Oestrogen and progesterone receptor immunoreactivity and *c-fos* expression in the ovine cervix

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Immunocytochemistry was used to detect the presence of oestrogen and progesterone receptors in the cervixes of prepubertal lambs, seasonally anoestrous ewes, cyclic ewes, and pregnant ewes of known gestational stages, to define the roles of gonadal steroids in cervical function. The presence of the immediate early gene product, c-Fos, a marker for cellular activation, was also investigated using immunocytochemistry and *in situ* hybridization. Oestrogen receptor immunoreactivity was restricted to the endometrium on days 0–3 of the oestrous cycle (day 0 = oestrus). In immature animals, very few scattered nuclei in the endometrium were immunoreactive. Oestrogen receptor immunoreactivity was not apparent in the endometrium during the remainder of the oestrous cycle or in this region in anoestrous animals. In pregnant ewes, oestrogen receptor immunostaining appeared as relatively few isolated nuclei in the connective tissue stroma. Progesterone receptor immunoreactivity was found in the endometrium at days 0–3 of the oestrous cycle and also in the luminal epithelium, the myometrium and the blood vessels. Progesterone receptor immunoreactivity was also found in these regions, with the exception of the endometrium, at all other stages examined. Immunostaining for c-Fos was present in the endometrium at days 0–3 of the oestrous cycle, and some scattered immunopositive nuclei were present in prepubertal animals. c-Fos immunoreactivity was also found in the myometrium and in blood vessels at all other stages examined. Visualization of *c-fos* gene expression by *in situ* hybridization showed that it occurred in the luminal epithelium and blood vessels at oestrus, but was restricted to the blood vessels in all other samples examined.

Introduction

The cervix acts as a protective barrier for the upper genital tract in non-pregnant females and prevents expulsion of the conceptus throughout pregnancy while permitting the passage of the offspring at parturition. Fulfilling these diverse roles involves major changes in both the function and the form of the cervix (Dobson, 1988). The factors that control the function of the ovine cervix are poorly understood, but like most of the female reproductive tract, it is thought that ovarian steroids are important.

Ovarian steroid hormones regulate development, growth, proliferation and differentiation of reproductive tissues through their specific receptors (Clark and Mani, 1994). The number of steroid receptors varies depending on the steroidal milieu. Oestradiol and progesterone are known to alter the expression of oestrogen receptors (Bergman *et al.*, 1992; Clark and Mani, 1994; Perrot-Appianat *et al.*, 1994).

Oestradiol also influences the expression of progesterone receptors (Blaustein and Turcotte, 1989; Chauchereau *et al.*, 1992; Clark and Mani, 1994). In the ovine uterus, the populations of oestrogen and progesterone receptors change markedly during the oestrous cycle (Cherny *et al.*, 1991; Wathes and Hamon, 1993). Consequently, the influence of oestradiol and progesterone varies in different stages of the oestrous cycle and also in different tissues (Wathes and Hamon, 1993).

The aim of this study was to investigate the role of oestrogen and progesterone in the function of the ovine cervix. Oestrogen and progesterone receptors in the cervix of animals with different steroidal backgrounds were mapped using immunocytochemistry. Tissue was examined from immature animals, seasonally anoestrous animals, cyclic animals, and animals in late pregnancy. c-Fos expression in the ovine cervix was also investigated in serial sections from the same animal by *in situ* hybridization and immunocytochemistry. Stimulation of cells by oestrogen has been linked to the expression of the immediate early gene product, c-Fos; oestradiol induces expression of *c-fos* mRNA in the rat uterus (Nephew *et al.*, 1995). In addition, *c-fos*

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expression correlates with the proliferative response in the oestrogen-stimulated immature uterus (Boettger-Tong *et al.*, 1995). The possible relationship between the presence and activation of oestrogen receptors and the induction of *c-fos* was investigated in the ovine cervix. The use of immunocytochemistry and *in situ* hybridization provides a direct comparison between tissues that express oestrogen and progesterone receptors and *c-fos*.

Materials and Methods

Animals

Ovine (Suffolk crossbred) cervical tissues were obtained from pre-pubertal lambs ($n = 2$), seasonally anoestrous ewes ($n = 4$) and cyclic ewes ($n = 14$) that had been killed at an abattoir. The stage of the oestrous cycle in the cyclic ewes was confirmed at death by visual inspection of the ovaries and corpora lutea as described by Oldham and Lindsay (1980). Pregnant ewes ($n = 6$) at a known gestational stage (125 and 135 days) were killed by barbiturate overdose (0.5 ml kg⁻¹ i.v.) and cervixes were collected. All procedures complied with the Animals (Scientific Procedures) Act of 1986. Tissue was rapidly frozen in isopentane over dry ice and stored at -70°C. Cryostat sections (20 µm) were taken from the mid-region of the cervix and thaw mounted onto gelatin-coated slides for immunocytochemistry or onto gelatin poly(L-lysine)-coated slides for *in situ* hybridization.

Immunocytochemistry

Cryostat sections were fixed overnight in acetone at -80°C, transferred to -20°C for 1 h, and incubated in 1% (w/v) BSA for 20 min at room temperature to reduce non-specific binding. Sections were then incubated for 18 h at 4°C with the primary antibody in 1% (w/v) BSA in 0.01 mol PBS I⁻¹. A polyclonal antibody raised against the rat oestrogen receptor (Department of Health and Human Services, National Institute of Health, Bethesda, MD) was used at a dilution of 1:1000. A polyclonal anti-human progesterone receptor (NCL-PGRp) (Novocastra Laboratories Ltd, Newcastle upon Tyne) was used at a dilution of 1:800 dilution, and a polyclonal antibody raised against a synthetic peptide derived from the N-terminal region of c-Fos (Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Fund, London) was used at a dilution of 1:500. Sections were then washed in 0.01 mol PBS I⁻¹ (2 × 5 min) and incubated with polyclonal anti-rabbit IgG peroxidase conjugate (Sigma International, Poole) diluted 1:500 in 1% (w/v) BSA in 0.01 mol PBS I⁻¹ for 2 h. Slides were then washed (2 × 5 min) in 0.01 mol PBS I⁻¹. The colour reaction was developed using a vector peroxidase substrate kit (Vector Laboratories, Peterborough). Control sections were incubated as described above with the exception that the primary antibody was replaced by normal rabbit serum. Sections were then rinsed in distilled water, lightly counterstained with neutral red, dehydrated and mounted.

In situ hybridization

Cryostat sections were fixed in 4% (w/v) paraformaldehyde in 0.1 mol PBS I⁻¹ for 20 min on ice. After washing in 0.5 mol PBS I⁻¹ (2 × 5 min), slides were treated in 0.1 mol triethanolamine (TEA) I⁻¹ for 2 min, followed by 0.1 mol TEA I⁻¹ and 0.25% (v/v) acetic anhydride for 10 min. Slides were then washed in PBS (2 × 2 min), dehydrated in a graded series of ethanol and dried under vacuum for 60 min. The slides were incubated with 80 µl hybridization solution for 18 h at 60°C. The cRNA probe was produced using a 684 bp fragment of the mouse *v-fos* gene (Beatson Cancer Research Institute, Glasgow) which was directionally cloned into pBluescript SK. The cRNA probe was labelled with [³⁵S]UTP and linearized with *Pst* I to generate antisense transcripts using T7 polymerase and linearized with *Sal* I to generate sense transcripts using T3 polymerase. The hybridization solution was composed of 50% (v/v) formamide, 0.3 mol NaCl I⁻¹, 1 × Denhardt's solution, 10 mmol Tris I⁻¹, 1 mmol EDTA I⁻¹, 0.05% (v/v) yeast transfer RNA, 10 mmol dithiothreitol I⁻¹ and 10% (w/v) dextran sulphate. After hybridization, sections were washed in several changes of 4 × saline-sodium citrate (SSC), 2 × SSC, 1 × SSC, 0.5 × SSC and 0.1 × SSC, treated with RNase A and dehydrated in ethanol. Air dried slides were then apposed to Hyperfilm β-max film (Amersham International, Little Chalfont, Amersham) for 5 days at room temperature, or coated with LM-1 liquid film (Amersham International, Little Chalfont, Amersham). The sections were then stained in toluidine blue and mounted for conventional photography. Photographic negatives were then scanned using a UMAX Power Look II scanner (UMAX Technologies Inc., Fremont, CA), processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and printed on a Tektronix Phaser dye sublimation printer (Tektronix Inc., Wilsonville, OR). All chemicals were supplied by Sigma International, Poole, unless otherwise indicated.

Results

Oestrogen receptors

There was marked variation in oestrogen receptor immunoreactivity in the cervix in the different groups of animals examined. During oestrus (day 0), strong nuclear staining was observed in the endometrium (Fig. 1a). The nuclear staining was also present on days 1, 2 and 3 of the oestrous cycle and appeared to be of a similar intensity and localization (not shown). Oestrogen receptor immunoreactivity was absent for the remainder of the oestrous cycle (not shown). Relatively few scattered immunopositive nuclei were observed in the endometrium of the prepubertal cervix (Fig. 1b) and no immunopositive nuclei were observed in the cervixes of seasonally anoestrous animals (Fig. 1c). Oestrogen receptor immunoreactivity was not observed in the endometrium on days 125 and 135 of pregnancy (Fig. 1d), but very few isolated nuclei were found in the myometrium and surrounding connective tissue (not shown).

Fig. 1. Oestrogen receptor immunoreactivity in the ovine cervix. (a) Immunopositive nuclei (blue-grey in colour; see arrow) occur in the endometrium (E) at oestrus. (b) Scattered immunopositive nuclei (arrows) are observed in the endometrium of an immature animal. No immunopositive nuclei are found in the endometrium (c) during seasonal anoestrus or (d) during late pregnancy (125 days of gestation). Results are representative of four independent experiments. Scale bar represents 200 μ m.

Progesterone receptors

Progesterone receptor immunoreactivity was present in the nuclei of the endometrium (Fig. 2a) at oestrus and up to day 3 of the oestrous cycle, and also appeared in the cytoplasm of the luminal epithelium, myometrium and blood vessels (not shown). Staining was not apparent in the endometrium during the later stages of the cycle, but remained present in the nuclei and cytoplasm of the myometrium and blood vessels (not shown). Positive staining was also observed in the myometrium, luminal epithelium and blood vessels in anoestrous ewes (Fig. 2b). During late pregnancy (days 125 and 135) positive staining was observed in myometrium, blood vessels (not shown) and the luminal epithelium (Fig. 2c).

c-Fos localization and gene expression

c-Fos localization and gene expression in the ovine cervix were investigated by immunocytochemistry and *in situ* hybridization. *c-Fos* immunoreactivity was restricted to the endometrium of the cervix on days 0–3 of the oestrous cycle

(Fig. 3a). No *c-Fos* immunoreactivity was apparent in this region during the later stages of the oestrous cycle or during anoestrus (not shown). In all samples examined, immunostaining could be observed in the myometrium and blood vessels (Fig. 3b). *In situ* hybridization revealed that *c-fos* mRNA occurred at oestrus in the luminal epithelium but not in the endometrium (Fig. 4a). No specific signal for expression of the *c-fos* gene was observed in the luminal epithelium on other days of the cycle, in immature animals, or during anoestrus or late pregnancy. However, a signal was apparent in endothelial cells of blood vessels in all samples examined (Fig. 4b–d).

Discussion

The structure and appearance of the cervix changes markedly during the oestrous cycle, pregnancy and parturition, and there appears to be a temporal relationship between these changes and the changes in circulating concentrations of oestradiol and progesterone (Fitzpatrick and Dobson, 1979). In sheep, oestradiol induces changes in the mechanical strength of the cervix (Fitzpatrick and

Fig. 2. Progesterone receptor immunoreactivity in the ovine cervix. (a) Immunopositive nuclei (blue–grey in colour; see arrows) occur in the endometrium at oestrus; the luminal epithelium is also positive. (b) Immunopositive staining in seasonal anoestrus in the myometrium and blood vessels resembles that found at all other

Fig. 3. c-Fos immunoreactivity in the ovine cervix. (a) Immunopositive nuclei (blue–grey in colour; see arrows) occur in the endometrium at oestrus. (b) Immunopositive staining in the myometrium (M) and blood vessels (BV) at seasonal anoestrus. Scale bar represents 150 μ m.

Dobson, 1981). However, the full role of steroid hormones in cervical function is not clear.

In the present study, oestrogen receptors were detected in the endometrium of the ovine cervix. This finding correlates strongly with the expected peak in serum oestradiol concentrations (Hauger *et al.*, 1977; Legan and Karsch, 1979), and is in accordance with the observation that oestrogen and progesterone receptors are at maximum concentrations in all regions of the ovine reproductive tract at oestrus (Wathes and Hamon, 1993). The absence of oestrogen receptor immunoreactivity in the ovine cervical endometrium during the remainder of the oestrous cycle may be the result of

stages examined. (c) Immunopositive staining in the luminal epithelium at day 125 of pregnancy. Results are representative of three independent experiments. BV, blood vessels; LE, luminal epithelium; M, myometrium. Scale bar represents 200 μ m.

Fig. 4. *c-fos* gene expression in the ovine cervix as revealed by *in situ* hybridization. (a) Dark-field micrograph showing *c-fos* expression (arrows) in (a) the luminal epithelium (LE) at oestrus and (b) in blood vessels at seasonal anoestrus. (c,d) Light-field micrographs showing silver grains (arrows) associated with the endothelial cells of blood vessels. Results are representative of three independent experiments. Scale bars represent (a) 70 µm, (b) 100 µm, (c,d) 50 µm.

reduced circulating oestradiol concentrations or increased progesterone concentrations. Oestradiol upregulates the number of oestrogen receptors (Bergman *et al.*, 1992; Clark and Mani, 1994), while progesterone downregulates the number of oestrogen receptors (Clark and Mani, 1994; Perrot-Applanat *et al.*, 1994).

Progesterone receptor immunoreactivity in the cervical endometrium at oestrus also correlates well with increased serum oestradiol and low serum progesterone concentrations at this stage (Legan and Karsch *et al.*, 1979). Progesterone receptor immunoreactivity persists in the

luminal epithelium, the myometrium and the vascular epithelium of the cervix and is not coincident with oestrogen receptor immunoreactivity, indicating a fundamental difference in the regulation of progesterone receptors between these regions and the endometrium. Oestradiol upregulates the number of progesterone receptors (Chauchereau *et al.*, 1992; Clark and Mani, 1994), and this effect is specific to oestrogen receptive cells in the brain (Blaustein and Turcotte, 1989). This implies that, in the cervical endometrium, progesterone receptor expression is increased by oestradiol and that progesterone and oestrogen receptors are present in the same cells within this region. An increase in progesterone concentration may also result in the downregulation of progesterone receptors in the cervical endometrium during the luteal phase (Clark and Mani 1994; Perrot-Applanat *et al.*, 1994). Progesterone receptors in the myometrium of the ovine uterus persist throughout the oestrous cycle, as in the cervix (Wathes and Hamon, 1993). The presence of progesterone receptors in muscle and blood vessels reflects the potential importance of progesterone in the growth and development of the cervix.

Expression of *c-fos* is induced by oestrogen (Loose-Mitchell *et al.*, 1988; Weisz and Bresciani, 1988; Weisz and Bresciani, 1993). Several groups have reported the presence of oestrogen response elements in the regulatory region of the *c-fos* gene (Weisz and Rosales, 1990; Hyder *et al.*, 1991). In the present study, immunoreactive c-Fos was found in the endometrium of the cervix at oestrus, which correlates with peak concentrations of circulating oestradiol and the presence of both oestrogen and progesterone receptors in this region. This observation indicates that the expression of c-Fos in the endometrium is related to the activation of oestrogen receptors. However, *c-fos* gene expression was not detected at oestrus by *in situ* hybridization. This is almost certainly due to the rapid turnover of *c-fos* mRNA, which is only expressed for a limited period, while c-Fos protein is more stable. These results are in agreement with a study of *c-fos* expression and immunoreactivity in the ovine uterus throughout the oestrous cycle, in which gene expression occurred only at oestrus, but the protein persisted for several days after gene expression became undetectable (Johnson *et al.*, 1997). *c-fos* mRNA was found consistently in blood vessels, and its presence may be linked to that of progesterone receptors. However, many endocrine and paracrine factors influence blood vessel function, particularly in tissues that have the potential to undergo rapid remodelling, such as the cervix. The presence of *c-fos* gene expression in the vasculature of the cervix could be the result of any one of these factors (Rivera and Greenburg, 1990; Hyman *et al.*, 1993).

In summary, the results of the present study show that the number of oestrogen and progesterone receptors in the endometrium of the ovine cervix is modulated during the oestrous cycle. Oestrogen receptors are mainly restricted to the endometrium and are consistently either absent or present in relatively low concentrations, with the exception of oestrus, during which period their expression is much more pronounced. Progesterone receptors are also present in blood vessels and in the myometrium. In the present study, progesterone receptors were expressed in these tissues at all

the stages examined. Therefore, the mechanisms controlling the expression of progesterone receptors in the endometrium differ from those controlling their expression in other areas of the cervix. The presence of oestrogen and progesterone receptors in the endometrium appears to correlate with c-Fos immunoreactivity.

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