Expression of CA-125 by progestational bovine endometrium: prospective regulation and function

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Cancer antigen 125 (CA-125) is expressed by malignant human ovarian surface epithelial cells and derivatives of the Müllerian duct system. This study explored the expression, regulation, and function of CA-125 in the bovine uterus. CA-125 was localized by immunohistochemistry to the apical surfaces of epithelial cells lining the endometrium and proximal glands of the late luteal phase and early pregnancy; antigen was not detected during oestrus or the postpartum period. Production of CA-125 by bovine endometrial cells *in vitro* was upregulated by progesterone and

interferon- τ . Immunopurified CA-125 from uterine flushes of dioestrous or pregnant cows was similar in biochemical composition (as determined by gel electrophoresis and amino acid content) to the human antigen isolated from incubation medium conditioned by the ovarian cancer cell line OVCAR-3. Bovine CA-125 inhibited complement-induced lysis of antibody-sensitized sheep erythrocytes. It is suggested that endometrial CA-125 exerts a progestational role in part by protecting maternal and embryonic cells from immune targeting and lysis.

Introduction

Cancer antigen 125 (CA-125) was first identified as an immunogenic determinant on an ovarian adenocarcinoma cell line (Bast *et al.*, 1981). The molecular characterization of CA-125 has since proved to be difficult. Most studies have indicated that CA-125 is a heterogeneous mucinous protein of high molecular mass. Size estimates of CA-125 have ranged from 200 to 2000 kDa, and smaller subunits have been reported (O'Brien *et al.*, 1998; Yin and Lloyd, 2001). The gene encoding CA-125 apparently yields a transmembrane molecule with a dominate extracellular motif composed of tandem repeat units, a plasmalemma-spanning domain and short cytoplasmic tail (O'Brien *et al.*, 2001). Serum measurements of secreted forms of CA-125 have proven useful in monitoring the progress of patients with ovarian cancer after cytoreductive surgery and chemotherapy (Meyer and Rustin, 2000).

Most ovarian cancers are thought to arise by clonal transformation of a surface epithelial cell containing DNA damaged by reactive oxidants generated during the process of ovulatory follicular rupture (Murdoch and McDonnel, 2002). Normal ovarian surface epithelial cells are of an uncommitted stem-like phenotype, and cell-surface expression of CA-125 occurs with metaplastic differentiation into a Müllerian-type epithelium

(Feeley and Wells, 2001). Indeed, CA-125 is not unique to ovarian cancer cells. Epithelia of the female genital tract also express CA-125 (Zeimet *et al.*, 1998). Circulatory CA-125 is higher in patients with endometriosis (Bast *et al.*, 1998).

Normal fluctuations in serum CA-125 (that is, those not associated with ovarian cancer or benign gynaecological disease) appear to be related to stage of the ovarian cycle and pregnancy status. Serum concentrations of CA-125 increase during the folliculo-luteal-menstrual transition of spontaneous human cycles (Bon *et al.*, 1999). Further increases in CA-125 occur in early pregnancy (Bast *et al.*, 1998). In fact, heightened serum CA-125 concentrations are indicative of successful implantation in women after *in vitro* fertilization and embryo transfer (Baalbergen *et al.*, 2000). Pregnancies equated with multiple corpora lutea after superovulation correlate with higher serum CA-125 than found in normal monovulatory pregnancies (Paoletti *et al.*, 1995). Production of CA-125 by malignant ovarian epithelial cells is upregulated by interferons (Marth *et al.*, 1998). A unique trophoblast-derived interferon (IFN), designated as IFN- τ , is involved in the local endometrial signalling process of pregnancy recognition in ruminants (Demmers *et al.*, 2001).

A patho-physiological function for CA-125 has not been established. In a recent study (A. C. McDonnel and W. J. Murdoch, unpublished), CA-125 of human ovarian cancer cell origin was found to exhibit (as a possible means to resist natural and therapeutic-based immune

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destruction) complement-inhibiting properties. Down-regulation of complement activation at the maternal-placental interface protects cells from immune targeting and lytic death (Morgan and Holmes, 2000).

Studies on CA-125 have been almost exclusively on specimens of human origin. The initial objective of this investigation was to determine the spatial distribution and temporal alterations of CA-125 in uterine samples of cyclic, pregnant and postpartum cows. Follow-up studies were designed to evaluate putative endocrine-paracrine (steroid hormonal and IFN) effectors of (endometrial) CA-125 production and to assess the biochemical and functional attributes of bovine CA-125.

Materials and Methods

Experiments were performed with the approval of the University of Wyoming Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless indicated otherwise.

Immunohistochemical localization of CA-125

Angus cross-bred cows were synchronized to oestrus (day 0) with prostaglandin $F_{2\alpha}$ (25 mg dinoprost tromethamine i.m.; Pharmacia and Upjohn, Kalamazoo, MI). Animals allotted to pregnancy groups were inseminated artificially 12 h after detection of oestrus. Reproductive organs were obtained at slaughter on day 0 (oestrus; $n=3$), day 18 of the oestrous cycle ($n=4$), and days 18 ($n=4$), 22 ($n=4$) and 50 ($n=4$) of pregnancy, and on day 45 after parturition (anoestrus; $n=3$). Uterine horns of inseminated females were inspected to confirm the presence of a conceptus or fetus.

Cross-sectional segments of uterine horns were fixed by immersion in Histochoice (Amresco, Solon, OH), washed in PBS, dehydrated in a graded series of ethanol, cleared in xylene, and infiltrated with and embedded in paraffin wax. Sections of 7 μm thickness were transferred on to microscope slides treated with subbing solution (0.025% (w/v) chromium potassium sulphate, 0.25% (w/v) gelatin), deparaffinized, rehydrated, microwaved in Retrieve-All-1 unmasking solution (Signet Pathology Systems, Dedham, MA), incubated in 10% normal blocking serum (5 min, 24°C), reacted with mouse monoclonal anti-CA-125 (4 nmol OC-125 l^{-1} for 1 h; Signet Pathology Systems), washed in PBS, stained using a streptavidin-alkaline phosphate-fast red detection system (Signet Pathology Systems), counterstained in Mayer's haematoxylin, rinsed in distilled water, and coverslips were added (Gel-Mount; Biomedica Corporation, Foster City, CA). Negative controls were performed without primary antibody and with primary antibody preadsorbed with human ovarian cancer cell-derived CA-125 (Research Diagnostics Inc., Flanders, NJ).

Selections of fields of microscopic inspection (three areas per animal; $\times 320$) were made at random and without knowledge of stage of cycle or pregnancy. Immunostaining along apical and basal aspects of endometrial luminal epithelium, proximal (within 0.6 mm of the lumen) glandular epithelium, and distal glandular epithelium were categorized on a scale of 0 to 4 (0 = non-detectable, 1 = scant, 2 = light, 3 = moderate, 4 = heavy).

In vitro effects of steroid hormones and interferon- τ on cellular CA-125 expression

Endometrial cells established from an epithelium-enriched bovine line (Staggs *et al.*, 1998) were transferred (1×10^6) on to poly-L-lysine coated coverslips (Becton Dickinson, Franklin Lakes, NJ) and incubated (24 h, 37°C, 5% CO_2) in Ham's-minimum essential medium (MEM; 1 ml) supplemented with 0.4 mmol D-valine l^{-1} and 10% fetal calf serum (control), plus 3 nmol oestradiol l^{-1} , 1 μmol progesterone l^{-1} , or 2.5 nmol recombinant bovine IFN- τ l^{-1} . Each treatment was replicated six times. Doses of steroid hormones and IFN- τ approximated to local concentrations typical of the oestrous cycle and early pregnancy (Henricks and Harris, 1978; Weems *et al.*, 1988; Benoit and Dailey, 1991; Roberts *et al.*, 1992). Cells immunostained for CA-125 were counted within five randomly selected areas ($\times 160$) on each coverslip. (In a preliminary study, the cell line was observed to contain a mixture of epithelial and stromal cells and only cytokeratin-positive (that is, epithelial) cells were co-reactive for CA-125.)

Immunopurification and amino acid analysis of CA-125

CA-125 was isolated from a pooled uterine flush of day 18 open (non-pregnant) and pregnant cows ($n=8$) and incubation medium conditioned by an ovarian carcinoma cell line (OVCAR-3; American Type Culture Collection, Rockville, MD) with the aid of a Seize-X Primary Immunoprecipitation Kit, according to the instructions of the manufacturer (Pierce, Rockford, IL). OC-125 antibody (100 μg) was coupled to amino-link gel for antigen retrievals. Eluted products were quantitated by spectrophotometric protein assay (Bio-Rad Laboratories, Hercules, CA), analysed by SDS-PAGE (8% (w/v) acrylamide; samples boiled for 5 min in Laemmli buffer without β -mercaptoethanol) with silver staining, and subjected to amino acid composition analysis by ion-exchange chromatography (Molecular Structure Facility, University of California, Davis, CA).

Effect of bovine CA-125 on complement-mediated haemolysis

A standard cytolytic (haemoglobin-release) activity assay incorporating sheep red blood cells, rabbit antisheep

erythrocyte antibody, and a complement source (guinea-pig serum) was used (Mayer, 1984). Briefly, erythrocytes (4 ml blood) were washed five times in gelatin veronal buffer (0.15 mmol $\text{CaCl}_2 \text{ l}^{-1}$, 141 mmol NaCl l^{-1} , 0.1% (w/v) gelatin, 1.8 mmol sodium barbital l^{-1} , 3.1 mmol barbituric acid l^{-1} ; pH 7.3) and sensitized with 1 U antisheep haemolysin (Colorado Serum Co., Denver, CO). Complement (196 CH50 ml^{-1} ; a CH50 unit is defined as the volume that causes 50% lysis of 5×10^8 antibody-sensitized erythrocytes at 37°C for 1 h in a 7.5 ml reaction mixture) was titrated to achieve 50% lysis during a 40 min incubation at 37°C . Samples were diluted sixfold with 0.15 mol NaCl l^{-1} ; cells were pelleted by centrifugation for 5 min at 450 *g*, and supernatants were analysed by spectrophotometry (412 nm). Triplicate reactions were performed in the presence of immunopurified CA-125 (0, 10, 20 or 30 μg).

Statistical analysis

Group and treatment mean comparisons were made by analysis of variance and Fisher's protected least significant difference test. Contrasts were considered significantly different at $P < 0.05$.

Results

An immunoreactive CA-125-like antigen was localized by histochemistry at the apical surfaces of progestational bovine endometrial epithelia associated with the uterine lumen and proximal glands. Endometrial glands distanced from the uterine lumen and basal regions of cells (regardless of the site of analysis) did not immunostain. Apical staining reactions ranged from nondetectable on day 0 (oestrus) and day 45 after parturition (anoestrus), increased on day 18, and were most intense during pregnancy ($P < 0.05$). Intensities of staining were similar in cyclic and pregnant cows on day 18 and on days 22 and 50 of pregnancy. There were no significant differences in apical staining intensities between cells bordering the uterine lumen and those cells associated with proximal glands (Figs 1 and 2).

Expression of CA-125 by bovine endometrial cells was induced by progesterone or IFN- τ ($P < 0.01$). There were no apparent differences due to *in vitro* treatments in intensity of immunostaining (that is, responses were positive or negative). The response to IFN- τ tended to be greater than that to progesterone; however, the contrast between these treatment groups was not statistically significant. Exposure of endometrial cells to oestradiol had no effect on CA-125 expression (Fig. 3).

Purified CA-125 from OVCAR-3 incubation medium and bovine uterine flush migrated in parallel during electrophoresis, revealing a dominant high molecular mass

Fig. 1. Representative light photomicrographs of bovine endometrial cancer antigen 125 (CA-125) immunoreactions (day 22 of pregnancy). (a) Cross-sectional view of luminal epithelium and glands. (b) Luminal epithelium illustrating apical localization. (c) Negative control section. DG: distal gland; L: lumen; LE: luminal epithelium; PG: proximal gland. Scale bars represent 0.1 mm.

Fig. 2. Quantification of cancer antigen 125 (CA-125) immunostaining (intensity scores) in bovine endometrium. Means + SE are plotted. Days = 0, 18, 22, 45, 50. N: non-pregnant; P: pregnant; PP, postpartum; LE: luminal epithelium; PGE: proximal gland epithelium; DGE: distal gland epithelium; a: apex; b: base.

Fig. 3. Effects of steroid hormones and interferon τ (IFN- τ) *in vitro* on cancer antigen 125 (CA-125) expression by bovine endometrial cells. E₂: oestradiol; P₄: progesterone.

Table 1. Amino acid contents (molecular weight fraction contributed; mol %) of immunopurified cancer antigen 125 (CA-125) derived from human epithelial ovarian cancer cells and bovine dioestrous–pregnant uterine fluid

Amino acid	Immunopurified CA-125 content (mol %)	
	Human epithelial ovarian cancer cells	Bovine dioestrous–pregnant uterine fluid
Asx	8.7	8.4
Glx	10.3	10.1
Ser	13.4	14.3
Gly	9.6	10.5
His	5.7	2.0
Arg	3.2	3.2
Thr	7.6	9.2
Ala	10.7	7.0
Pro	5.0	6.1
Tyr	3.4	3.6
Val	7.7	9.1
Iso	1.8	2.4
Leu	5.5	6.4
Phe	2.4	2.8
Lys	4.9	5.0

Fig. 4. Gel electrophoresis of immunopurified cancer antigen 125 (CA-125) (0.1 μ g loaded per lane) from conditioned medium of human epithelial cancer cells (centre) and bovine uterine flush (right). Molecular mass markers (kDa) are shown in the left lane.

fraction of approximately 200 kDa; proteins from the uterine flush appeared somewhat more heterogeneous than the human antigen. An additional protein band of about 80 kDa was apparent in the human sample (Fig. 4). Amino acid compositions of the human and bovine samples were similar (Table 1).

Uterine CA-125 invoked a linear dose-dependent inhibition ($P < 0.01$) of complement-induced lysis of antibody-sensitized sheep erythrocytes (Fig. 5).

Fig. 5. Effect of bovine cancer antigen 125 (CA-125) on complement-mediated haemolysis of sensitized sheep red blood cells.

Discussion

Results of these experiments indicate that a human-like CA-125 antigen is expressed at the apical surfaces of epithelia contiguous with the uterine cavity and proximal glands of cows. Concentrations of CA-125 increased during the shift from the late luteal phase of the oestrous cycle through the period of luteal rescue and into pregnancy, intimating a progestational role. Progesterone and IFN- τ appear to act as inducers of CA-125 production.

This is the first report to the present authors' knowledge demonstrating the presence of CA-125 within the genital tract of a farm species. As well as in humans, positive immunostaining for CA-125 has been identified in Müllerian-derived epithelia of rabbits, dogs and

monkeys (Nouwen *et al.*, 1990). Circulatory fluctuations in CA-125, which reflect endometrial dynamics during menstrual cycles and human pregnancy, were not predictive of reproductive status in cows; concentrations of CA-125 in jugular serum of cyclic and pregnant animals (as measured by commercial radio- and enzyme-linked immunoassays) were undetectable or highly variable (A. C. McDonnell and W. J. Murdoch, unpublished).

Molecular avenues of regulation of CA-125 biosynthesis and secretion are evidently multifaceted. The classical mode of progesterone action involves hormonal binding to an intracellular (nuclear) receptor, initiating gene transcription (Beato and Sánchez-Pacheco, 1996). However, cyclic expression of progesterone receptors by bovine uterine epithelial cells *in vivo* was relatively low during the late luteal phase (Robinson *et al.*, 2001); thus, the physiological significance of the present results *in vitro* (indicating upregulation of CA-125 by progesterone) remain equivocal. Type-I interferons activate a plasma membrane receptor coupled to the Jak-Stat transduction pathway (Prejean and Colamonici, 2000). Proteolytic release of CA-125 from the membrane surface (for example, during cellular death or in response to epidermal growth factor) is dependent upon a phosphorylation event within its cytoplasmic domain (Fendrick *et al.*, 1997; Beck *et al.*, 1998; O'Brien *et al.*, 2001).

The biochemical character of bovine uterine CA-125 seems closely related to the human cancer antigen. Electrophoretic migration patterns and amino acid compositions of CA-125 observed in this study are in agreement with previous descriptions of the human antigen (O'Brien *et al.*, 2001; Yin and Lloyd, 2001).

The findings of the differential expression of CA-125 along mucosal surfaces particularly susceptible to mechanical or chemical injury implies an intrinsic protective function perhaps related to its mucinous nature. Complement inactivation–entrapment is a possibility. A battery of diverse proteins (for example, decay-accelerating factor, membrane cofactor protein, protectin, complement receptor 1), which operate in the fluid phase and on plasma membranes prevent inappropriate complement fixation and autolysis; this is certainly the case at the endometrial–placental interface (Morgan and Holmes, 2000). Preliminary findings of the present investigation indicate that CA-125 is a prospective regulator of the complement system in cattle. CA-125 could also potentially alter the adhesive properties of glandular epithelia (maintaining patency) and of the endometrium toward the developing conceptus. Highly glycosylated mucins with large exodomains (for example, Muc-1) are generally thought to mask adhesion molecules (DeSouza *et al.*, 1999). Endometrial expression of Muc-1 by luminal epithelia of mice, pigs, and sheep decreased near the time of blastocyst apposition (Surveyor *et al.*, 1995; Bowen *et al.*, 1996; Johnson *et al.*, 2001); overall Muc-1 concentrations

increased in rabbits and humans, but were attenuated at localized sites of trophoctoderm attachment (Hoffman *et al.*, 1998; Aplin, 1999). Moreover, Muc-1 is a potent glandular morphogen (Hudson *et al.*, 2001). Formation during the luteal phase of endometrial glands, which become the source of nutrients (histotrophe) for the preimplantation embryo, is essential for the establishment of pregnancy; this is especially important in species (including cows) that do not implant intrusively (Gray *et al.*, 2001). Future studies should be directed at ascertaining the biological significance of CA-125 in uterine functions.

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