Immunohistological localization of human endometrial secretory protein, 'pregnancy-associated endometrial α_2 -globulin' (α_2 -PEG), during the menstrual cycle*

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Summary. The distribution of α_2 -PEG, a human analogue of β -lactoglobulin, in endometrium at different phases of the cycle was determined using immunohistochemistry with monoclonal and polyclonal antibodies. In the epithelial cells of glands in the functional zone of the endometrium, α_2 -PEG was first detectable from Days 19 to 21 during the mid-luteal phase and maximal immunostaining was observed during the end of the late luteal phase. Intense staining in the glandular secretions and weaker staining in surface luminal epithelial cells during this period were observed. A minor population of basal glands contained α_2 -PEG during the follicular phase. These results suggest that α_2 -PEG synthesis by the glandular epithelium of the regenerated endometrium is hormonally regulated. Maximal staining occurring during the late luteal phase suggests that regulation may be related to the hormonal requirement for pre-decidualization rather than that required for histologically defined glandular epithelial secretion.

Keywords: endometrium; human; menstrual cycle; pregnancy-associated endometrial α_2 -globulin; pregnancy proteins

Introduction

Pregnancy-associated endometrial α_2 -globulin (α_2 -PEG) is a dimeric glycoprotein of native molecular weight 56 000 and average isoelectric point of pH 4.6 which exhibits microheterogeneity of molecular weight and isoelectric point (Bell, 1986a, 1988). This protein, as its subunit endometrial protein 15, was originally identified as the quantitatively major secretory protein product synthesized *de novo* and secreted *in vitro* by the human endometrium obtained during the early first trimester of pregnancy (Bell *et al.*, 1985a, b). Subsequently, it was also identified as the major *in vitro* secretory protein product of the endometrium during the second half of the luteal phase of the menstrual cycle, although basal values were measurable throughout the cycle (Bell *et al.*, 1986a). α_2 -PEG has been detected in intraluminal uterine flushings during the menstrual cycle (Bell & Doré-Green, 1987) and in amniotic fluid during pregnancy (Bell *et al.*, 1986b), both at higher concentrations than in peripheral serum (Bell *et al.*, 1987a), suggesting that it is principally secreted *in vivo* into the uterine lumen and transported across the amniochorion. It has been suggested that the temporal pattern of synthesis and secretion of this protein reflects the development and regression of the zona and decidua spongiosa regions of the endometrium (Bell, 1988).

α_2 -PEG has been reported to be identical to the placental protein, PP14 (Bell & Bohn, 1986) in spite of the dissimilar properties previously reported for PP14 (Bohn *et al.*, 1982). Subsequent reports concerning the detection of synthesis and secretion of PP14 by the endometrium during the

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menstrual cycle (Julkunen *et al.*, 1986a) and at term (Julkunen, 1986) appear to confirm this relationship. PP14 also has been reported to be serologically identical to progesterone-dependent endometrial protein, PEP (Julkunen *et al.*, 1986b; Joshi, 1983) (see Bell, 1986a). Both α_2 -PEG and PP14 have been reported to exhibit sequence homology with the β -lactoglobulin protein family (Bell *et al.*, 1987b; Huhtala *et al.*, 1987). In the present study we have used monoclonal and polyclonal antibody reagents to determine the cellular origin of α_2 -PEG by immunohistological localization of α_2 -PEG in dated endometrial specimens obtained during the normal menstrual cycle.

Materials and Methods

Specimens. Strips of endometrial tissue were obtained by curettage or from whole uteri of regularly cycling women. All these patients were operated on for non-endocrine conditions. Tissues were immersed in 4% formaldehyde in saline, a fixative that has been shown to give good α_2 -PEG antigen preservation as detected using monoclonal antibodies, and were routinely processed. Serial 5 μ m paraffin-wax sections were used for routine haematoxylin and eosin staining and for immunohistochemistry. The endometrial specimens were dated from the last menstrual period and were only used in the study if there was corroboration by independent histological dating which followed standard histological criteria (Hendrickson & Kempson, 1980). A total of 54 specimens, including 6 hysterectomies, were collected: there were 5, 5 and 10 during the early, mid- and late proliferative stages respectively; 11, 4 and 15 samples during the early, mid- and late secretory stages; and 4 samples during the menstrual stage.

Antibodies. Routinely, a mouse monoclonal antibody (code 2C6H11) was used as a culture supernatant at a dilution of 1:20. On selected specimens another monoclonal antibody (code 1D4G11) was used at a dilution of 1:2 and a rabbit polyclonal antibody at a dilution of 1:50. Antibodies were raised against α_2 -PEG purified from first trimester endometrial cytosol (Bell, 1986b), using standard procedures.

Immunohistological techniques. The mouse monoclonal antibodies were used in an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining method (Cordell *et al.*, 1984). All incubations were carried out at room temperature with rocking in a moist chamber and all dilutions and washes were done in Tris-buffered saline (TBS) pH 7.6 with 10% BSA added. After dewaxing, rehydration and washing of sections, normal rabbit serum (Dako Ltd, High Wycombe, Bucks, U.K.) was applied diluted 1:5 for 30 min to block non-specific binding. The primary monoclonal antibody to α_2 -PEG was applied for 1 h, a rabbit anti-mouse immunoglobulin antiserum (Dako Ltd) at a dilution of 1:50 for 30 min, followed by the mouse alkaline phosphatase-anti-alkaline phosphatase complex (Dako Ltd) at a dilution of 1:100 for 30 min. Alkaline phosphatase was developed for 1 h using naphthol AS BI phosphoric acid and fast red in veronal acetate buffer, pH 9.2 containing 2 mM-levamisole (Sigma, Poole, Dorset, U.K.). Sections were counterstained with Mayer's haematoxylin. Negative controls used were hybridoma growth medium, supernatant from NSO parent myeloma cell culture and monoclonal antibodies absorbed with purified α_2 -PEG. An inappropriate monoclonal antibody of the same immunoglobulin class as the 2C6H11 was also used.

The rabbit polyclonal antibody was used in an indirect immunoperoxidase staining method (Sternberger, 1979) under conditions similar to those described for the monoclonal antisera. Endogenous peroxidase activity was inhibited by addition of 0.3% hydrogen peroxide in methanol for 30 min and non-specific binding of the secondary reagent was blocked by normal goat serum (Miles Lab Ltd, Slough, U.K.) at a 1:5 dilution for 30 min. The primary antibody was applied overnight at 4°C followed by goat anti-rabbit immunoglobulin peroxidase conjugate (Miles) at a dilution of 1:20 for 30 min at room temperature. Peroxidase activity was demonstrated by the diaminobenzidine reaction and counterstaining was with Mayer's haematoxylin. Controls used were anti- α_2 -PEG absorbed with purified protein at a predetermined optimal ratio and non-immune rabbit sera at a dilution of 1:50.

The positive α_2 -PEG immunostaining, assessed using the monoclonal antibody 2C6H11, was graded, by two independent observers, with an arbitrary 4-point scale taking into account the intensity of glandular staining and the proportion of positively stained cells and glands. To control assessment of staining intensity between days, a pregnancy specimen was routinely included which was given the maximum score. A score of 4 indicates intense staining of more than 95% of glands compared to a score of 1 which is indicative of occasional and/or very weak glandular epithelial staining.

Results

Initially all specimens obtained by curettage were stained for α_2 -PEG using the monoclonal antibody 2C6H11 and the following results refer to this study. However, experiments performed on selected specimens with monoclonal 1D4G11 and the polyclonal antisera revealed similar cellular localization and modulation during the cycle.

Early proliferative stage

During the 5th to 7th days of the menstrual cycle the luminal epithelium did not exhibit any immunoreactivity. In these specimens most glands were negative, but 3 samples from the 6th and 7th days contained a small number of glands in which a proportion of the cells had diffuse cytoplasmic staining of variable intensity. No protein was detected in the endometrial stroma.

Mid-proliferative stage

During the 8th to 10th days the luminal epithelium and stroma remained negative for the protein as did the majority of glands (Fig. 1a). Only one or two glands were found with very weak epithelial cytoplasmic staining in some of the cells from 3 specimens.

Late proliferative stage

During the 11th to 14th days no immunoreactive protein was detected in the luminal epithelium or the endometrial stroma. However, in a number of specimens, one obtained on each day, immunoreactive protein was detected in epithelial cells of a small number of glands. The glands also contained negative cells and staining ranged from being restricted to the apical cytoplasm to being uniform throughout the cytoplasm. These glands were never found in endometrial areas adjacent to the luminal epithelium.

Early secretory stage

In all 11 specimens during the 15th to 18th days the luminal epithelium and stroma were devoid of protein (Fig. 1b). In only 2 specimens on the 16th day were positive glands identified, 2 in each specimen. These differed from the majority of negative glands in not exhibiting the subnuclear vacuolation of the epithelial cells a characteristic feature of this stage.

Mid-secretory stage

During this stage (Days 19–23) unequivocal staining for α_2 -PEG was detected in a proportion of luminal epithelial cells. The staining varied from being diffuse through the cytoplasm to a more granular appearance and the intensity was not uniform. Some areas remained negative. More than 50% of the glands in each specimen (one from nearly every day) exhibited immunoreactivity and where such glands opened into the lumen the neighbouring luminal epithelial cells were more positive than in those distal to the gland opening (Fig. 1c). The intensity of staining in the glandular epithelium was greater than in the luminal epithelium cells. Not all glandular epithelial cells showed immunoreactivity and those that did varied from being diffuse cytoplasmic to being localized to the apical cytoplasmic area (Fig. 1c). Positive secretions were observed in the lumen of a number of glands (Fig. 1d). In some glandular and luminal epithelial cells staining seemed to be restricted to the region of the Golgi apparatus (Fig. 1e). Several of these types of staining could be seen within the same specimen. Although positive glands were present in most areas of each specimen some regions were overall more intense than others. The proportion of glands and epithelial cells staining for α_2 -PEG increased throughout the mid-secretory stage. Some small regions within the stroma exhibited weak immunoreactivity.

Late secretory stage

From Days 24 to 28 the staining pattern of the luminal epithelium was similar to that observed in the mid-secretory stage and although the intensity increased, paralleling the changes in the glands, it was always less than that observed in the glandular epithelium of the same specimen.

Between Days 24 and 26 some degree of protein staining was observed in at least 50% of glands and, although the intensity of staining varied widely between glands in the same specimen (Fig. 1f),

Fig. 1. Micrographs of endometrial specimens immunostained with a monoclonal antibody (2C6H11) against α_2 -PEG protein using the APAAP method and counterstained with haematoxylin. (a) Mid-proliferative phase (Day 8). Glands and stroma negative. $\times 70$. (b) Early secretory phase (Day 16). Glandular epithelial cells and stroma negative for α_2 -PEG. $\times 70$. (c) Mid-secretory phase (Day 23). Gland positive for α_2 -PEG secreting protein into the lumen. Luminal epithelial cells are positive near the gland opening. Staining of glandular epithelial cells can be seen either throughout the cytoplasm or in the apical cytoplasmic area. $\times 280$. (d) Mid-secretory phase (Day 21). Gland showing positively stained secretions within its lumen. $\times 280$. (e) Mid-secretory phase (Day 23). Discrete staining, possibly of Golgi, in glandular epithelial cells (arrowed). $\times 700$. (f) Late secretory phase (Day 24). Variability seen in the α_2 -PEG staining between glands. The arrow shows neighbouring glands, one intensely positive (left) and one negative (right). $\times 70$. (g) Late secretory phase (Day 24). Glands showing intense staining throughout the cytoplasm of a proportion of cells with neighbouring cells negative. $\times 280$. (h) Late secretory phase (Day 28). Intense staining in the majority of cells of most glands. $\times 70$. (i) Late secretory phase (Day 28). Higher power of positive cells shown in Fig. 1h. The whole cytoplasm is stained. $\times 280$. (j) Late secretory phase (Day 28). Weak staining in predecidual cells (arrowed). $\times 280$. (k) Menstrual phase (Day 2). Degenerating gland showing dense granular subnuclear bodies (SB) and α_2 -PEG staining structures (α_2) some containing SB. $\times 280$. (l) Late proliferative phase (Day 12). Hysterectomy specimen showing positive glands towards the endometrial-myometrial junction (dotted lines). Lu=lumen. $\times 22$.

overall the staining was greater than in the mid-secretory stage. In weakly staining glands the protein tended to be localized to the apical cytoplasmic area and in the more intensely stained glands it appeared throughout the cytoplasm (Fig. 1g). On Day 28 of the menstrual cycle intense staining in the majority of epithelial cells in most glands was observed (Figs 1h & i), with diffuse cytoplasmic reactivity apart from occasional vacuoles that were negative. Only the occasional large gland was negative at this time.

Intensely staining secretions within glands were observed throughout the late secretory stage, being most prominent in the Day 26 specimens. During this stage α_2 -PEG-positive staining

Fig. 2. Schematic representation of the intensity of anti- α_2 -PEG staining in the glandular epithelium during menstrual cycle. The degree of change in intensity was graded with an arbitrary 4-point scale (see 'Materials and Methods'). For specimens obtained on the same day a mean value is represented.

appeared in many of the predecidual cells located around spiral arteries and beneath the luminal epithelium although this staining was weak relative to glandular epithelial staining (Fig. 1j). The intensity and degree of this stromal staining became more pronounced on Day 27/28.

Menstrual stage

Most menstrual specimens contained endometrium in the process of shedding and necrosis. In these areas the majority of glandular epithelium exhibited weak to moderate cytoplasmic staining. A characteristic feature of the glands exhibiting cytological evidence of degeneration was the presence of densely immunostaining bodies within the subnuclear (basal) cytoplasm (Fig. 1k). In specimens containing regions of basal endometrium a few glands containing positive epithelial cells were noted.

Hysterectomy specimens

In 3 specimens obtained on Days 6, 7 and 12, although most glands were negative, glands containing positive epithelial cells were observed towards the endometrial–myometrial junction (Fig. 1l). In a hysterectomy specimen obtained on Day 24 most glands were positive throughout the endometrial layer although there was a marked variability in the intensity of staining between both glands and individual cells. In a specimen obtained on Day 28 all glands were positive for the protein and weak diffuse staining was also detected in the myometrium at the endometrial–myometrial junction.

Figure 2 summarizes these observations, based on staining with the 2C6H11 monoclonal antibody in a semi-quantitative manner, and emphasizes the modulation of the protein staining within the glandular epithelial population. Negative controls for the polyclonal antiserum (non-immune rabbit serum and antiserum absorbed with purified α_2 -PEG) and for the monoclonal antisera (hybridoma growth medium, supernatant from parent myeloma cell culture and absorbed with α_2 -PEG) exhibited no immunostaining. Use of an inappropriate monoclonal antibody confirmed that the staining seen with 2C6H11 was due to reactivity against α_2 -PEG and not non-specifically related to the immunoglobulin class.

Discussion

This study, using monoclonal and polyclonal reagents, clearly demonstrates that α_2 -PEG is principally associated with glandular epithelium and its secretions and is modulated during the menstrual

cycle. The pattern of modulation of α_2 -PEG staining during the cycle closely reflects the alteration of in-vitro rates of synthesis and secretion of this protein by the endometrium (Bell *et al.*, 1986a). This latter study revealed that α_2 -PEG represented the major secretory protein product of the endometrium during the late luteal phase and therefore it is probable that α_2 -PEG represents the major product of the glandular epithelium during the late luteal phase of the cycle. Two related proteins, PP14 (Julkunen *et al.*, 1986a) and AUP (Horne *et al.*, 1982), have also been localized to this population during the luteal phase and PEP to the endometrial glands during the first trimester of pregnancy (Mazurkiewicz *et al.*, 1981) by techniques using polyclonal antisera. However, it is clear from this study that α_2 -PEG is maximally expressed during the late luteal phase, which is characterized by decidualization of the stroma and falling oestrogen/progesterone concentrations, and not during the period of maximal histologically apparent secretory activity, i.e. mid-luteal phase which parallels the luteal phase profiles of oestrogen and progesterone concentrations. This implies that the biochemical nature of glandular secretions alters during the luteal phase with α_2 -PEG secretion being initiated well after the histologically defined secretory phase has begun. This appears to rule out a direct steroid hormonal control and possibly reflects a dependence either upon extended progesterone exposure or a link with predecidualization of the stroma. The temporal pattern of intensity of α_2 -PEG most strikingly reflects the production of the decidual cell-derived prolactin during the menstrual cycle (Maslar & Riddick, 1979).

The localization of the protein intracellularly, to the apical membrane, and to glandular lumen secretions supports the original identity of this protein as a major secretory protein synthesized by the mid- to late secretory endometrium *in vitro* (Bell *et al.*, 1986a). These secretions presumably gain access to the uterine lumen as indicated by recovery of α_2 -PEG from luminal flushings (Bell & Doré-Green, 1987). The initiation of synthesis of this protein is suggested to be from Days 19 to 21 in this study and thus would be unlikely to contribute to the intraluminal milieu of the pre-implantation embryo, although it would be expected to be present during the implantation process.

α_2 -PEG was detectable in a small proportion of luminal epithelial cells, albeit at low levels, and followed the same pattern of change during the menstrual cycle as observed in the glands. Clusters of stained cells appeared to be associated with the opening of glands on the luminal surface. This pattern was dramatically different from that observed for the oestrogen-regulated cytosolic protein of M_r 24 000 which is maximally expressed by these cells during the mid-secretory stage (Ciocca *et al.*, 1983).

The inconsistent detection of a small number of α_2 -PEG stained glands in proliferative endometrial biopsy specimens could be related to random biopsy of positive glands in the basal layer of the endometrium. This distribution was apparent in the hysterectomy specimens and may suggest that a basal layer of glands constitutively express α_2 -PEG in contrast to the hormonally regulated expression of α_2 -PEG in the glands of the remaining stroma. Since the glands must regenerate from this basal layer, these observations indicate that oestradiol-dependent proliferation produces epithelial cells in which α_2 -PEG is not synthesized, but thereafter is induced by the luteal-phase hormonal milieu. Alternatively glands in the basal layer may respond during the luteal phase, since all glands were positive throughout the endometrium, but after menstruation production in these basal glands falls, reflecting the lack of hormonal stimulation during the follicular phase. The presence of weak staining in decidual cells supports an hormonal dependence of α_2 -PEG expression since decidualization is principally progesterone-dependent and may imply that α_2 -PEG production is a property of any progesterone-responsive cell type. The implication of this study that α_2 -PEG is the major secretory product of the endometrial glandular epithelium is of interest with reference to its sequence homology with a major secretory product of glandular epithelium in the mammary gland of certain species, i.e. β -lactoglobulin, and suggests that these tissues may subserve similar functions.

In conclusion, this distribution of α_2 -PEG emphasizes its potential importance as a unique marker of endometrial function, particularly that of the secretory glandular epithelium, and suggests that examination of its expression in endometrial biopsies from abnormal menstrual cycles

and infertile patients may be of value in determining the potential contribution of glandular epithelial dysfunction to these conditions.

We thank Miss A. Cave for secretarial assistance and Ms B. Jordan for photographic expertise. This study was supported by a grant from the MRC (G8519778 SB).

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Received 13 July 1987