OvUS is typically collected at Days 100–140 of gestation and it is possible that much of the protein in uterine fluid was secreted weeks or months before collection. Uterine fluid contains OvUS-immunoreactive proteins of lower molecular weight less than 55,000 that are probably proteolytic breakdown products (16, 20). The OvUS can also form large aggregates in the uterus (15). While intact, monomeric OvUS would be separated from most lower-molecular-weight breakdown products and aggregates of OvUS during purification, some proteolytically processed OvUS would be expected to copurify with intact OvUS because of noncovalent forces holding the digested protein together. Indeed, OvUS subjected to limited digestion with trypsin comigrates with intact OvUS on gel filtration columns, although formation of proteolytic cleavage products can be detected by SDS-PAGE (5). In addition to proteolytic degradation, it is also possible that OvUS undergoes other conformational changes *in utero* that reduce its biological activity. The protypical serpin exists in a stressed conformation that is required for proteinase inhibitory activity (25). It is questionable whether OvUS exists in such a conformation (5), but conformational requirements for retention of antiproliferative activity by OvUS cannot be ruled out.

The putative role of OvUS is to inhibit lymphocyte proliferative responses directed against fetal antigens (26). Given the ability of the protein to inhibit other cells, OvUS may also play a role in regulation of trophoblast proliferation to limit invasiveness of trophoblast. The inhibition of preimplantation embryonic development is unlikely to be of physiological relevance because OvUS is not synthesized until well after cleavage states of embryonic development. OvUS messenger RNA is first detected in the endometrium at Day 13–14 of pregnancy and the protein can first be identified in uterine secretions at Day 16 of pregnancy (27, 28).

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