Immunohistological localization of human pregnancy-associated endometrial α_2 -globulin (α_2 -PEG), a glycosylated β -lactoglobulin homologue, in the decidua and placenta during pregnancy*

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Summary. Monoclonal and polyclonal antibodies to pregnancy-associated endometrial α_2 -globulin (α_2 -PEG), a glycosylated human β -lactoglobulin homologue, were used in an immunohistological technique to determine the cellular localization of this protein in the decidua and placental tissues during pregnancy. During the first trimester the protein was principally localized to the glandular epithelium of the decidua spongiosa region of the endometrium with only weak staining associated with glands of the decidualized decidua compacta region. No significant cellular staining was detected in the decidua capsularis. At term in the decidua of the amniochorion and the placental bed weak staining for α_2 -PEG was only associated with the epithelium of attenuated glands. No significant staining was detected in the placenta during pregnancy. These results suggest that the epithelium of glands associated with non-decidualized stroma represents the primary source of α_2 -PEG during the first trimester and that a function of the decidua spongiosa in early pregnancy may be related to production of α_2 -PEG. The decline in production of α_2 -PEG during pregnancy is suggested to result from involution of the decidua spongiosa and at term the attenuated glands of the decidua represents the source of α_2 -PEG.

Keywords: endometrium; decidua; placenta; pregnancy-associated proteins; β -lactoglobulin homologue; man

Introduction

Pregnancy-associated endometrial α_2 -globulin (α_2 -PEG) was identified from de-novo synthesis and secretion *in vitro* as the quantitatively major secretory protein of the human endometrium during the second half of the luteal phase of the menstrual cycle and early first trimester of pregnancy (Bell *et al.*, 1985, 1986a). The protein purified from pregnancy endometrium is a non-covalently linked dimeric glycoprotein of native molecular weight 56 000 whose subunits exhibit microheterogeneity of molecular weight and pI (Bell *et al.*, 1985; Bell, 1986a). It is detected in intraluminal uterine flushing during the menstrual cycle (Bell & Dore-Green, 1987) and amniotic fluid during pregnancy (Bell *et al.*, 1986b) at levels greatly exceeding those detected in peripheral serum during these periods (Bell *et al.*, 1987a; Wood *et al.*, 1989). That the principal route of secretion of this protein *in vivo* is into the uterine lumen is supported by its localization by immunohistological techniques to the glandular epithelium and its secretion during the late luteal phase of the cycle (Waites *et al.*, 1988). These studies have suggested that α_2 -PEG represents a major product of the secretory glandular epithelium during the luteal phase of the menstrual cycle (Bell, 1986b, 1988).

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α_2 -PEG appears to be identical to progesteragen-dependent endometrial protein (PEP: Joshi, 1983), alpha-uterine protein (AUP: Sutcliffe *et al.*, 1980), and placental protein 14 (PP14: Bohn *et al.*, 1982), and very similar N-terminal amino acid sequences for α_2 -PEG (Bell *et al.*, 1987b) and PP14 (Huhtala *et al.*, 1987; Westwood *et al.*, 1988) have been reported which exhibit sequence homologies with the β -lactoglobulin-related secretory protein family (Ali & Clark, 1988).

Synthesis and secretion of α_2 -PEG, PEP and PP14 by the glandular epithelium of the non-gestational endometrium have been suggested to result from the action of progesterone, a view supported by the in-vivo effects of exogenous progesterone (Seppala *et al.*, 1987a) and progestagens (Seppala *et al.*, 1987b; Wood *et al.*, 1988). Paradoxically, although α_2 -PEG represents the major secretory product of the endometrium during the early first trimester, synthesis and secretion *in vitro* and serum concentrations rapidly decline despite continued steroid hormone production. In the present study monoclonal and polyclonal antibodies have been used to determine the cellular localization of α_2 -PEG in the uterus during pregnancy.

Materials and Methods

Specimens. During the first trimester of pregnancy tissues were obtained from women undergoing therapeutic termination of pregnancy (8–13 weeks). Endometrial tissue (identified as decidua parietalis), placental trophoblast, and membranous tissue, the latter identified as decidua capsularis and chorion laevae, were dissected from 35, 14 and 10 specimens respectively. Tissues were washed in physiological saline (0.154 M-NaCl) and fixed in 10% buffered neutral formalin. Several pieces of tissue from each specimen were normally fixed. Four placental bed biopsies were obtained at Caesarean section and 5 specimens of placental and 9 of amniochorion with attached endometrial tissue were obtained at Caesarean section or after spontaneous delivery. All tissues were embedded in paraffin wax and 4–5- μ m sections were cut. Sections from all tissues were routinely stained with haematoxylin and eosin and examined. Similar tissues were also obtained from an anembryonic pregnancy hysterectomy specimen (8 weeks after last menstrual period).

Antibodies. Murine monoclonal antibodies (MAbs) were raised against α_2 -PEG purified from first trimester endometrial cytosol (Bell, 1986a), using standard procedures (Kohler & Milstein, 1975). Routinely an IgG1 antibody designated 2C6H11 was used as a culture supernatant (immunoglobulin (Ig) content 0.42 μ g/ml) at dilutions ranging from 1:4 to 1:200. To confirm observations another MAb (designated 1D4G11) and a rabbit polyclonal antibody to α_2 -PEG were used on replicate sections of selected specimens. The MAb 1D4G11 was used as a culture supernatant (IgG1, Ig content 1.2 μ g/ml) and the polyclonal antibody at a dilution of 1:50 or 1:100. Murine MAb (2B2H10) raised against α_1 -PEG (an insulin-like growth factor binding protein) was used as a culture supernatant (IgG1, Ig content 4.4 μ g/ml) at a dilution of 1:50 as described previously (Waites *et al.*, 1989). The murine MAb to human milk-fat globulin protein-1 (HMFG-1) (Unipath Ltd, Bedford, UK) was used as purified mouse immunoglobulin (Ig content 200 μ g/ml) at a dilution of 1:10. This epithelial-cell membrane marker was used to locate glands within specimens since it had been demonstrated that residual gland structures in term placental bed are not clearly identified by haematoxylin and eosin staining (Bulmer *et al.*, 1986).

Immunohistochemical techniques. The murine MAbs were used in an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining method (Cordell *et al.*, 1984) as described previously (Waites *et al.*, 1988). Briefly, after blocking with 1:5 (v/v) normal rabbit serum (Dako Ltd, High Wycombe, Bucks, UK) the MAb was applied for 1 h and rabbit anti-mouse immunoglobulin antiserum (Dako Ltd) at 1:50 for 30 min, followed by mouse alkaline phosphatase-anti-alkaline phosphatase complex (Dako Ltd) at 1:100 (v/v) for 30 min. The reaction was developed for 1 h using naphthol ASBI phosphoric acid and Fast Red in veronal acetate buffer containing levamisole (Sigma, Poole, Dorset, UK). Sections were counterstained with Mayer's haematoxylin. Selected tissue specimens to be stained with each MAb were treated with 0.1% trypsin (Sigma) and 0.1% calcium chloride in Tris-buffered saline, pH 7.8, for 20 min at 37°C before staining. Negative controls used were hybridoma growth medium, supernatant from NSO parent myeloma cell culture and an inappropriate MAb (against Leukocyte Common Antigen: Dako Ltd) of the same immunoglobulin class and isotype as 2C6H11. In addition mouse IgG (Sigma) was used as a control at a range of dilutions equivalent to the Ig content of the MAbs 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 (ICN Biomedical Ltd, High Wycombe, Bucks, UK) at a 1:5 (v/v) dilution for 30 min. The primary antibody was applied overnight at 4°C followed by goat anti-rabbit immunoglobulin peroxidase conjugate (ICN) 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 serum at a dilution of 1:50 (v/v).

Results

First trimester

Decidua. Thirty-five (35) first-trimester decidua parietalis specimens were stained for α_2 -PEG (using antibody dilutions from 1:30 to 1:200, v/v) and 19 of these also for HMFG-1. All specimens contained areas of decidua compacta whilst decidua spongiosa was present in 69%. Both MAb against α_2 -PEG (2C6H11 and 1D4G11) and the polyclonal antiserum exhibited a similar staining pattern, the most intense immunoreactivity being detected in the decidua spongiosa region in association with the glands (Figs 1a, b; results with polyclonal antibodies and monoclonal antibody 1D4G11 not shown). This is in direct contrast to the localization of staining for α_1 -PEG which was most intense within decidual cells in the compacta region (Fig. 1c). Several histologically distinct gland types were observed, within and between tissue specimens, which gave the impression of different staining patterns. The epithelial cells of many glands exhibited uniform cytoplasmic staining (Fig. 1d) whilst in other glands the cells contained unstained vacuoles within this positive cytoplasm, often, although not solely, at the apical aspect (Fig. 1e). This latter 'hypersecretory' gland-type was also associated with apparent budding of the glandular epithelial cells. In some regions the glands were heavily vacuolated and unstained for α_2 -PEG (Fig. 1f). Two types of secretions were identified, one granular, often intensely positive for α_2 -PEG, and the other of a globular nature which was unstained (Fig. 1g). There was a tendency towards an inverse correlation between staining for α_2 -PEG within the cytoplasm of epithelial cells and of α_2 -PEG-positive secretory material in the lumen.

All glandular epithelial cell membranes in the decidua spongiosa stained intensely for HMFG-1 (Fig. 1h). Granular but not globular secretions were also intensely positive for HMFG-1.

In the decidua compacta region open glands were distributed sparsely throughout (Fig. 1i) and although the epithelial cell layer lost its columnar appearance and secretions were rarely seen in the lumen, the glands were identifiable histologically and this was confirmed by HMFG-1 staining which was localized to the epithelial cell membrane of all glands. The intensity of staining for α_2 -PEG in the compacta was lower than in the spongiosa of the same specimen. Where staining in the spongiosa was intense the majority of glands within the associated compacta were also positive for α_2 -PEG, although with an overall low cytoplasmic intensity and was not detectable in all cells of glands (Fig. 1j). Where staining in the spongiosa of a specimen was weaker, reactivity of the compacta was also reduced often with fewer than half of the glands showing any reactivity for α_2 -PEG. Where secretions were evident these were of the granular type and were positive for α_2 -PEG. Decidual cells showed a diffuse cytoplasmic reaction with HMFG-1 MAb but this was demonstrated to be due to mouse Ig-binding which occurred above an Ig concentration of 5 μ g/ml. This reactivity could be removed by prior trypsinization, whilst epithelial staining remained unaffected. Some small areas in the compacta did show weak cytoplasmic staining within decidual cells and the surrounding matrix for α_2 -PEG.

Membranes. Using antibody dilutions of 1:4 or 1:20 all 10 specimens showed variable degrees of extracellular matrix α_2 -PEG reactivity, often very intense, and where present in the specimen some cytoplasmic staining within the epithelial cells. With HMFG-1 a thin layer of reactivity was seen on the amniotic epithelial cell membrane and also very rare glands in the decidual layer were revealed which were weakly stained for α_2 -PEG.

Placenta. Even at a dilution of 1:4, 14 specimens stained for α_2 -PEG were essentially negative, except for very weak reactivity in the syncytiotrophoblast and mesenchyme of a small proportion of villi (Fig. 1k).

Tissues obtained from an 8-week anembryonic pregnancy hysterectomy exhibited immunoreactivity very similar to those from normal first trimester pregnancy as regards decidua, membranes and trophoblast.

Fig. 1a-f

Fig. 1. Photomicrographs of pregnancy endometrium, membranes and placenta immunostained with monoclonal antibodies against α_2 -PEG (2C6H11 at 1:30–1:200 MAb dilution unless stated), HMFG-I or α_1 -PEG (2B2H10) using the alkaline phosphatase–anti-alkaline phosphatase method and counterstained with haematoxylin. The bar represents 50 μ m except in (a) and (c) where it represents 200 μ m. **(a)** First trimester endometrium showing decidua spongiosa (ds) and decidualized decidua compacta regions (dc). Intense immunoreactivity for α_2 -PEG is seen within ds glands while those in the dc region (arrowed) are more weakly stained. **(b)** First trimester decidua spongiosa showing intense staining for α_2 -PEG within the epithelium of glands. **(c)** As (a) but stained for α_1 -PEG showing the most intense reactivity within decidual cells in the compacta (dc) region. **(d)** First trimester decidua spongiosa exhibiting uniform cytoplasmic staining for α_2 -PEG within glandular epithelial cells. Granular material in the lumen is positive for α_2 -PEG. **(e)** First trimester decidua spongiosa showing α_2 -PEG positive glandular epithelial cells containing apical vacuoles which do not stain. Note non-granular secretory material in lumen does not stain for α_2 -PEG. **(f)** First trimester decidua spongiosa showing heavily vacuolated glandular epithelial cells which are unstained for α_2 -PEG. Gland on lower right contains intensively α_2 -PEG staining secretions.

Third trimester and term

Glands in these specimens were difficult to identify histologically and HMFG-I reactivity was particularly useful therefore as a marker of gland structures.

Fig. 1g-l

Fig. 1 continued: (g) First trimester decidua spongiosa region showing two types of secretions within the glandular lumen, one granular and immunoreactive for α_2 -PEG (see also (d)) and the other (arrowed) of a globular nature and unstained (see also (e)). (h) Heavily vacuolated glands in decidua spongiosa stained for HMFG-1. The epithelial cell membrane is clearly defined. (i) First trimester decidua compacta showing reactivity for α_2 -PEG in some glandular epithelial cells (arrowed gland shown in (j)). Note paucity of glands and loss of columnar appearance of cells compared to spongiosa glands in (b). (j) Higher magnification of gland in (i) showing α_2 -PEG staining. (k) First trimester placental villi showing only weak (note 1:4 MAb dilution) patchy staining for α_2 -PEG associated with syncytiotrophoblast membranes. (l) Placental bed biopsy obtained at Caesarean section stained for HMFG-1 to reveal residual glands, frequently attenuated (arrowed). Note further reduction in epithelial cell height and size of glands compared to those in first trimester compacta (i).

Decidua. Staining of 4 placental bed biopsies with HMFG-1 revealed many residual glands, some which were frequently attenuated, with the cell height further reduced compared to those glands detected in first trimester compacta (Fig. 1l). These gland structures have been previously noted in the study of Bulmer *et al.* (1986). In addition, occasional isolated cells, identified as mononuclear and multinucleate interstitial trophoblast cells by Bulmer *et al.* (1986), were immunoreactive with HMFG-1. Cytoplasmic staining for α_2 -PEG (at a 1:4 dilution of MAb) was associated

Fig. 1m-r

Fig. 1 continued: (m) As (l) but stained for α_2 -PEG (note 1:4 MAb dilution). The majority of glands revealed by HMFG-1 in (l) are seen to be positive also for α_2 -PEG, although not all (arrowed attenuated gland in (l) is negative). (n) As (m) showing α_2 -PEG reactivity in a small number of epithelial cells and of low intensity (note 1:4 MAb dilution). (o) Term membrane stained for HMFG-1 to reveal long, attenuated glands in the decidua (d). In addition membrane staining within the cytotrophoblast cell layer (t) and the amniotic epithelial cell layer (e) for HMFG-1 can be seen although weak in this specimen. (p) as (o) but stained for α_2 -PEG at a 1:4 dilution. The only immunoreactivity seen was of the glands revealed by HMFG-1 in (o) within the decidua where most were also α_2 -PEG positive, but only weakly stained. (q) Higher magnification to show weak α_2 -PEG staining (1:4 MAb dilution) within some glandular epithelial cells of a gland shown in (p). (r) Term placenta showing no staining for α_2 -PEG at a 1:4 MAb dilution.

with the majority of glands identified (Fig. 1m), but this was of low intensity and was not present in all cells of individual glands (Fig. 1n).

Membranes. A few often extremely long, attenuated glands were revealed by HMFG-1 in the decidua layer of 3/7 specimens (Fig. 1o). Most of these were also positive for α_2 -PEG when the MAb was used at a 1:4 dilution, but this was of weak intensity (Figs 1p & q). Intense membrane staining within the cytotrophoblast cell layer and also in the amniotic epithelial cell layer was apparent with HMFG-1 but not α_2 -PEG.

Placenta. No staining for α_2 -PEG was detectable in 5 specimens (Fig. 1r) even at a 1:4 dilution of MAb, except for weak patchy syncytiotrophoblast membrane reactivity which was found with mouse IgG at the same concentration.

The antibody used to detect α_2 -PEG in third trimester and term specimens was at least 8-fold more concentrated than that used for first trimester specimens and the staining intensity of α_2 -PEG was generally greater in the latter specimens.

The staining pattern obtained with an inappropriate IgG1 antibody (against LCA) was entirely different from that of α_2 -PEG or HMFG-1. Except as indicated, mouse IgG matched with α_2 -PEG, and HMFG-1 MAbs for Ig content did not exhibit any reactivity. No staining was seen when MAb was replaced with NSO parent myeloma cell culture supernatant or hybridoma growth medium, or when the polyclonal reagent was absorbed with purified α_2 -PEG or replaced with non-immune rabbit serum.

Discussion

This study using monoclonal reagents clearly demonstrates that α_2 -PEG is principally associated with the glandular epithelium and its secretions during the first and third trimesters of pregnancy. It was also apparent that most intense reactivity was detected in glands of the decidua spongiosa region of endometrium during the first trimester, a region where the stroma is not decidualized. No evidence for production by placental tissues was obtained in this study. PEP has also been localized by means of polyclonal reagents to the glandular and luminal epithelium of the endometrium during the first trimester (Mazurkiewicz *et al.*, 1981), but these observations appeared restricted to the decidualized decidua compacta. Similarly, alpha-uterine protein has been localized to the epithelium during the first trimester (Horne *et al.*, 1982). Tatarinov *et al.* (1980) and Inaba *et al.* (1987) have reported that related proteins, placenta-specific α_2 -microglobulin and placental protein 14, are localized to cells within the placenta including the cytotrophoblast and syncytiotrophoblast, but no such localization was observed in the present study. The restricted localization of α_2 -PEG to the epithelium of attenuated glandular structures in the decidua of fetal membranes and in the placental bed at term probably accounts for the designation of PP14 as a placental protein since it was isolated from placenta and fetal membranes. The localization of the protein intracellularly as well as to the apical membrane of glandular epithelial cells and to the glandular luminal secretions supports the original identity of α_2 -PEG as a major secretory protein synthesized by the first trimester endometrium and suggests that this cell, present in morphologically distinguishable glandular structures, represents its major cellular source during pregnancy, as proposed during the menstrual cycle.

Studies on rates of in-vitro synthesis and secretion of this protein by the endometrium during pregnancy demonstrated a decline of synthesis during the first trimester, which corresponded to an increase in synthesis and secretion of the decidua compacta-derived protein, pregnancy-associated endometrial α_1 -globulin (α_1 -PEG) (Bell *et al.*, 1985). This switch from α_2 -PEG to α_1 -PEG as major secretory proteins of the gestational endometrium has been interpreted as reflecting a relative change in composition of the endometrium, from principally α_2 -PEG-secreting decidua spongiosa to α_1 -PEG-secreting decidua compacta (Bell *et al.*, 1985; Bell, 1988). The present studies support this view, with epithelium of glands in the decidua spongiosa exhibiting greater cytoplasmic staining intensity and their lumina containing positive secretions. In the compacta region, where the stroma had undergone decidualization, glands were more sparse, the glandular epithelium more flattened, cytoplasmic staining was weak in intensity and α_2 -PEG positive secretion uncommon. In direct contrast α_1 -PEG is not detected in the non-decidualized stroma of the spongiosa but is localized to decidual cells of the compacta region (Waites *et al.*, 1989). At term the attenuated glands of the decidua associated with the amniochorion and, in the placental bed, which could in some cases only be identified immunohistologically, α_2 -PEG staining declined as indicated by the

necessity to use the antibodies at higher concentration. The in-vitro studies on whole tissue could therefore be accounted for by the involution of the α_2 -PEG-producing decidua spongiosa region and the appearance of attenuated glands associated with decidualized regions which produce and secrete only low levels of α_2 -PEG. These studies provide no evidence for direct hormonal regulation of α_2 -PEG production by glands during pregnancy but rather a direct relationship to the morphology and tissue localization of glands. The factors that control the morphology of glands may result from a direct hormonal effect or be mediated via the stroma. The co-existence of α_2 -PEG secretory glands of the spongiosa and attenuated glands of the compacta in the same specimen could suggest that differentiation and decidualization of the stroma is incompatible with support of the secretory glands and these undergo transformation to the flattened gland. That progressive decidualization is associated with involution of the spongiosa-type glands is supported by in-vitro organ culture in which continued progesterone exposure results in conversion of secretory endometrium to non-gland-containing decidualized endometrium (Maslar *et al.*, 1986). Glands in the decidua compacta are often associated with a mononuclear stromal population and it has been proposed that the loss of these glands may be associated with an autoimmune rejection (Bulmer & Johnson, 1985), a proposal linked with the reported altered antigenic expression of glands in the first trimester, i.e. they have been reported to lose expression of Class I major histocompatibility antigens and to acquire a trophoblast-associated antigen (Johnson & Bulmer, 1984).

Serum concentrations of α_2 -PEG, PEP and PP14 during pregnancy are maximal during the first trimester (Joshi *et al.*, 1982; Julkunen *et al.*, 1985; Bell *et al.*, 1987a) and the present study supports the contention that the serum protein is derived from the glandular epithelium. The question remains as to the route by which a glandular secretory protein gains access to the maternal circulation when histological evidence suggests that the secretion is directed toward the glandular and uterine lumen. Protein may gain access by diffusion from the basal capillary membrane through the stroma to the capillary bed, but more probable is that α_2 -PEG is liberated by involution of the glands or their disruption by the developing placenta. CA125, an epithelial-associated protein detected on glandular epithelium during the menstrual cycle but not at significant levels in peripheral serum, exhibits a serum profile during pregnancy similar to that of α_2 -PEG (Seki *et al.*, 1986; Jacobs *et al.*, 1988). The marked decrease in intensity of α_2 -PEG staining in glands of the decidualized compacta region and the proposal that decidualization is involved with gland involution suggests that serum concentrations of α_2 -PEG may inversely correlate with decidualization, as suggested from in-vitro synthesis studies (Bell *et al.*, 1985), and abnormalities in decidualization be reflected by α_2 -PEG serum values.

In conclusion this histological distribution further supports the glandular epithelial origin of this protein and the principal contribution of glands within the decidua spongiosa to tissue synthesis and serum concentrations. Serum values of this protein during the first trimester may represent a unique functional marker of secretory glandular epithelium of the decidua spongiosa but not of decidualization.

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