Clusterin is expressed in the anterior and intermediate lobes, but not in the posterior pituitary of sheep

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ABSTRACT
We have examined the expression of the ovine clusterin gene in the sheep pituitary gland, with the aim of determining its site of synthesis in this tissue. Northern blotting analysis of extracted polyadenylated RNA, using a 32P-labelled rat clusterin cDNA probe, detected the greatest amounts of clusterin mRNA in the anterior part of dissected pituitary glands. In situ hybridisation studies showed clusterin mRNA in anterior and intermediate pituitary cells, with lower amounts in vascular endothelium and posterior pituicytes. Clusterin protein, detected by immunohistochemistry, was observed in some single secretory cells, within the capillary lumen and in cells around capillaries in the anterior and intermediate lobes, but no immunoreactivity was observed in posterior pituitary tissue. The pattern of clusterin expression in anterior and intermediate pituitary cells suggests possible roles for the protein in secretory cell turnover and/or hormone secretion or lipid uptake. Clusterin does not appear to be involved in ovine posterior pituitary hormone neurosecretion.

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INTRODUCTION
Clusterin is a highly sulphated glycoprotein that is expressed in a wide range of tissues, often in association with tissue remodelling or apoptosis, in response to injury or pathology, or connected with lipid transport or atherosclerotic vascular disease (Fritz & Murphy 1993). It is expressed in many endocrine tissues, including ovary, testis, prostate and uterus (Sylveste & Griswold 1995), pancreas (Scaglia et al. 1995) and breast tissue (Tenniswood et al. 1992, Welsh 1994). Clusterin is known to be an adrenal chromaffin granule constituent and its expression increases in the adrenal in response to histamine or forskolin treatment (Palmer & Christie 1990, Laslop et al. 1993). In gonadal tissue, large amounts of clusterin mRNA have been measured in extracts of ovine corpora lutea (Fleming et al. 1992) and high concentrations of clusterin protein are synthesised in the ram rete testis (Tung & Fritz 1985).

There is further evidence for a role for this protein in search of a function (Fritz 1995) in neural tissue, where its expression is upregulated in pathologies such as Alzheimer’s disease (Finch & May 1995). Clusterin is induced in reactive astrocytes in response to neurotoxic injury (Zwain et al. 1994, Walton et al. 1996, Tornquist et al. 1997) and appears to have a protective role in the brain, possibly acting as a local inhibitor of the complement cascade (Tornquist et al. 1996).

Clusterin expression is known to be suppressed by gonadal steroids in prostate, mammary gland and uterus (Bettuzzi et al. 1992, Wunsche et al. 1998) and by glucocorticoids in brain and kidney cells (Finch & May 1995, Gutacker et al. 1996). These observations led us to investigate clusterin expression in the ovine pituitary gland, with the aim of determining the distribution of clusterin mRNA and protein in the different parts of this tissue. In previous studies of bovine pituitary, the protein was detected in both the anterior and posterior lobes (Laslop et al. 1993). However, the site of clusterin mRNA transcription remains unclear. Aronow et al. (1993) did not detect clusterin mRNA in mouse anterior pituitary, whereas Laslop et al. (1993) detected it by Northern blotting in both anterior and posterior.
lobes. We have used RNA expression analysis, immunohistochemistry and in situ hybridisation to investigate clusterin synthesis in the ovine pituitary gland.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals.

For Northern blotting experiments the heads of adult ewes were obtained from the local abattoir and the brain and pituitary gland removed as rapidly as possible. Pituitary glands were dissected into the stalk median eminence, a dorsal strip containing neuronal axons, a posterior portion and the remaining anterior portion. Samples of medial basal hypothalamus, cerebral cortex, optic chiasma, spinal cord and cerebellum were collected for comparison. All tissues were frozen on dry ice and stored at −80°C until extraction. Ram lamb pituitary mRNA (Fleming et al. 1997) was used as a positive control on Northern blots probed with the growth hormone gene probe.

Pituitary glands for immunohistochemistry and in situ hybridisation were obtained from five 4- to 6-year-old Romney ewes killed with Euthatal (Southern Veterinary Supplies, Christchurch, New Zealand; 100 mg/kg, i.v.), at the mid-luteal phase of the oestrous cycle. Whole glands were removed from the skull within 10 min of death, embedded in OCT compound (Tissue Tek, Bayer Diagnostics Ltd, Christchurch, New Zealand), frozen on dry ice and stored at −80°C until required.

Poly(A)-enriched RNA extraction

Poly(A)-enriched RNA was extracted from 0.3-0.5 g samples, as previously described (Fleming et al. 1992). Poly(A)-depleted RNA remaining in solution after affinity chromatography on oligo(dT) cellulose was ethanol precipitated and resuspended in RNase-free water. The yield of RNA was estimated spectrophotometrically from absorbance at 260 nm (A260) and the purity of the poly(A)-enriched RNA from the A260/A280 ratio.

Gene probes

Rat clusterin (Cheng et al. 1988) and ovine growth hormone (Fleming et al. 1997) cDNA probes were verified by restriction analysis. Isolated insert was labelled with [α-32P]dCTP to specific activities >1 × 10^6 c.p.m./µg using random primers (Feinberg & Vogelstein 1983). Labelled probes were denatured at 95–100°C for 5 min before hybridisation.

For in situ hybridisation studies, the rat clusterin cDNA was digested with BstXI (New England Biolabs Inc., Beverly, MA, USA) and 32P-labelled cRNA riboprobes were transcribed with T7 (antisense) and SP6 (sense) RNA polymerases using the Riboprobe Gemini System II (Promega, Dade Behring Diagnostics Ltd, Auckland, New Zealand).

Northern blotting and hybridisation

Poly(A)-enriched and -depleted RNA samples (2 µg) were denatured, electrophoresed and blotted onto Hybond-N nylon filters (Amersham International, Amersham, Bucks, UK) as previously described (Fleming et al. 1992). The RNA was stained routinely with 1 µg ethidium bromide per 2 µg sample, added before denaturation (Gong 1992). In order to assess the amounts of RNA added to each lane, the ethidium bromide-stained gel photographs were scanned using reflectance densitometry (Bio-Rad Video Densitometer Model 620; Bio-Rad, Richmond, CA, USA) (Fleming et al. 1992).

Blots were hybridised with an [α-32P]dCTP-labelled cDNA probe for 20 h at 65°C and washed to a final stringency of 0·1×SSC (1×SSC is 15 mM sodium citrate plus 150 mM sodium chloride) and 0·1% (w/v) SDS at 65°C. Autoradiographs were scanned on the densitometer and the density of the signal from each sample was normalised by dividing it by the density of the ethidium bromide staining at 2 kb for that sample.

In situ hybridisation

The method of in situ hybridisation described by Leeuwenberg et al. (1995) was modified for use with ovine pituitary glands. Frozen OCT-embedded pituitary glands were sectioned to a thickness of 10 µm and thaw-mounted onto Superfrost Plus slides (BDH Ltd, Poole, Dorset, UK). Frozen sections were fixed in 4% (v/v) diethyl pyrocarbonate (DEPC; BDH)-treated paraformaldehyde/PBS (0·14 M NaCl, 2·7 mM KCl, 10 mM Na2HPO4, 1·8 mM KH2PO4), pH 7·4 for 20 min and washed in DEPC-treated PBS (the second wash included 0·1 M glycine). The tissue was treated for 10 min with 1 µg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) in 0·1 M Tris–HCl (pH 8·0), 50 mM EDTA at 37°C, washed in
DEPC-treated PBS and acetylated in 0·25% (v/v) acetic anhydride in 0·1 M triethanolamine (pH 8) at room temperature, before dehydration. Prior to hybridisation, negative controls were treated for 30 min at 37 °C with 20 µg/ml RNase A (Boehringer Mannheim) in 0·5 M NaCl, 10 mM Tris–HCl (pH 8·0) and 1 mM EDTA.

Sections were hybridised under coverslips overnight at 42 °C with 30 µl hybridisation buffer containing approximately 30 000 c.p.m. 32P-labelled clusterin antisense or sense riboprobe, 50% (v/v) deionised formamide, 0·3 M NaCl, 10 mM Tris–HCl (pH 6·8), 10 mM NaPO4, 5 mM EDTA, 1 × Denhardt’s solution (0·02% (w/v) Ficoll 400 (Sigma-Aldrich, St Louis, MO, USA), 0·025% (w/v) polyvinlypyrrollidone (Sigma), 0·02% BSA), 10% (w/v) dextran sulphate (Sigma), 50 mM dithiothreitol (Boehringer Mannheim), 1 mg/ml E. coli tRNA (Boehringer Mannheim). The slides were washed twice for 30 min in 50% formamide in 2 × SSC, twice for 30 min in 2 × SSC and twice for 30 min in 1 × SSC at 50 °C. Sections were then incubated in 20 µg/ml RNase A (Boehringer Mannheim) in 0·5 M NaCl, 10 mM Tris–HCl (pH 8·0), 1 mM EDTA for 30 min at 37 °C, washed and dehydrated through increasing concentrations of ethanol containing 0·3 M ammonium acetate.

The slides were then exposed overnight to Kodak BioMax film (Eastman Kodak, Rochester, NY, USA) to detect hybridisation signals, before being dipped in Hypercoat emulsion (Amersham) and exposed for 5–7 days at 4 °C in light-tight boxes containing silica gel. The sections were lightly counterstained with haematoxylin and eosin after anti-clusterin immunohistochemistry. Controls included serial dilution of anti-clusterin, omission of anti-clusterin and application of mouse IgG instead of anti-clusterin. The anti-clusterin monoclonal antibody (Blaschuk & Fritz 1984, Tung & Fritz 1985, Rosenior et al. 1987) was effective in dilutions up to 1:20 000 and was used at 1:10 000. There was no staining in the absence of anti-clusterin or with the application of mouse IgG at a concentration of five times that used for anti-clusterin.

RESULTS

Northern blot analysis of poly(A)-enriched RNA from all brain and pituitary samples revealed three clusterin mRNA transcripts of 2·3, 3·0 and 4·4 kb. The 2·3 kb band was absent from poly(A)-depleted extracts and the larger bands were stronger in poly(A)-enriched RNA than in poly(A)-depleted samples (Fig. 1a). The rat clusterin cDNA probe bound strongly to the ribosomal RNA bands at 5·0 and 2·1 kb in Northern blots of total cellular RNA extracts (data not shown).

The ovine growth hormone cDNA probe detected a single mRNA transcript at 1·0 kb (Fig. 1b), as previously reported (Fleming et al. 1997). Growth hormone hybridisation was detected only in pituitary extracts (Fig. 1b). There was considerable growth hormone hybridisation with poly(A)-enriched RNA extracted from the dorsal edge and posterior pituitary, confirming that these dissected regions contained significant amounts of anterior and intermediate lobe. Pituitary mRNA extracts from ram lambs gave a stronger hybridisation signal than those from adult ewes. No attempt was made to quantitate growth hormone hybridisation.

The density of the clusterin signal was highest in mRNA extracts from the anterior pituitary, spinal cord and hypothalamus and lowest in the dorsal strip and posterior pituitary extracts and white matter from the optic chiasma. Clusterin hybridisation densities obtained from three to five different
ewes and normalised with respect to the ethidium bromide staining (arbitrary units) are shown in Fig. 2 (mean ± s.d.).

**In situ hybridisation**

*In situ* hybridisation with the clusterin antisense riboprobe revealed a high density of silver grains throughout the anterior and intermediate pituitary lobes (Fig. 3B and E). Silver grains did not appear to be associated with any particular cell type or structures such as capillaries. A lower grain density was apparent in the posterior pituitary, where the cell density was also lower (Fig. 3B and E). A low grain density was also observed when the tissue was probed with the control sense riboprobe (Fig. 3C and F).

**Immunohistochemistry**

Immunohistochemistry with ovine anti-clusterin showed strong immunoreactivity present in the capillary endothelium and lumen of the anterior and intermediate lobes of the pituitary. Clusterin protein was evident within the capillaries in many sections (Fig. 3D and Fig. 4A and B). Isolated single cells within the anterior and intermediate lobes were also stained with the anti-clusterin antibody (Fig. 3D and Fig. 4A and B). In contrast, the pituicytes and capillary endothelium of the posterior lobe of the ovine pituitary were unreactive (Fig. 3A and Fig. 4A). Clusterin-immunopositive cells did not appear to be a specific anterior pituitary cell type. A small number of both acidophilic and basophilic cells (common in the intermediate lobe)

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**Figure 1.** Northern analysis of mRNA from extracts of dissected ovine pituitary glands and brain samples. (a) Clusterin hybridisation signals in samples of poly(A)-enriched and -depleted RNA from the dorsal edge of the pituitary (DP), the posterior pituitary (PP), the anterior pituitary (AP), median eminence (ME), medial basal hypothalamus (MBH), optic chiasma (OC), spinal cord (SC) and cerebellum (CB) of adult ewes. (b) Growth hormone mRNA hybridisation in extracts of brain and dissected pituitary glands from adult ewes and from two ram lamb pituitaries (RLP).
were immunopositive for clusterin (results not shown). There did not appear to be specific groups or clusters of immunopositive cells, or areas of the anterior and intermediate sheep pituitary gland in which cells stained preferentially (Fig. 3A). Cells that contained clusterin protein did not show an accumulation of silver grains after in situ hybridisation of adjacent sections, suggesting that the stained cells were not expressing high amounts of clusterin mRNA (Fig. 3A–C).

**DISCUSSION**

Northern analysis of pituitary extracts showed that clusterin expression occurred predominantly in the ovine anterior pituitary gland, with low concentrations of clusterin mRNA found in the posterior lobe extracts. This finding is similar to that of Laslop et al. (1993), who observed higher amounts of mRNA in bovine anterior pituitary extracts, but clusterin protein in both pituitary lobes. In the sheep brain extracts analysed, the highest clusterin hybridisation densities were found in the medial basal hypothalamus and spinal cord, both areas in which neuronal clusterin gene expression has been reported (Messmer-Joudrier et al. 1996, Wener et al. 1997). In previous studies on clusterin expression in sheep (Fleming et al. 1992), much higher amounts of clusterin mRNA were observed by Northern analysis in the liver and gonads than in the pituitary.

Three bands of clusterin hybridisation at 2.3, 3.0 and 4.4 kb were observed on Northern blots of ovine brain and pituitary poly(A)-enriched RNA, using a double-stranded, $^{32}$P-labelled cDNA probe. Previous studies with single-stranded, antisense riboprobes detected a single hybridisation band at 2.2 kb in a variety of sheep tissue extracts (Fleming et al. 1992). Wunsche et al. (1998) also reported a second band of clusterin hybridisation at 3.9 kb, in Northern blots of rat endometrium or endometrial adenocarcinoma cell lines. Levels of both mRNA transcripts were upregulated by oestradiol-17β treatment. In the study reported here, poly(A)-depleted RNA showed a substantially reduced signal for all three bands and especially for the 2.3 kb band, which suggests that the higher bands were not generated by non-specific binding to ribosomal RNA. Because the identity of the larger species was not verified, only the density of the 2.3 kb band was used in the semi-quantitative comparison of clusterin expression in different tissues.

The in situ hybridisation studies confirmed the findings of the Northern blotting analysis, but did not clearly define which anterior and intermediate pituitary cells expressed clusterin mRNA. Although it could be argued that the density of cell bodies in the posterior pituitary was lower than in the anterior or intermediate lobes, the density of silver grains over the posterior pituitary sections was similar to that seen with the control sense riboprobe in all pituitary lobes and there were no noticeable clusters of silver grains over specific posterior pituitary cells. In the anterior and intermediate lobes it appeared that many cells were synthesising clusterin mRNA. There was no strong evidence that the cells which stained with anti-clusterin immunoreactivity were synthesising more mRNA (Fig. 3A–C).
Clusterin expression in sheep pituitary gland
Clusterin therefore appears to be synthesised by the majority of cells in the ovine anterior and intermediate pituitary gland, but most of the protein appears to be in or around the capillaries. Evidence for synthesis of clusterin by capillary endothelial cells is somewhat contradictory. Clusterin is expressed in porcine vascular smooth muscle cells in culture and appears to be required for the formation of multicellular nodules in these cultures (Thomas-Salgar & Millis 1994), suggesting that the protein functions as a cell adhesion molecule in vitro differentiation. While clusterin expression is upregulated in studies of hypertension-induced microcapillary apoptosis in skeletal muscle, clusterin mRNA is not found to be localised in the endothelial cells (Gobe et al. 1997). Clusterin is also known to act as a serum complement inhibitor, rendering the terminal complement complex soluble and unable to insert into cell membranes (Tschopp et al. 1993). Berge et al. (1997) found some evidence of clusterin synthesis in human umbilical vein endothelial cells which was regulated by various proinflammatory cytokines (interleukins IL-1 and IL-6, and tumour necrosis factor), as well as interferon-gamma. The presence of clusterin immunoreactivity in endothelial cells does not necessarily imply its synthesis here, since clusterin has been shown to facilitate transport of soluble amyloid beta (1–40)–clusterin complexes across the cerebral vascular endothelium and choroid epithelium, via glycoprotein 330 (gp330)/megalin, the proposed receptor for clusterin (Zlokovic et al. 1996). Megalin is a member of the low-density lipoprotein receptor gene family and is expressed on the apical surfaces of epithelial tissues, where it mediates the endocytotic uptake of diverse macromolecules, such as cholesterol-carrying lipoproteins (including clusterin, also known as apolipoprotein J (Kounnas et al. 1997), proteases and serum proteins including albumin (Cui et al. 1996). The question therefore arises as to whether the strong immunoreactivity observed in the lumen of capillaries in the ovine anterior and intermediate pituitary indicates secretion of the protein, or the uptake of clusterin–lipid or clusterin–protein complexes from the serum. It is possible that clusterin and gp330/megalin act together as a cellular waste management system within tissues, but studies on the distribution of the gp330/megalin molecule in this tissue are needed before this question can be answered.

In the pig, clusterin has been shown to form a high proportion of the colloid deposits that occur in the anterior pituitary gland of older animals (Ogawa et al. 1997). These insoluble complexes are found associated with non-secretory folliculo-stellate cells and increase in number with age in the pig and in some species after hibernation (Ogawa et al. 1997). Clusterin gene expression has been shown to be negatively controlled in vivo and in vitro by glucocorticoids, testosterone, progesterone and oestrogen, in a range of hormone-dependent tissues (Frassoldati et al. 1995, Furlong et al. 1996, Gutacker et al. 1996, Wunsche et al. 1998), but there is little evidence for steroid control of clusterin expression at the level of the gene promoter (Wilson et al. 1995). Because clusterin synthesis is induced in many hormone-dependent tissues after hormone ablation, the presence of pituitary colloid deposits in situations of decreased circulating steroid, such as senescence or hibernation, implies steroid control of clusterin uptake and/or synthesis in the anterior pituitary gland.

Alternatively, clusterin may act as a granin in the anterior pituitary and aid in hormone secretion in response to Ca2+ flux. Clusterin is found in the dense core secretory granules of endocrine tissues such as the adrenal medulla and pituitary gland, associated with granins such as secretogranin II and chromogranin B (Laslop et al. 1993), which are aggregated by increased Ca2+ concentrations and a pH of around 5·5 (Gerdes et al. 1989). Prolactin granulogenesis and secretion is upregulated by oestradiol-17β treatment in a GH4C1 pituitary tumour cell line (growth hormone cells) and this is associated with increased secretogranin II and chromogranin B expression and aggregation in the Golgi bodies and secretory granules (Thompson et al. 1995). Evidence for synthesis of clusterin by capillary endothelial cells is somewhat contradictory. Clusterin is expressed in porcine vascular smooth muscle cells in culture and appears to be required for the formation of multicellular nodules in these cultures (Thomas-Salgar & Millis 1994), suggesting that the protein functions as a cell adhesion molecule in vitro differentiation. While clusterin expression is upregulated in studies of hypertension-induced microcapillary apoptosis in skeletal muscle, clusterin mRNA is not found to be localised in the endothelial cells (Gobe et al. 1997). Clusterin is also known to act as a serum complement inhibitor, rendering the terminal complement complex soluble and unable to insert into cell membranes (Tschopp et al. 1993). Berge et al. (1997) found some evidence of clusterin synthesis in human umbilical vein endothelial cells which was regulated by various proinflammatory cytokines (interleukins IL-1 and IL-6, and tumour necrosis factor), as well as interferon-gamma. The presence of clusterin immunoreactivity in endothelial cells does not necessarily imply its synthesis here, since clusterin has been shown to facilitate transport of soluble amyloid beta (1–40)–clusterin complexes across the cerebral vascular endothelium and choroid epithelium, via glycoprotein 330 (gp330)/megalin, the proposed receptor for clusterin (Zlokovic et al. 1996). Megalin is a member of the low-density lipoprotein receptor gene family and is expressed on the apical surfaces of epithelial tissues, where it mediates the endocytotic uptake of diverse macromolecules, such as cholesterol-carrying lipoproteins (including clusterin, also known as apolipoprotein J (Kounnas et al. 1997), proteases and serum proteins including albumin (Cui et al. 1996). The question therefore arises as to whether the strong immunoreactivity observed in the lumen of capillaries in the ovine anterior and intermediate pituitary indicates secretion of the protein, or the uptake of clusterin–lipid or clusterin–protein complexes from the serum. It is possible that clusterin and gp330/megalin act together as a cellular waste management system within tissues, but studies on the distribution of the gp330/megalin molecule in this tissue are needed before this question can be answered.

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**Figure 3.** Expression of clusterin mRNA and protein in serial sections of the ovine pituitary gland. (A–C) Serial sections at the transition between the intermediate lobe (I) and the posterior lobe (P). (A) Phase contrast immunohistochemistry, with a specific monoclonal antibody immunoglobulin against sheep clusterin. A single intermediate pituitary cell is stained with the anti-clusterin antibody (arrowhead). (B) In situ hybridisation with the 33P-labelled ovine clusterin antisense riboprobe, showing higher silver grain density in the intermediate lobe than in the posterior pituitary. The immunoreactive cell in (A) does not appear to be expressing more clusterin mRNA. (C) In situ hybridisation with the control sense riboprobe. (D–F) Serial sections within the ovine anterior pituitary. (D) Ovine anti-clusterin immunohistochemistry, demonstrating staining within a capillary and within some cells. (E) In situ hybridisation with the 33P-labelled ovine clusterin antisense riboprobe. Most anterior pituitary cells have silver grains associated with them. (F) In situ hybridisation with the control sense riboprobe. Scale bars in the bottom left of panel (C) and (F) represent 50 µm with minor divisions of 10 µm.
Clusterin immunoreactivity in the ovine pituitary gland. (A) A section through the posterior (P) and intermediate (I) lobes, showing immunoreactive deposits in the capillary lumen (arrowheads) and in the occasional single cell (arrows). Scale bar represents 50 µm with minor divisions of 10 µm. (B) Anterior pituitary gland anti-clusterin immunohistochemistry, counterstained with eosin, demonstrating staining in or on the capillary endothelium (arrow head) and within the capillaries. Isolated secretory pituitary cells are also stained (arrows). Scale bar represents 50 µm with minor divisions of 10 µm.

We conclude that clusterin is expressed by many cells in the ovine anterior and intermediate pituitary lobes. The high clusterin immunoreactivity observed in isolated single cells may be indicative of rare apoptotic events occurring within the anterior and intermediate lobes. Strong clusterin immunoreactivity in and around the capillaries, on the other hand, may be associated with megalin-mediated lipid or lipoprotein uptake into the pituitary cells, or with hormone secretion from this endocrine tissue.

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