Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells

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The effect of oestrogen and progesterone on the proliferation of cultured bovine uterine epithelial and stromal cells was assessed. Epithelial and stromal cells recovered from cows at day 1 to day 3 of the oestrous cycle were cultured in RPMI medium supplemented with 5% steroid-free fetal calf serum for 4 and 8 days. The addition of progesterone to the culture medium altered the morphology of stromal cells. Oestradiol ($0.1\text{--}10\text{ nmol l}^{-1}$) and progesterone (50 nmol l^{-1}) significantly increased the total DNA (from 9.6 ± 0.96 to $25.6 \pm 0.99\text{ }\mu\text{g}$ per well, $P < 0.001$) and protein content (from 76.6 ± 2.6 to $125.8 \pm 2.6\text{ }\mu\text{g}$ per well, $P < 0.001$) and decreased the ratio of protein to DNA (from 8.0 ± 0.24 to 4.9 ± 0.24 , $P < 0.01$) in stromal cells during the first 4 days. During the second 4 days, the relative percentages of increase in DNA content were not affected by steroids, indicating that the major effect of steroids on stromal cell proliferation was exerted during the first 4 days of incubation. The morphology of epithelial cells was not influenced by the addition of steroids. DNA content of epithelial cells was reduced by the addition of oestrogen (from 22.9 ± 2.1 to $15.0 \pm 2.0\text{ }\mu\text{g}$ per well, $P < 0.01$), but not progesterone (from 22.9 ± 2.1 to $25.8 \pm 2.0\text{ }\mu\text{g}$ per well, $P > 0.05$). Total protein content of epithelial cells was reduced by oestradiol by day 4 (from 111.0 ± 6.2 to $71.0 \pm 6.2\text{ }\mu\text{g}$ per well, $P < 0.01$), but not by day 8 (from 305.0 ± 10.5 to $296.0 \pm 10.5\text{ }\mu\text{g}$ per well, $P > 0.05$). Progesterone increased the total protein content (from 305.0 ± 10.5 to $366.0 \pm 10.5\text{ }\mu\text{g}$ per well, $P < 0.01$). Oestradiol significantly enhanced the ratio of protein to DNA in epithelial cells at day 8 (from 10.1 ± 1.0 to 16.8 ± 1.0 , $P < 0.01$). These results show that oestradiol and progesterone have different effects on the proliferation and morphology of epithelial and stromal cells *in vitro*.

Introduction

The endometrium undergoes major histological and biochemical changes during the oestrous cycle. These changes are induced by progesterone and oestradiol and are necessary to provide a suitable environment for embryo development in the pregnant animal. There are marked morphological changes in the endometrium, such as an increase in the thickness and growth of the glands during the oestrous cycle in ruminants (Nalbandov, 1976). Progesterone and oestradiol are also responsible for maintaining synchrony between the embryo and the endometrium (Wilmot *et al.*, 1985) and play an important role in regulating prostaglandin release at luteolysis (McCracken *et al.*, 1984; Lafrance and Goff, 1988). Understanding the mechanisms involved in the action of progesterone and oestradiol in these processes is difficult due to the complexity of the interactions involved.

Recent work in sheep has shown that growth of epithelial and stromal cells is correlated with systemic concentrations of progesterone and oestradiol (Johnson *et al.*, 1997). The

proliferation and differentiation of uterine epithelial and stromal cells are controlled primarily by oestradiol and progesterone (Conti *et al.*, 1981; Irwin *et al.*, 1991; Watson *et al.*, 1994). However, the growth and differentiation responses to these hormones in the different endometrial cell types are not yet fully understood. Furthermore, the effect of oestradiol and progesterone on uterine endometrial proliferation observed *in vitro* is inconsistent and depends on experimental conditions, such as the presence of serum and the purity of the isolated cell population. Oestradiol, progesterone or their combination stimulate the growth of human endometrial cells (Pavlik and Katzenellenbogen, 1978) and isolated stromal cells (Irwin *et al.*, 1991) cultured in the presence of a steroid-free calf serum. In combination, but not separately, oestradiol and progesterone significantly increased [^3H]thymidine incorporation by human endometrial stromal cells in serum-free medium (Chegini *et al.*, 1992). A lack of effect by oestradiol and progesterone on the proliferation of endometrial, epithelial and stromal cells from rodents and humans has been reported (Iguchi *et al.*, 1983; Tomooka and McLachlan, 1986; Irwin *et al.*, 1989; Chegini *et al.*, 1992; Rossi *et al.*, 1992). It has been suggested that this lack of oestradiol may be due to the absence of growth factors,

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such as insulin (Van der Burg *et al.*, 1988), insulin-related polypeptides (Murphy *et al.*, 1987) and epidermal growth factor (Mukku and Stancel, 1985; Di Augustine *et al.*, 1988). These factors are considered to be potential mediators of oestrogen-induced proliferation. Inhibitory effects of oestrogen on the proliferation of guinea-pig (Alkhalaf *et al.*, 1991) and human (Marshburn *et al.*, 1994) endometrial glandular epithelium have also been reported.

We have previously shown that progesterone and oestradiol can alter prostaglandin secretion from epithelial and stromal cells isolated from bovine endometria (Bergeron and Goff, 1993; Asselin *et al.*, 1996). However, little information is available on the effect of these hormones on bovine endometrial cell proliferation. Although oestradiol is uterotrophic *in vivo*, no effect of oestradiol has been observed on the growth of bovine endometrial cells *in vitro* (Tiemann *et al.*, 1994; Asselin and Fortier, 1996). The objective of the study reported here was to determine if oestradiol and progesterone are able to alter the DNA and protein content of endometrial stromal and epithelial cells grown *in vitro*.

Materials and Methods

Chemicals and reagents

Tissue culture medium (RPMI 1640), Hank's Buffered Saline Solution (HBSS, calcium and magnesium free), fetal calf serum (FCS), antibiotics and Trypan blue were purchased from Gibco (Grand Island, NY). Collagenase (Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), gentamicin, calf thymus DNA, Hoechst No. 33258, BSA, oestradiol, progesterone and goat anti-mouse immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St Louis, MO). Monoclonal antibodies to cytokeratin (No. 18) and fibronectin were obtained from Boehringer (Mannheim). Matri-gel was obtained from VWR Scientific (Ontario). Bio-Rad protein assay-dye reagent concentrate was obtained from Bio-Rad Laboratories (Mississauga, Ontario).

Preparation and culture of cells

Bovine uteri from cows at day 1 to day 3 of the oestrous cycle were collected at an abattoir and transported on ice to the laboratory. Days 1–3 were selected because the stage of oestrus can be determined accurately from slaughterhouse material owing to the presence of the corpus haemorrhagicum in the ovary and, thus, variability among uterine samples is reduced. The endometrial epithelial and stromal cells were separated by a modification of the procedure described by Fortier *et al.* (1988). Briefly, the two horns of the uterus were placed in sterile HBSS containing 10 µl antibiotics ml⁻¹ (100 units of penicillin, 100 µg of streptomycin and 0.25 µg of amphotericin B). The myometrial layers were dissected from the two horns, and the remaining endometrial tissue was then everted to expose the epithelium. The everted horns were first digested for 2 h in HBSS with 0.3% (w/v) trypsin at room temperature to obtain epithelial cells. At the end of incubation, the digested horns were scraped slightly with forceps and washed twice in HBSS. The cells from the digestion and

washings were pooled and FCS was added (10% final volume) to block the action of trypsin. The horns were then further digested to obtain stromal cells by incubating in HBSS with 0.064% (w/v) trypsin III, 0.064% (w/v) collagenase II and 0.032% (w/v) DNase I for 45 min at 37°C. FCS was added (10% final volume) to block the action of trypsin at the end of incubation.

Epithelial cells. Most of the epithelial cells were in clumps after trypsin digestion. Therefore, it was possible to separate them from single stromal cells by low-speed centrifugation (60 *g* for 5 min). The centrifugation pellet was then washed three more times with HBSS. The epithelial cells were purified further by suspension in 20 ml RPMI medium supplemented with 5% FCS and 50 µg ml⁻¹ of gentamicin and plated onto 100 mm × 20 mm Petri dishes (Nunc, Grand Island, NY) and incubated at 37°C with 5% CO₂, 95% air for 3 h. At the end of the incubation, any contaminating stromal cells that had adhered to the dish and the floating epithelial cells were collected. The cells were counted and their viability determined by Trypan blue exclusion. At this stage >95% of cells were viable. The epithelial cells were then plated onto Matri-gel coated six-well plates at a concentration of 5 × 10⁵ cells per well (Corning, NY). Since the epithelial cells took about 48 h to attach to the plates, they were cultured for 4 more days in RPMI-1640 medium containing 5% FCS that had been depleted of steroids by dextran–charcoal extraction at 37°C in humidified air (5% CO₂). The cells were then cultured for 4 or 8 days in the presence or absence of various concentrations of oestradiol and progesterone.

Stromal cells. The cell suspension of stromal cells was centrifuged at 60 *g* for 5 min to remove cell clumps and the supernatant was then centrifuged at 1000 *g* for 10 min. The pelleted stromal cells were washed twice with HBSS and plated onto six-well plates at a concentration of 2 × 10⁶ cells per well and incubated for 3 h. The floating cells were washed away by gentle pipetting. The attached stromal cells were then cultured in RPMI-1640 medium containing 5% FCS, which was depleted of steroids by dextran–charcoal extraction, in the presence or absence of various concentrations of oestradiol and progesterone. At the time of plating, the viability of the stromal cells was >95%.

Cell proliferation experiment

All cells were cultured in phenol red-free RPMI-1640 medium containing 5% FCS (depleted of steroids by dextran–charcoal extraction) in the presence or absence of steroids at 37°C in humidified air (5% CO₂). The charcoal extraction procedure decreased added [³H]oestradiol (25 pg ml⁻¹) by at least 100-fold. The stromal and epithelial cells had reached about 60% confluency at the beginning of culture. Cells were cultured for 4 or 8 days in medium alone or in medium containing oestradiol (0.1, 1 or 10 nmol l⁻¹), progesterone (50 nmol l⁻¹) or oestradiol (1 nmol l⁻¹) plus progesterone (50 nmol l⁻¹). The medium was changed every 2 days. The 4- and 8-day culture periods presented in the results represent the time the cells were exposed to the hormone treatment.

Determination of DNA content

At the end of the culture period, the medium was removed and the cells were rinsed twice with HBSS and then detached with 1 mmol EDTA l^{-1} in HBSS and the use of a rubber scraper. Cells were pelleted by centrifuging at 1000 g for 5 min, and 100 μl of 0.2% (w/v) SDS in ETN buffer (10 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl, 100 mmol l^{-1} NaCl, pH 7.0) was added to the pellet. The pellet was sonified 10 times for stromal cells and 15 times for epithelial cells using a Branson sonifier-450 (VWR Scientific, Ontario) at 10% power. The DNA content in 10 μl sonified cell suspension was determined using the bisbenzimidazole fluorescent dye method of Labarca and Paigen (1980). Calf thymus DNA was used as standard at concentrations of 4, 8, 16, 32, 64, 128 and 250 ng ml^{-1} . In some experiments ($n = 3$), the numbers of cells were counted after detachment from the culture dish, and a linear relationship between the number of cells and the DNA content was observed ($y = (35 + 179.8x)10^3$, $r^2 = 0.999$, where y is the number of cells and x is the DNA content (μg)).

Immunocytochemistry

Homogeneity of the cells was examined by indirect immunofluorescent staining for specific markers of epithelial cells (cytokeratin) and stromal cells (fibronectin). Cells were cultured in 24-well plates, washed with PBS and fixed using 2% formaldehyde in PBS for 15 min at room temperature. The cells were permeabilized by treatment with 0.5% Triton-X100 overnight, rinsed with PBS and then incubated for 1 h with either anticytokeratin or antifibronectin at a concentration of 10 $\mu\text{g ml}^{-1}$. Negative controls were incubated with bovine serum. The cells were washed with PBS and incubated with the second antibody, goat anti-mouse IgG conjugated to FITC, for 1 h at 37°C. The slides were examined with a Universel Zeiss epifluorescence microscope.

Measurement of total protein

Total protein was measured in 10 μl of sonified cell suspension using the Bradford method (Bio-Rad Laboratories). BSA was used as standard (0.0875 to 1.4 mg ml^{-1}).

Statistical analyses

Each treatment was carried out in triplicate using the cells from one uterus and each experiment was repeated with 6–10 different uteri. The effects of treatment on DNA content and on the ratio of total protein to DNA content of uterine cells were evaluated by least-squares analysis of variance. Treatments were analysed in multifactorial design (ANOVA) which included the main effects of experiments, cell type, duration of culture and hormone treatments, and the appropriate interactions. Simple linear contrasts were used to determine differences between individual means. All differences were considered significant at $P < 0.05$.

Results

Effect of oestradiol and progesterone on cell morphology

Clumps of epithelial cells attached to the culture surface as small colonies within 48 h, and were polygonal or spherical in

shape. When incubation time was extended, the cells became a monolayer and exhibited cuboidal or columnar morphology characteristic of epithelial cells (Fig. 1a). Stromal cells presented a monolayer of flat, spindle- and fibroblast-like cells. The homogeneity of the cell populations was examined by immunocytochemistry. Epithelial cell contamination of stromal cells was about 3% and stromal cell contamination of epithelial cells was less than 1% (Fig. 1).

The effect of oestradiol and progesterone on the morphology of epithelial and stromal cells was evaluated after 4 days of culture. The general pattern of growth was observed using an inverted phase-contrast microscope. Neither oestradiol nor progesterone treatment resulted in a change in the appearance of the epithelial cells. The morphology of the stromal cells was not altered by oestradiol at the different concentrations used. However, progesterone (50 nmol l^{-1}) and the combination of progesterone (50 nmol l^{-1}) plus oestradiol (1 nmol l^{-1}) significantly changed the morphology of stromal cells (Fig. 2). Instead of the homogeneous distribution of cells seen in control or oestradiol-treated groups (Fig. 2a, b), the progesterone-treated cells migrated together to form many star-shaped colonies (Fig. 2c). The appearance of these colonies was more dense (Fig. 2d) in the oestradiol plus progesterone treatment group than in the progesterone alone treatment group.

Effect of oestradiol and progesterone on the DNA and protein content of stromal cells

Both oestradiol and progesterone significantly stimulated proliferation of stromal cells after incubation for 4 days ($P < 0.001$) (Fig. 3a). The effect of oestradiol was dose dependent with the maximum dose being between 1 and 10 nmol l^{-1} . Progesterone stimulated DNA content to a greater degree than oestradiol ($P < 0.001$) and the effect of progesterone was not modified by oestradiol, indicating no interaction between these two hormones.

The total protein content of stromal cells measured after 4 days of incubation followed the same pattern as the DNA content (Fig. 3b). The stimulating effect of oestradiol was dose dependent and there were no differences among the treatments of 10 $\text{nmol oestradiol l}^{-1}$, 50 $\text{nmol progesterone l}^{-1}$ and 1 $\text{nmol oestradiol l}^{-1}$ plus 50 $\text{nmol progesterone l}^{-1}$. Oestradiol and progesterone significantly decreased the ratio of total protein to DNA content ($P < 0.05$, Fig. 3c). The ratio declined 19%, 29%, 25%, 36% and 39% for cells treated with 0.1, 1, 10 $\text{nmol oestradiol l}^{-1}$, 50 $\text{nmol progesterone l}^{-1}$, and 1 $\text{nmol oestradiol l}^{-1}$ plus 50 $\text{nmol progesterone l}^{-1}$, respectively.

The effects of hormone treatment and duration of culture on cell growth were highly significant ($P < 0.0001$), and there was an interaction between treatments and duration ($P < 0.01$) for DNA content, protein content, and protein to DNA ratio (Fig. 4). In medium alone, the DNA content of stromal cells increased 34.4% within the following 4 days ($P < 0.01$) (Fig. 4a). Although the absolute increases in DNA content (value at day 8 minus value at day 4) during the second 4 days of culture (5.4, 7.6, 6.2 $\mu\text{g per well}$, for 1 $\text{nmol oestradiol l}^{-1}$, 50 $\text{nmol progesterone l}^{-1}$, and 1 $\text{nmol oestradiol l}^{-1}$ plus 50 $\text{nmol progesterone l}^{-1}$, respectively) were higher than that

Fig. 1. Immunofluorescent staining of purified bovine epithelial and stromal cells. Epithelial cells were stained with anticytokeratin (a), but not with antifibronectin (b) or non-immune serum (c). Stromal cells were stained with antifibronectin (d), but not with anticytokeratin (e) or non-immune serum (f). Scale bar represents 25 μm .

of the control (3.3 μg per well), the relative increase in DNA (the increase in DNA between days 4 and 8 expressed as a percentage of the content at day 4) of the hormone-treated cells decreased when compared with the control (34.4%, 28.9%, 30.9% and 24.2%, for control, 1 nmol oestradiol l^{-1} , 50 nmol progesterone l^{-1} , and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} , respectively). This result indicated that DNA synthesis had slowed during the second 4 days of culture and that oestrogen and progesterone were less effective in stimulating DNA synthesis during this time. In contrast to the change in DNA content, oestradiol and progesterone increased both the absolute (44, 81, 88, 104 μg per well for control, 1 nmol oestradiol l^{-1} , 50 nmol progesterone l^{-1} , and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} , respectively) and the relative protein content in stromal cells (58%, 76%, 70%, and 82% for control, 1 nmol oestradiol l^{-1} , 50 nmol

progesterone l^{-1} , and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} , respectively). At day 8, the ratios of total protein to DNA content increased significantly compared with those at day 4 (17.5, 36.8, 31.4, and 46.9 for control, 1 nmol oestradiol l^{-1} , 50 nmol progesterone l^{-1} , and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} , respectively, Fig. 4c). Thus, the effect of hormone treatment on the protein content of the cells was enhanced with the extended incubation time.

Effect of oestradiol and progesterone on the DNA and protein content of epithelial cells

Treatment with oestradiol alone inhibited the proliferation of endometrial epithelial cells in a dose-dependent manner

Fig. 2. Influence of oestradiol and progesterone on the morphology of bovine endometrial stromal cells. Stromal cells were cultured in RPMI medium supplemented with 5% steroid-free fetal calf serum (a) alone, or in presence of (b) 10 nmol oestradiol l^{-1} , (c) 50 nmol progesterone l^{-1} , or (d) 10 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} for 4 days. The photographs were taken using an inverted phase contrast microscope. Scale bar represents 50 μm .

(Fig. 5a). When 0.1 nmol oestradiol l^{-1} was used, DNA content decreased by 11.8%, but was not statistically different from the control. However, when increased concentrations of oestradiol (1 and 10 nmol l^{-1}) were used, the inhibitory effect of oestradiol on epithelial proliferation was significant (27.9% and 34.5%, respectively, $P < 0.001$). In contrast, progesterone had no significant effect on the proliferation of epithelial cells, but did block the inhibitory effect of oestradiol on epithelial proliferation.

The changes in total protein content of endometrial epithelial cells measured at day 4 (Fig. 5b) were similar to those observed for the DNA content. The total protein contents for the various concentrations of oestradiol (0.1, 1, 10 nmol l^{-1}) were 15.9%, 28.8%, and 36% lower than that of the control ($P < 0.05$). Progesterone alone or progesterone plus oestradiol had no significant effect on total protein content.

The results of the ANOVA for DNA content, protein content and the ratio of protein to DNA showed that the effects of hormone treatments, duration of culture, and the interaction between treatments and duration were significant ($P < 0.01$) (Fig. 6). The DNA content did not change between days 4 and 8 in the epithelial cells treated with oestradiol (1 nmol l^{-1}) or progesterone (50 nmol l^{-1}) plus oestradiol (1 nmol l^{-1}) ($P > 0.05$) (Fig. 6a). Thus, there was no obvious cell proliferation in the presence of 1 nmol oestradiol l^{-1} during this time. However, the DNA content increased 46.7% in the control and 45.3% in the presence of progesterone alone (50 nmol l^{-1}) ($P < 0.01$). Also, after more prolonged culture

(8 days), progesterone was not able to prevent the inhibitory effect of oestradiol on proliferation of epithelial cells. Therefore, the effect of oestradiol and progesterone on epithelial proliferation was time dependent.

After 8 days of incubation, total protein content in epithelial cells was increased by progesterone (50 nmol l^{-1}) and progesterone plus oestradiol (19.9%, 17.3%, respectively, over control, $P < 0.01$) and the inhibitory effect of oestradiol (1 nmol l^{-1}) was not observed (Fig. 6b). After 4 days of incubation there was no effect of treatment on the protein: DNA ratio (Fig. 6c). However, after 8 days of incubation, this ratio was increased significantly by the presence of oestradiol (1 nmol l^{-1}), either alone, or in combination with progesterone (Fig. 6c). This result indicates that even though oestradiol inhibited DNA synthesis in endometrial epithelial cells, it did not inhibit protein synthesis, and even stimulated protein synthesis with time.

Discussion

In the present report, a method is described for studying the effects of progesterone and oestradiol on the proliferation and morphology of bovine uterine endometrial epithelial and stromal cells using an *in vitro* primary cell culture system. The purification procedures used resulted in a homogeneous stromal cell population with very little epithelial cell

Fig. 3. Effects of oestradiol and progesterone on (a) DNA content, (b) protein content and (c) the ratio of protein to DNA in bovine endometrial stromal cells. Primary bovine endometrial stromal cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of various doses of oestradiol (E0.1: 0.1 nmol l^{-1} ; E1: 1 nmol l^{-1} ; E10: 10 nmol l^{-1}) or progesterone (P: 50 nmol l^{-1}) or oestradiol plus progesterone (P + E1: $1 \text{ nmol oestradiol l}^{-1}$ plus $50 \text{ nmol progesterone l}^{-1}$) for 4 days. Data represent least-square means $\pm$ SEM. Bars with different letters are significantly different ($P < 0.01$).

contamination. Stromal cell contamination of the epithelial cells was approximately 3%. The effects of oestradiol on bovine endometrial cell proliferation have been shown for the first time using this system.

Oestradiol did not significantly change the morphology of either cell type. However, progesterone, either alone or in

Fig. 4. Time-dependent effect of oestradiol and progesterone on (a) DNA content (b) protein content and (c) ratio of protein to DNA in bovine endometrial stromal cells. Primary bovine endometrial stromal cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of either oestradiol (E1: 1 nmol l^{-1}), or progesterone (P: 50 nmol l^{-1}), or oestradiol plus progesterone (P + E1: $1 \text{ nmol oestradiol l}^{-1}$ plus $50 \text{ nmol progesterone l}^{-1}$) for 4 days ($\square$) or 8 days (hatched). Data represent least-square means $\pm$ SEM.

combination with oestradiol, altered the morphology of stromal, but not epithelial cells. Significant changes in the morphology of cultured uterine stromal cells, induced by the addition of ovarian steroids or progesterone, have been observed in humans (Gurpide and Holinka, 1987; Holinka, 1988), and it has been suggested that progestins may affect cytoskeletal proteins (Gurpide and Holinka, 1987).

Nuclear incorporation of [^3H]thymidine is a conventional and widely used measurement of DNA synthesis and cell proliferation. However, some discrepancies between

Fig. 5. Effects of oestradiol and progesterone on (a) DNA content and (b) protein content in bovine endometrial epithelial cells. Primary bovine endometrial epithelial cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence or presence of various doses of oestradiol (E0.1: 0.1 nmol l⁻¹; E1: 1 nmol l⁻¹; E10: 10 nmol l⁻¹) or progesterone (P: 50 nmol l⁻¹) or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l⁻¹ plus 50 nmol progesterone l⁻¹) for 4 days. Data represent least-square means $\pm$ SEM. Bars with different letters are significantly different ($P < 0.01$).

[³H]thymidine incorporation and true cell proliferation have been reported (Friedman *et al.*, 1981; Jozan *et al.*, 1983, 1985). Jozan *et al.* (1983) noted increased incorporation of [³H]thymidine after oestradiol treatment, but no change in the number of cells, no increase of the size of nuclei, and no increase in DNA content. This discrepancy was also shown in primary cultures of rat hepatocytes that incorporated [³H]thymidine but did not divide (Friedman *et al.*, 1981). The explanation for this discrepancy is that the addition of [³H]thymidine during a short period of time could miss the S phase of DNA synthesis. If the incubation time was extended, there would be more time for the cell to degrade labelled thymidine and to spread the label in its general metabolism (Murat *et al.*, 1990). The direct measurement of DNA content

Fig. 6. Time-dependent effect of oestradiol and progesterone on (a) DNA content (b) protein content and (c) ratio of protein to DNA in bovine endometrial epithelial cells. Primary bovine endometrial epithelial cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of either oestradiol (E1: 1 nmol l⁻¹), or progesterone (P: 50 nmol l⁻¹), or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l⁻¹ plus 50 nmol progesterone l⁻¹) for 4 days (□) or 8 days (▨). Data represent least-square means $\pm$ SEM.

used in the present study is highly correlated with cell number ($r^2 = 0.999$), which is consistent with a previous report (Inaba *et al.*, 1988), and therefore, should be a better indication of cell proliferation. However, even with the use of DNA and protein measurement, the possibility that treatment of the cells affects cell viability cannot be eliminated.

In vivo, oestradiol causes hypertrophy and hyperplasia of the endometrium and progesterone is thought to act on an oestradiol-primed uterus to stimulate growth of the endometrium. Progesterone is known to modify the action of oestrogen on the uterus by redirecting the proliferative response from the epithelium to the stroma. Martin and Finn (1969) reported that oestradiol stimulated the proliferation of the luminal epithelial cells, but not the stromal cells in mice uteri. However, after pretreatment with progesterone, oestradiol stimulated mitosis in the stroma, but not in the epithelium.

Recent results in sheep have shown that highest rates of cell proliferation occur at the beginning of the oestrous cycle, with proliferation being greater in the luminal epithelium and glands than in the stroma (Johnson *et al.*, 1997). In ovariectomized sheep, oestradiol stimulates proliferation in the epithelium and stroma, whereas progesterone predominantly stimulates the proliferation of epithelial cells (Johnson *et al.*, 1994).

In the present study, the proliferation of bovine endometrial stromal cells was stimulated by oestradiol and progesterone, either alone or in combination. Similar effects have been reported for oestradiol and progesterone on the proliferation of human endometrial stromal cells (Irwin *et al.*, 1991; Chegini *et al.*, 1992). The observed stimulation by oestradiol is also consistent with the effect of oestradiol on ovariectomized sheep (Johnson *et al.*, 1997). However, progesterone had little effect in the ovariectomized sheep in that study, whereas, in the present study, it stimulated stromal cell proliferation *in vitro*. Therefore, it is possible that the response to progesterone *in vivo* is modified by the epithelial cells. The present results differ from previously published data on the effects of steroids on bovine endometrial cells. Tiemann *et al.* (1994) found that oestradiol and progesterone were ineffective in stimulating [³H]thymidine incorporation by epithelial and stromal cells in a serum-free medium. Asselin and Fortier (1996) recently reported that oestradiol had no effect on cell proliferation, while progesterone inhibited [³H]thymidine incorporation in cultured bovine epithelial and stromal cells. There are three possible explanations for these differences: (1) as explained above, thymidine incorporation may give different results to DNA or protein measurement. (2) In the study of Tiemann *et al.* (1994), cells were isolated from the uterus at the mid-luteal phase, whereas in the present study, cells were isolated early in the cycle. Thus, the stage of the cycle at which the samples were taken could affect the response *in vitro*. There is evidence to support this suggestion. In sheep, proliferation of both the epithelial and stromal cells is greatest at the beginning of the cycle (Johnson *et al.*, 1997). Oestradiol stimulates the proliferation of stromal cells in immature rodents (Eide, 1975; Kirkland *et al.*, 1979; Pollard, 1990), but not in adult rats and mice (Quarmby and Korach, 1984; Pollard, 1990). Fleming and Gursipide (1982) also demonstrated that human endometrial stromal cells, obtained at different phases of the oestrous cycle, grow differently in culture. There is clear evidence of cyclical changes in the expression of steroid receptors in endometrial tissue (Bergqvist, 1991; Prentice *et al.*, 1992). Thus, the time that tissue samples are taken might result in differences in the expression of steroid and epidermal growth factor receptors (Mellor and Thomas, 1994) and alter the response *in vitro*. (3) Serum or growth factors may be necessary for oestradiol to exert its effect. The ineffectiveness of oestradiol on the proliferation of stromal cells (Fleming and Gursipide, 1982; Mellor and Thomas, 1994; Tiemann *et al.*, 1994; Asselin and Fortier, 1996) could be due to a lack of unknown serum factors or growth factors required for cultured cells to respond to oestrogen stimulation (Kano-Sueoka *et al.*, 1979; Page *et al.*, 1983; Irwin *et al.*, 1991; Tiemann *et al.*, 1994). The medium used in this study was supplemented with 5% FCS to provide the required serum factors or growth factors. The choice of 5% FCS was based on the consideration that higher concen-

trations of serum may mask any effects of treatment since cell proliferation increases as serum concentration in the medium increases.

Oestradiol and progesterone increased the total protein content of stromal cells. However, the ratios of protein to DNA were significantly decreased by treatment with oestradiol and progesterone for 4 days. It is possible that the cells became multi-nucleated (this was not examined). These results do agree with a previous study in rats (McCormack, 1980), which demonstrated that the ratios of protein to DNA in stromal cells remained unchanged for 12 h after an injection of oestradiol, and then decreased significantly by 24 h. In addition, in sheep, the ratio of protein to DNA decreases at mid-cycle suggesting an *in vivo* effect of steroid hormones (Johnson *et al.*, 1997).

Oestradiol alone inhibited proliferation in epithelial cells in a dose-dependent manner, and the addition of progesterone prevented this inhibitory effect of oestradiol. In mouse epithelial cells (Huet-Hudson *et al.*, 1989), *c-myc* induction and enhanced proliferation are oestradiol-dependent and in sheep, oestradiol stimulates the proliferation of the luminal epithelium (Johnson *et al.*, 1994). However, Marshburn *et al.* (1994) showed that DNA synthesis in cultured human endometrial epithelial glands was decreased by 40% after treatment with oestradiol (1×10^{-8} mol l⁻¹) for 5 days. A similar inhibitory effect of oestradiol on DNA synthesis was also observed by Alkhalaf *et al.* (1991) in the endometrial epithelial cells of guinea-pigs. The inhibitory effect of oestradiol on epithelial cell proliferation may be due to the lack of stromal-epithelial interaction. Stroma can mediate the hormonal responsiveness of epithelial cells in humans (Brenner *et al.*, 1990) and mice (Inaba *et al.*, 1988; Haslam and Counterman, 1991) and a paracrine interaction between stromal and epithelial cells may be required before an increase in proliferation in response to oestradiol will occur (Cunha *et al.*, 1983).

The ratios of protein to DNA of epithelial cells remained unchanged for 4 days after treatment with oestradiol and progesterone. However, oestradiol significantly increased the ratios in epithelial cells after 8 days of treatment to much higher values than in stromal cells. Smith *et al.* (1970) demonstrated that oestradiol enhances protein synthesis in the epithelial cells of adult mice, and that protein synthesis was higher in epithelial cells than in stromal cells. However, McCormack and Glasser (1980) showed that oestradiol administration did not significantly change the ratios of protein to DNA in rat epithelial cells.

In conclusion, progesterone altered the morphology of stromal cells, but not of epithelial cells. The present study demonstrates for the first time that both oestradiol and progesterone can enhance the proliferation of bovine stromal cells. Oestradiol inhibited the growth of epithelial cells in a dose-dependent manner, while progesterone appeared to have no effect on the proliferation of epithelial cells. Oestradiol and progesterone decreased the ratios of protein to DNA in stromal cells, whereas oestradiol enhanced the ratios in epithelial cells. Stromal cells were more responsive to steroids than epithelial cells. It is possible that an interaction between stromal and epithelial cells is necessary for the normal response of epithelial cells *in vivo*.

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