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The production of tumour necrosis factor α (TNF-α) by human endometrial cells in culture

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The production of tumour necrosis factor α (TNF-α) by cultured human endometrial epithelial and stromal cells prepared from endometrium obtained at different stages in the menstrual cycle has been investigated. TNF-α was not detectable in the supernatants of stromal cell cultures prepared from endometrial tissue obtained at any time in the menstrual cycle. TNF-α production by endometrial epithelial cells in culture varied depending on the time in the cycle at which the endometrial tissue was taken. Cells prepared from tissue obtained during the late proliferative phase of the cycle produced more TNF-α than those prepared from tissue obtained at other times in the cycle. In addition, a small increase in TNF-α production was seen by cells prepared from tissue obtained during the mid-secretory phase of the cycle. Interleukin 1 (IL-1) (1.4–140 pmol/l) caused a dose-dependent increase in TNF-α production by cells prepared from both proliferative and secretory endometrium. Maximum IL-1-stimulated increases in TNF-α production were similar in cells from both proliferative and secretory endometrium and typically reached from four to 10 times basal values. High doses of progesterone, either alone or in the presence of oestradiol, also affected TNF-α production by epithelial cells. TNF-α production by cells prepared from proliferative endometrium was increased by progesterone. In contrast, TNF-α production by cells prepared from secretory endometrium was decreased in the presence of progesterone. The effects of steroids on TNF-α production were less marked than that of IL-1, with values increasing or decreasing to a maximum of three times the basal value. Placental protein 14 (PP14) (0.18 and 1.8 nmol/l) also increased TNF-α production by cells prepared from proliferative tissue, but had no effect on its production by cells prepared from secretory endometrium. PP14-stimulated TNF-α levels typically only reached a maximum of two times basal values.

Key words: human endometrium/interleukin 1/placental protein 14/steroids/tumour necrosis factor α

Introduction

Various studies have shown the importance of cytokines in endometrial function and in particular in embryo implantation. The mRNA and protein of numerous cytokines have been found in the human endometrium and are known to originate from both the epithelial and stromal cells, and also from populations of leukocytes (Saito *et al.*, 1993; Tabibzadeh, 1994). The exact role of these cytokines in the endometrium is unknown but it has been suggested that they may be involved in the local modulation of the maternal immune system during implantation. In particular, evidence from rodent pregnancies has suggested that T_H1 cytokines, such as interferon-γ and interleukin 2, which control cell-mediated immune responses are deleterious for successful implantation, while T_H2 cytokines, such as interleukin 1 (IL-1), interleukin 4 (IL-4) and interleukin 6 (IL-6), which control the humoral immune response are beneficial for successful implantation (Wegmann *et al.*, 1993).

Tumour necrosis factor α (TNF-α) is a cytokine with similar functions to IL-1 and IL-6 within the systemic immune system (Dinarello, 1989). It was originally described as a factor produced by macrophages in response to agents such as bacterial lipopolysaccharide and its major role in the systemic immune system is thought to be in the control of inflammatory responses and in protection from bacterial infection (Old, 1985). Like other cytokines, its presence in many other cell types has now been reported (Ruco *et al.*, 1989) and immunocytochemical and in-situ hybridization analyses have shown the presence of TNF-α within both the stromal/epithelial cells and the leukocytes of the human endometrium (Tabibzadeh, 1991a,b; Hunt *et al.*, 1992). These studies have suggested that most TNF-α is present within the epithelial glandular cells and the cells of the artery walls (Garcia *et al.*, 1994; Philippeaux and Piguet, 1993).

The factors which control TNF-α production within the endometrium are not clearly understood. Some studies have suggested that TNF-α mRNA and protein production by the endometrium is increased during the secretory phase of the menstrual cycle (Philippeaux and Piguet, 1993; Tabibzadeh *et al.*, 1995a,b), while others have also shown an additional increase during the late proliferative phase of the cycle (Hunt *et al.*, 1992). The design of these in-vivo experiments meant that a direct effect of factors on the production of TNF-α by either endometrial stromal or epithelial cells could not be studied. This study uses an established cell culture method (Laird *et al.*, 1993, 1994) to investigate the effects of known modulators of endometrial function, steroids, IL-1 and placental protein 14 (PP14), on TNF-α production.

Materials and methods

All reagents except the IL-1 and PP14 were obtained from Sigma Chemical Co. Ltd, Poole, UK. IL-1 was obtained from British

Biotechnology Ltd, Abingdon, Oxon, UK. PP14 was obtained from Intermune Life Sciences, Toronto, Canada. All cell culture plastics were obtained from Fisons, (Loughborough, UK) except for 96-well plates which were obtained from Costar (High Wycombe, UK).

Human subjects

The endometrial tissues were obtained from subjects undergoing laparoscopy or hysterectomy at the Jessop Hospital for Women, Sheffield, UK. Informed consent was obtained from all women participating in the study. All 27 subjects were aged 20–40 years; four were being investigated for pelvic pain and nine for primary infertility; 10 were undergoing laparoscopic sterilization and four hysterectomy. All had regular menstrual cycles of 25–35 days and endocrinological investigation showed evidence of ovulation. The specimens were divided into two groups depending on the time of the cycle that the biopsy was obtained. There were 12 proliferative samples (days 3–14) and 15 secretory samples (days 15–35). The day of the cycle was calculated from the date of the last menstrual period.

Cell culture

Human endometrial cells were prepared and cultured as described previously (Laird *et al.*, 1993). The endometrial biopsy samples were collected in Hanks' balanced salt solution containing streptomycin and penicillin (50 IU/ml). The tissue was chopped finely with scissors and incubated at 37°C for 45 min in 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.2% collagenase (type 1a) (DMEMC). During the incubation and again at the end of the incubation the tissue was pipetted gently to disperse the cells. The epithelial cells were separated from the stromal cells by centrifugation at 100 *g*. The supernatant containing the stromal cells was removed and the pellet, which contained mainly epithelial cells present as glands, was incubated at 37°C for a further 45 min in 5 ml of DMEMC. The cells were again dispersed by gentle pipetting and the epithelial cells pelleted by centrifugation at 100 *g*. The stromal cells present in the supernatant were pooled with those from the first supernatant and pelleted at 300 *g*.

The epithelial and stromal cells were further purified by unit density sedimentation. Each cell type was resuspended in 2 ml DMEM containing fetal calf serum (FCS) (10%), glutamine (4 mmol/l) and penicillin and streptomycin (50 IU/ml) (CDMEM), and gently pipetted onto 8 ml of CDMEM in a test tube and left for 30 min at room temperature. For stromal cell cultures the cells in the top 8 ml were used. For the epithelial cells the top 8 ml was discarded and the cells in the lower 2 ml were used. Each cell type was plated into 96-well plates at ~0.8×10^5 cells per well. The cells were grown to confluency (usually 48–72 h) at 37°C in an atmosphere of 5% CO_2 and 95% air. At confluency the medium was changed and replaced with medium containing either no supplements or IL-1 (1.4–140 pmol/l), PP14 (0.18–9 nmol/l), oestradiol (1 μmol/l), progesterone (1 μmol/l) or oestradiol and progesterone together (each at 1 μmol/l). Additions were made to between 10 and 15 replicate wells for each concentration of each factor added. The cells were grown for a further 48 h, after which the supernatants were removed and stored at –20°C for analysis of TNF-α. Supernatants from two or three wells with the same additions were pooled to give a sample of 500 or 750 μl for analysis. This gave five replicate samples for each treatment for cells prepared from each biopsy sample.

Immunocytochemistry

Immunocytochemical analysis of cytokeratin (epithelial cell marker), vimentin (stromal cell marker) and CD 45 (leukocyte marker) was performed on the cells after 5 days in culture.

The cells were fixed in acetone/methanol (50:50) for 90 s. After this and all subsequent incubations the cells were washed in Tris-buffered saline (pH 7.6) (TBS) for 5 min. The cells were then incubated with anti-cytokeratin (Dakopatts) (30 μl of a 1:50 dilution), anti-vimentin (Dakopatts, High Wycombe, UK) (30 μl of a 1:100 dilution) or anti-CD 45 (Becton Dickinson, San Jose, CA, USA) (30 μl of a 1:100 dilution) in TBS for 2 h at room temperature. After washing, rabbit anti-mouse IgG (Dakopatts) (30 μl of a 1:20 dilution in TBS) was added to the cells which were incubated for 30 min. After a further wash, the cells were incubated with alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (Dakopatts) (30 μl of a 1:20 dilution in TBS) for 30 min at room temperature followed by a further wash. The incubations with rabbit anti-mouse IgG and APAAP were repeated to amplify the reaction. Finally, the cells were incubated with the enzyme substrate Fast Red TR (1 mg/ml) for 15 min. The cells were then washed in running water and counter-stained in haematoxylin.

TNF-α assay

The TNF-α assay was carried out in the Institute of Cancer Studies at the University of Sheffield Medical School, UK, using the method of MacIntyre *et al.* (1992). Flexible 96-well assay plates were coated with a TNF-α polyclonal antibody (Genzyme, Cambridge, MA, USA) (1:1000 dilution in carbonate buffer, pH 9.6) for 2 h at 37°C and then blocked overnight at 4°C with 2.5% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS). Plates were washed with TBS containing 0.1% Tween 20. Standards (0.014–44 pmol/l) were diluted in CDMEM, and 50 μl aliquots of diluted standards or samples in duplicate were added to each well, followed by a 1 h incubation at 37°C. Plates were washed as before and then incubated for 1 h at 37°C with TNF-α monoclonal antibody (Boehringer Mannheim, Lewes, East Sussex, UK) [1:1000 dilution in TBS containing 1% heat-inactivated fetal calf serum (TBSF)]. After a further wash, the plates were incubated at 37°C for 1 h with biotinylated sheep anti-mouse immunoglobulin (Amersham) (50 μl of a 1:1000 dilution in TBSF). After washing streptavin-conjugated alkaline phosphatase (Amersham, Bucks, UK) (50 μl of a 1:1000 dilution in TBSF) was added to the wells and the plates incubated for a further 30 min at 37°C. After a final wash, 50 μl of alkaline buffer (1.5 mol/l 2-amino-2-methyl-1-propanol, pH 10.3) was added to each well followed by 50 μl of phosphatase substrate. After colour development the optical density was determined at 414 nm.

Results

Immunocytochemical staining of human endometrial epithelial cells after 5 days in culture was carried out with anti-cytokeratin (epithelial cell marker), anti-vimentin (stromal cell marker) and anti-CD 45 (leukocyte marker). The positive staining for cytokeratin and the negative staining for CD 45 and vimentin show that the cultures consisted essentially of epithelial cells free from contamination with stromal cells and leukocytes. Stromal cells showed similar positive staining for vimentin and negative staining for CD 45 and cytokeratin, showing that these cultures were also essentially free of leukocytes and epithelial cells.

Figure 1 shows the production of TNF-α by human endometrial epithelial cells in culture prepared from endometrial tissue obtained throughout the menstrual cycle after 5 days in culture. TNF-α was not detectable in the culture supernatants from stromal cells prepared from endometrial tissue obtained at any time in the menstrual cycle. The results show that there was

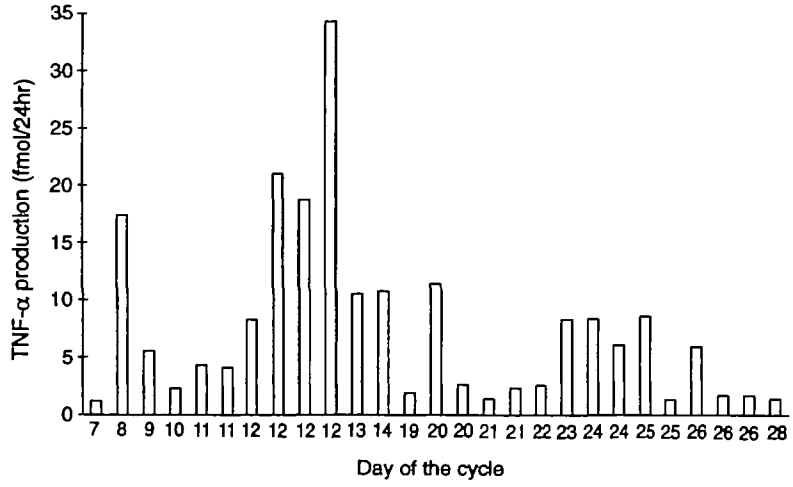

Figure 1. Tumour necrosis factor α (TNF-α) production by cultured human endometrial epithelial cells after 5 days in culture. Cells were prepared from endometrial biopsy samples obtained at different times in the menstrual cycle.

some variation in the amounts of TNF-α produced by epithelial cells prepared at different times in the cycle. Maximum TNF-α production was seen by cells prepared from late proliferative endometrium. Production of TNF-α by cells prepared from secretory endometrium was generally lower than that prepared from proliferative endometrium, but a small increase in production was observed by epithelial cells prepared from endometrium obtained between days 23 and 26 of the cycle.

Figure 2 shows the effects of IL-1 on TNF-α production by endometrial epithelial cells prepared from both proliferative and secretory endometrium. IL-1 caused a dose-dependent increase in TNF-α production by cells from both proliferative and secretory endometrium. Significant increases were seen at all doses of IL-1 (1.4–140 pmol/l) and maximum stimulated values were typically four to 10 times the basal values. No difference in the effect of IL-1 on the response of cells prepared from proliferative and secretory endometrium was seen.

The effect of high doses of the steroids oestradiol and progesterone on TNF-α production by epithelial cells prepared from both proliferative and secretory endometrium is shown in Figure 3. A different response to steroids was seen depending on whether the cells were prepared from proliferative or secretory endometrium. Progesterone, either alone or in the presence of oestradiol, caused an increase in TNF-α production by cells prepared from proliferative endometrium. In contrast, progesterone, either alone or in the presence of oestradiol, caused a decrease in the production of TNF-α by cells prepared from secretory endometrium. Oestradiol alone had no significant effect on TNF-α production by cells from either proliferative or secretory endometrium. The changes in TNF-α production seen in the presence of steroids were less dramatic than those seen in the presence of IL-1, with maximal increases or decreases being three times basal values.

Figure 4 shows the effect of placental protein 14 (PP14) on TNF-α production by epithelial cells prepared from proliferative and secretory endometrial tissue. PP14 caused an increase in TNF-α production by cells prepared from proliferative tissue but had no significant effect on TNF-α production by cells prepared from secretory tissue. Significant increases in TNF-α production by cells from proliferative endometrium

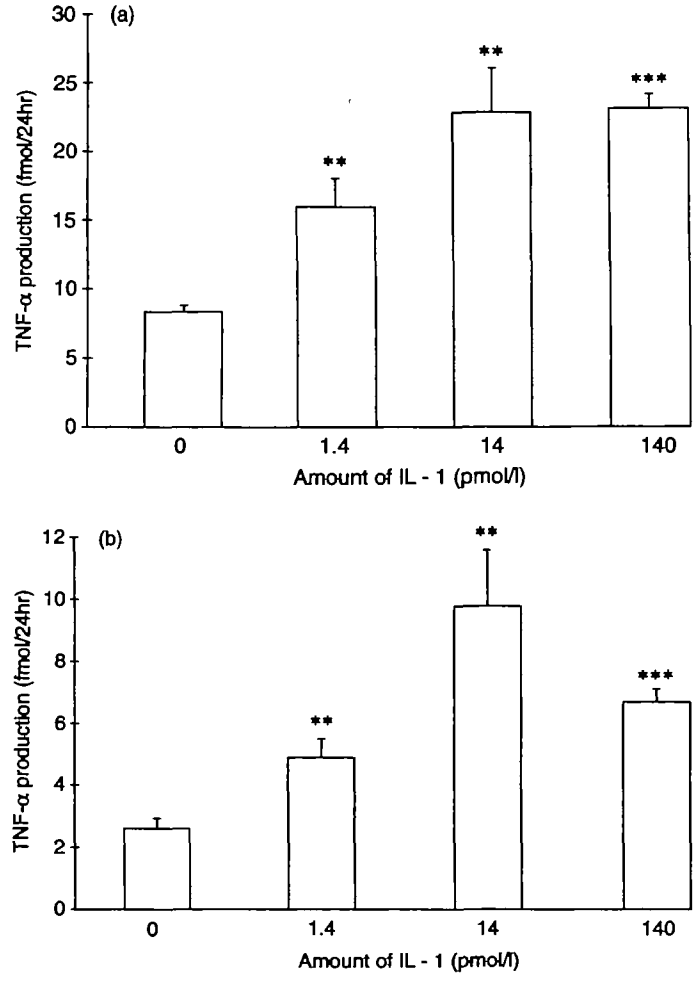

Figure 2. The effect of interleukin 1 (IL-1) on the production of tumour necrosis factor α (TNF-α) by epithelial cells prepared from endometrium obtained during the proliferative (**a**) and secretory (**b**) phases of the cycle. Values are mean ± SEM (n = 5) Significant differences from controls shown by: $^{**}P < 0.01$, $^{***}P < 0.001$. The results shown were obtained from a single biopsy and are typical of those obtained in at least three different experiments.

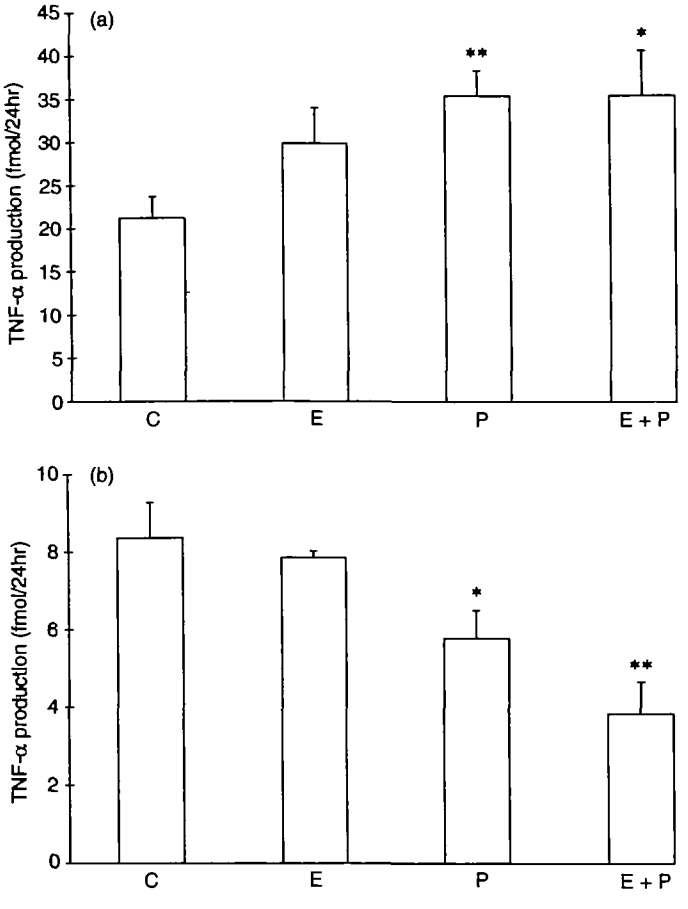

Figure 3. The effect of steroids on tumour necrosis factor α (TNF-α) production by epithelial cells prepared from endometrium during the proliferative (**a**) and secretory (**b**) phases of the cycle. Values are mean + SEM (n = 5). C = control, E = oestradiol (μmol/l), P = progesterone (μmol/l), E + P = oestradiol and progesterone (each at μmol/l). Significant differences from controls are shown by: $*P < 0.05$, $**P < 0.01$. The results shown were obtained from a single biopsy and are typical of those obtained in at least three different experiments.

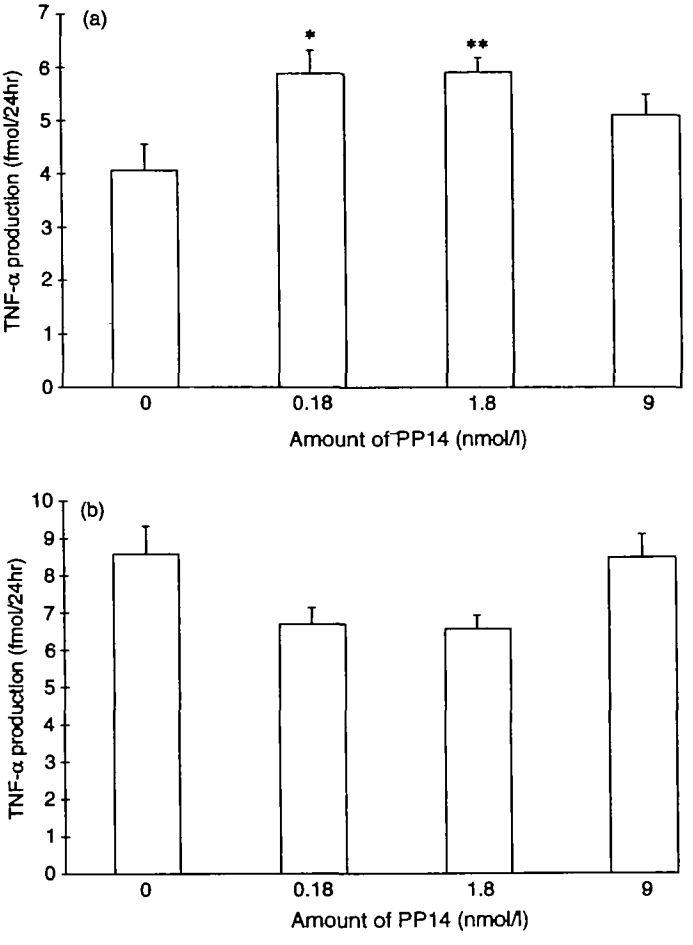

Figure 4. The effect of placental protein 14 (PP14) on tumour necrosis factor α (TNF-α) production by epithelial cells prepared from endometrium obtained during the proliferative (**a**) and secretory (**b**) phases of the cycle. Values are mean ± SEM (n = 5). Significantly different to controls at: $*P < 0.05$, $**P < 0.01$. The results shown were obtained from a single biopsy and are typical of those obtained in at least three different experiments.

were seen at PP14 concentrations of 0.18 ($P < 0.05$) and 1.8 ($P < 0.01$) nmol/l, but not at 9 nmol/l. Stimulatory effects of PP14 were small with maximum stimulated levels typically reaching two times the basal values.

Discussion

Previous studies using immunocytochemistry, Northern blot analysis and explant cultures have shown the production of TNF-α mRNA and protein by the human endometrium (Hunt *et al.*, 1992; Philippeaux and Piguet, 1993; Garcia *et al.*, 1994; Tabibzadeh *et al.*, 1995a). These studies did not allow, because of their design, a study of the factors which control endometrial production of TNF-α. The experiments described in this paper were carried out using an established endometrial cell culture system to investigate directly the effect of known modulators of endometrial function on the endometrial production of TNF-α. Immunocytochemical analysis of the expression of cytokeratin (epithelial cell marker), vimentin (stromal cell marker) and CD 45 (leukocyte marker) have shown that the cultures consist essentially of either pure epithelial or stromal cells and were not contaminated by leukocytes.

In this study TNF-α was only detected in the supernatants from epithelial cells and not in the supernatants from stromal cells prepared from endometrial tissue obtained at any time in the cycle. This agrees with immunocytochemical and in-situ hybridization studies which have described staining for TNF-α mainly within the epithelial cells (Hunt *et al.*, 1992; Philippeaux and Piguet, 1993). The production of TNF-α by epithelial cells in the absence of added factors varied depending on the time in the cycle at which the endometrial biopsy was taken (Figure 1), with maximum amounts produced by cells prepared from tissue taken in the late proliferative phase. The cycle dating for the endometrium was calculated from the start of the last menstrual period, which is less accurate than dating from the time of the luteinizing hormone (LH) surge and could therefore result in inaccurate cycle dating. However, the tissue was obtained from normally cycling women in whom the LH surge would be expected to fall on day 14 of the cycle, and therefore any inaccuracies in dating will be minor. Immunocytochemistry and explant culture studies have suggested that

TNF-α production *in vivo* is maximal during the secretory phase of the cycle (Philippeaux and Piguet, 1993; Tabibzadeh *et al.*, 1995a). However an increase in TNF-α mRNA levels during the proliferative phase has also been described (Hunt *et al.*, 1992).

The experiments described in this paper investigate the effect of IL-1, steroids and PP14 on TNF-α production by endometrial epithelial cells in culture. Previous studies have shown that amounts of endometrial IL-1 mRNA increase during the secretory phase of the cycle (Kauma *et al.*, 1990). The presence of IL-1 receptors on both epithelial and stromal cells has been shown (Tabibzadeh, 1991a) and IL-1 has been shown to increase IL-6 protein and mRNA production by both stromal and epithelial cells (Semer *et al.*, 1991; Laird *et al.*, 1994). Our experiments show that IL-1 also stimulates TNF-α production by endometrial epithelial cells. TNF-α production by endometrial epithelial cells was also modulated by steroids, particularly progesterone. High doses of progesterone and oestradiol were used in this study in order to overcome the binding of steroid to binding proteins present in the fetal calf serum. The effect of these high doses of progesterone, either alone or in the presence of oestradiol, differed depending on the time in the cycle that the tissue was taken. TNF-α production by cells prepared from proliferative tissue was increased by progesterone, while production by cells from secretory tissue was decreased in the presence of progesterone (Figure 3). In these experiments TNF-α production was expressed as fmol/24 h, which does not take into account any effect these factors may have on cell growth. However, in all experiments the effect of factors on cell growth was assessed simultaneously by measuring uptake of ^{3}H-thymidine into the cells. Neither IL-1 nor steroids had any effect on the uptake of ^{3}H-thymidine (data not shown), suggesting that these factors affect TNF-α secretion directly. The effects of both IL-1 and steroids on endometrial epithelial cell TNF-α production are similar to their effect on epithelial cell IL-6 production previously reported (Laird *et al.*, 1993, 1994).

The endometrial protein PP14 was also able to modulate production of TNF-α by human endometrial epithelial cells (Figure 4). Again differences were seen in the response to PP14 by cells prepared from proliferative and secretory endometrium. PP14 increased TNF-α production by cells from proliferative endometrium but had no effect on TNF-α production by cells from secretory endometrium. PP14 is known to decrease the growth of human endometrial cells in culture (Tuckerman *et al.*, 1994) and therefore the effect of PP14 on TNF-α production could potentially be modulated by its effect on cell growth. However, PP14 either increased or had no effect on TNF-α production, suggesting that these responses were independent from the effects on growth. Previous reports have shown that PP14 at the same concentrations increases IL-6 production by human endometrial epithelial cells (Laird *et al.*, 1994) and suppresses lymphocyte proliferation (Pockley *et al.*, 1988).

Maximally stimulated TNF-α production by epithelial cells was different in the presence of IL-1, steroids or PP14. IL-1 caused the greatest stimulation of TNF-α with maximum stimulated values reaching four to 10 times basal levels. Steroids had less of an effect, with changes in TNF-α concentrations being three times those of basal values. PP14 caused only small changes in TNF-α production with maximum stimulated values reaching only two times basal values. These differences in response suggest that the mechanisms by which these factors affect cytokine production differ.

The role of TNF-α in endometrial function is unknown. TNF-α receptors have been found in the endometrium throughout the menstrual cycle (Tabibzadeh *et al.*, 1995a) and TNF-α has been shown to increase IL-6 and parathyroid-related protein gene expression by human endometrial stromal cells in culture (Semer *et al.*, 1991; Casey *et al.*, 1993). It has also been shown to cause cell–cell dissociation of an endometrial epithelial cell line and to increase lymphoid cell entry into the endometrial epithelial cell compartment (Tabibzadeh *et al.*, 1993, 1995b).

In summary, these results show that TNF-α production by epithelial cells in culture varies depending on the time in the menstrual cycle that the endometrial sample was taken, and that IL-1, steroids and PP14 can all affect TNF-α production. Its change in production in response to both steroids and IL-1 is similar to that described for IL-6 (Laird *et al.*, 1993, 1994), another pro-inflammatory cytokine. Although care must be taken in extrapolating data from the in-vitro to the in-vivo situation, the fact that steroids, which are the principal external factors involved in the control of endometrial function, can modulate TNF-α production by endometrial cells suggests that TNF-α may be important in the paracrine control of endometrial function.

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