Heterogeneity of progesterone receptors A and B expression in human endometrial glands and stroma

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The human progesterone receptor (PR) is expressed as two isoforms, PRA and PRB, which function as ligand-activated transcription factors. In-vitro studies suggest that the isoforms differ functionally and that their relative expression in a target cell may determine the nature and magnitude of response to progesterone. We have shown recently that PRA and PRB are co-expressed in target cells of the human endometrium. The purpose of this study was to investigate the homogeneity of expression of PRA and PRB in target cells of the human uterus throughout the menstrual cycle. In the functionalis, PRA and PRB were expressed in comparable levels in glandular epithelium during the proliferative phase of the cycle, whereas there was persistence of PRB but not PRA in the glands during mid-secretory phase. In the stroma, there was predominance of the PRA isoform throughout the cycle. There was remarkable homogeneity in the relative expression of PRA and PRB in adjacent cells within the same tissue compartment, suggesting that the mechanisms regulating relative PR isoform expression are similarly active in these cells. By contrast, heterogeneity between glands was observed under some circumstances in the functionalis of the endometrium, suggesting PR isoform down-regulation by progesterone to be asynchronous. Heterogeneity was also seen between the glands of the basalis and functionalis of the endometrium implying region-specific responses to hormonal stimuli. This study demonstrates adjacent cell homogeneity in the relative expression of PRA and PRB in normal human endometrial tissue and a differential response to ovarian steroid hormones between cell types and between different regions within the same tissue.

Key words: endometrium/immunofluorescent histochemistry/progesterone receptor isoforms

Introduction

The endometrium is composed of two layers: the basalis, the layer from which it regenerates after menstrual shedding, and the overlying functionalis. Each layer is comprised of two major components, the epithelium, that is present as either glandular elements or superficial epithelium, and the mesenchymal component of stromal cells. In the course of the normal menstrual cycle, the human endometrium demonstrates a regular sequence of proliferation, differentiation and degeneration in response to fluctuations in steroid hormone concentrations. Oestrogens induce proliferation of the epithelial and stromal elements of the endometrium during the pre-ovulatory proliferative phase and, post-ovulation, progesterone is involved in glandular differentiation and glycogenesis, as well as stromal proliferation and the development of predecidual cells (Graham and Clarke, 1997). The effects of oestrogen and progesterone are mediated by specific nuclear receptor proteins, oestrogen receptor (ER) and progesterone receptor (PR),...
which are present in endometrial stromal and epithelial cells (Clark, 1979).

It is well established that PR concentrations vary with menstrual cycle phase (Janne et al., 1975; Bergeron et al., 1988; Garcia et al., 1988; Lessey et al., 1988; Press et al., 1988; Snijders et al., 1992; Fung et al., 1994; Zeimet et al., 1994; Moutsatsou and Sekeris, 1997). PR content increases in both the epithelial and stromal compartments during the proliferative phase and remains high during the early secretory phase. In mid- to late secretory phase there is a decline in PR expression, which is marked in glandular cells and less evident in the stroma (Bergeron et al., 1988; Lessey et al., 1988; Fung et al., 1994; Moutsatsou and Sekeris, 1997; Press et al., 1988). These changes are likely to be related to the known effects of oestrogen and progesterone on PR expression, with high concentrations of oestrogen in the proliferative phase inducing PR synthesis and progesterone down-regulating expression of its own receptor post-ovulation (Graham and Clarke, 1997). The maintenance of stromal PR throughout the secretory phase of the menstrual cycle is suggestive of constitutive PR expression and implies the continued need for progesterone to support further growth and development in this tissue (Bergeron et al., 1988; Lessey et al., 1988).

The human PR is expressed as two isoforms, PRA and PRB (Horwitz and Alexander, 1983; Savouret et al., 1990) that differ only in that the smaller isoform, PRA, lacks 164 amino acids from the N-terminus (Kastner et al., 1990). PRA and PRB are products of a single gene and are translated from individual mRNA species under the control of distinct promoters (Kastner et al., 1990). Both PRA and PRB function as ligand-activated transcription factors, but it has been suggested on the basis of in-vitro studies that the two proteins are not functionally equivalent. Transient co-transfection of PRB or PRA and progestin-sensitive reporter genes has shown that, in general, PRB is transcriptionally more active of the two isoforms (Wen et al., 1994; Giangrande et al., 1997). Furthermore, PRA can act as a dominant repressor of PRB activation of progestin-sensitive reporter genes (Tung et al., 1993; Vegeoto et al., 1993; Giangrande et al., 1997), and similarly inhibits the transcriptional activity of receptors for androgens, glucocorticoids and mineralocorticoids (Vegeto et al., 1993; McDonnell et al., 1994). In addition, PRA has been implicated in inhibition of ER activity: co-transfection of PRA, ER and oestrogen-sensitive reporters has shown a striking diminution of ER transactivation (McDonnell and Goldman, 1994; Wen et al., 1994; Kraus et al., 1995).

The implications of these findings in-vitro, if borne out in-vivo, are that the relative expression of PRA and PRB within target cells may determine the nature and magnitude of functional responses to progesterone and oestrogen (Vegeto et al., 1993). We have shown recently that PRA and PRB normally reside within the same cell in-vivo, in the human endometrium (Mote et al., 1999). The aim of this study was to evaluate the homogeneity of PRA and PRB expression in glands and stroma of the endometrium and to compare the expression of these isoforms in the functionalis and basalis of the endometrium.

Materials and methods

Tissue samples

Archival, formalin-fixed, paraffin-embedded endometrial tissue from 26 women and histological dating of tissue were described previously (Mote et al., 1999). Samples were from the proliferative (n = 8); early secretory (n = 3); mid-secretory (n = 6), and late secretory (n = 9) phases of the menstrual cycle. The ages of the women ranged from 18 to 49 years (mean 38 years).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded sections were cut at 2 μm using a standard rotary microtome, mounted onto Superfrost Plus slides (Lomb Scientific, NSW, Australia) to which Mayer Albumen adhesive (Humason, 1979) had been applied, and dried at 37°C for 72 h. This was followed by storage at 4°C for no longer than 3 weeks.

Antigen retrieval

A combination of heat and pressure was used for antigen retrieval as previously described (Mote et al., 1998). Briefly, immediately prior to
staining, sections were deparaffinized, rehydrated to distilled water and placed in 0.01 mol/l sodium citrate solution (pH 6.0). Pairs of slides were positioned back to back into Corning 50 ml polypropylene centrifuge tubes (Crown Scientific, NSW, Australia) with sufficient 0.01 mol/l sodium citrate solution to cover the tissue. The tubes were fitted with loose-fitting screw caps, placed vertically into a foil-covered 500 ml beaker and heated in a Tuttnauer 2540 EKA autoclave at 121 °C, 15 psi for 30 min. After autoclaving, the sections were allowed to remain in the sodium citrate solution for a minimum of 30 min, followed by washing in three 5 min changes of phosphate-buffered saline (PBS).

**Dual immunofluorescent staining**

Dual immunofluorescent staining was carried out as previously described (Mote et al., 1999). Briefly, sections were incubated overnight with a mouse anti-human PR monoclonal antibody that detects PRB alone (hPRa6) (Clarke et al., 1987) diluted 1:5. PRB protein was detected by incubation with a biotinylated goat anti-mouse antibody (Dako, NSW, Australia), diluted 1:100, and with Texas red (TXR)–avidin (Vector Laboratories, CA, USA) diluted 1:250. To block sites of potential cross-reactivity between the two staining sequences, sections were incubated overnight with goat anti-mouse Ig Fab (Cappel Antibodies, ICN Biomedical, NSW, Australia) diluted 1:200. Subsequently, sections were incubated with the mouse monoclonal antibody to detect human PRA (Mote et al., 1999) diluted 1:10. The PR protein was detected by incubation with a biotinylated goat anti-mouse antibody (Dako, NSW, Australia), diluted 1:100, and with fluorescein isothiocyanate (FITC)–avidin (Calbiochem, Australia) diluted 1:200.

**Control sections**

Control sections were treated and stained in the same way as the test sections. Controls included adjacent sections to each endometrial sample stained using PBS/0.5% Triton X-100 (i) in place of both primary antibodies to control for non-specific staining and (ii) to replace the second sequence primary antibody to ensure no cross-reactivity between the two staining sequences, and dual staining of the transfected cells expressing only PRA or PRB (Mote et al., 1999).

**Fluorescent analysis**

PR staining was examined using an Olympus BX40 fluorescent microscope fitted with filters to detect both TXR (Band pass 545–580) and FITC (Band pass 450–480) fluorescence simultaneously, and each of the two fluorochromes separately. All of the sections were examined in detail, under individual fluorochrome excitation and also using the dual filter, by three observers. The sections were scored ‘blind’ and intensity per field recorded. Staining of the epithelial and stromal elements of the endometrium across the whole section were specifically examined and compared. The intensities of the TXR and FITC signals in each field were scored according to a five-point scale: very high (4–5); high (3); moderate (2); low (1), negative (0). The mean intensity of each fluorochrome for each section was calculated. The same arbitrary scales were used to show the relative intensity of signals for PRA and PRB in both stromal and glandular tissue.

**Results**

Dual immunofluorescent staining of the two PR isoforms was accomplished by sequential staining with a primary antibody that detects PRB alone (hPRa6), followed by a second primary antibody (hPRa7) which recognises PRA but not PRB in paraffin sections (Mote et al., 1999).

**Expression of PRA and PRB in the endometrium**

We have shown recently that PRA and PRB isoforms were co-expressed in the nuclei of most PR positive cells (Mote et al., 1999). PRB was visualised as a red colour under TXR excitation, whereas FITC fluorescent excitation of the same section revealed PRA proteins to be green. When the same section was viewed with simultaneous excitation of both fluorochromes, nuclei that expressed predominantly PRA proteins were green, whereas nuclei that expressed primarily PRB proteins were orange. Nuclei co-expressing both PRA and PRB proteins in similar concentra-
Expression of PRA and PRB proteins in human endometrium during the menstrual cycle.

Sections of endometrial tissue from each phase of the menstrual cycle were stained by dual immunofluorescence as described in the Materials and methods and visualised by dual excitation of both Texas red and fluorescein isothiocyanate (fluorochromes). (A) Proliferative phase, original magnification ×400; (B) mid-secretory phase, original magnification ×400; (C) mid-secretory phase endometrium, original magnification ×200 and (D) mid-secretory phase endometrium, original magnification ×400, showing heterogeneity between glands in relative PRA and PRB expression; (E) basalis and (F) functionalis from menstrual endometrium, original magnification ×400.

Expression of PRA and PRB in the functionalis during the menstrual cycle

During the proliferative phase of the menstrual cycle, high expression of both PRA and PRB was noted in the glands (Figure 1A; Table I). The intensity of PR staining increased during the proliferative phase, reaching maximal expression in mid-late proliferative phase, and a similar increase in the concentrations of PRA and PRB

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<th>Table I. Progesterone receptor (PR)A and B expression in endometrial glands and stroma during the menstrual cycle</th>
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PRA and PRB concentrations were determined by examination under fluorescein isothiocyanate and Texas red excitation respectively and scoring of numbers of stained cells and intensity of staining, as described in Materials and methods.
was evident. Stromal PR staining was less intense than glandular staining in the proliferative phase (Table I) and PRA was consistently observed to be the predominant isoform expressed in the stroma. The intensity of PR staining in the stroma increased during the proliferative phase; however, in contrast with the glands, there was a greater relative increase in the PRA isoform. During the early secretory phase, PR protein expression in the glands was lower than during the proliferative phase, consistent with a reduction in PRB expression in some cells (Table I). However, by the mid-secretory phase of the menstrual cycle, although overall PR protein concentrations were still further reduced, the majority of glands contained a predominant expression of the PRB isoform (Figure 1B). By the late secretory phase the majority of glands were negative. PR was expressed in the stroma throughout the secretory phase of the menstrual cycle, and PRA was the predominant isoform present in these cells (Table I).

**Homogeneous expression of PRA and PRB in glands and stroma of the functionalis**

In both proliferative and secretory phases of the cycle, adjacent cells within the glands contained very similar levels of PRA and PRB. In the proliferative phase, cells contained both PRA and PRB and all cells within a gland appeared to contain the same concentration of each protein (Figure 1A). In the mid-secretory phase, PRB was the predominant isoform in the glands, and all cells within the gland were similarly stained (Figure 1B). The same homogeneity of PRA and PRB expression in adjacent cells was also noted within the stroma.

However, although the homogeneity was striking within glands, there was between-gland heterogeneity observed within some areas of the functionalis of the endometrium, with neighbouring glands displaying a predominance of either PRB (orange staining) or both isoforms (yellow staining) within the same field (Figure 1C and D). This was observed in the secretory phase, when rising serum progesterone concentrations were associated with loss of PR isoforms (Mote et al., 1999).

**Expression of PRA and PRB in the basalis of the endometrium**

The functionalis and upper basalis layers of the endometrium largely expressed similar relative concentrations of PRA and PRB. However, in some cases there was a discrepancy, with the functionalis expressing both PRA and PRB whereas the deeper layers of the basalis expressed a distinct predominance of PRA (Figure 1E and F). In general, if there was a discordance between the basalis and functionalis, it tended to be reflected in a relative lack of PRB expression in the basalis in comparison with the functionalis.

**Discussion**

In-vitro studies show the A and B isoforms of PR to demonstrate unique properties and to activate different target genes (Tora et al., 1988; Kastner et al., 1990). Transfection experiments have shown that PRA can act as a dominant negative inhibitor of PRB, dependent upon cell and promoter context (Tung et al., 1993; Vegeto et al., 1993), and that it is generally considered to be the weaker of the two PR isoforms in activation of target gene transcription (Vegeto et al., 1993). Additionally, PRA can inhibit the transcriptional efficiency of ER (McDonnell and Goldman, 1994; Wen et al., 1994), a repression dependent on the absolute concentration of PRA (Wen et al., 1994). We have shown recently that PRA and PRB are co-expressed in nuclei of PR positive cells (Mote et al., 1999), suggesting that the relative concentrations of PRA and PRB within endometrial cells may determine the nature and magnitude of functional responses to progesterone.

**Regulation of PRA and PRB in the proliferative phase**

During glandular proliferation, expression of both PRA and PRB proteins increased markedly, reaching similar high concentrations by late proliferative phase and suggesting that the known induction of PR by oestrogen (Janne et al., 1975; Katzenellenbogen, 1980; Horwitz, 1981; Okulicz et al., 1989; Savouret et al., 1990) in the glandular epithelium during the proliferative phase is reflected by increases in both PRA and PRB. Similarly, in the stroma, concentrations of both PRA and PRB
proteins were increased during the proliferative phase, but in this tissue, the increase in PRA expression was greater than that of PRB, and throughout the proliferative phase PRA was the dominant isoform.

**Regulation of PRA and PRB in the secretory phase**

In the glandular epithelium, expression of both PRA and PRB was reduced during the secretory phase of the cycle, under the influence of rising serum progesterone concentrations, consistent with the known down-regulating effect of progestins on PR expression (Graham and Clarke, 1997). Interestingly, dual immunofluorescent localisation of PR revealed that there was discordance in the down-regulation of each isoform. In early secretory phase, loss of PRB was evident and glands expressing a predominance of PRA were noted, whereas in mid-secretory phase, PRB was the predominant isoform expressed in most glands. These results are consistent with the possibility that there was an initial down-regulation of PRB protein by progesterone in early secretory phase, but that this loss was compensated for during the mid-secretory phase by the second oestrogen peak that is known to be present at this time (Thorneycroft et al., 1971). Conversely, loss of PRA protein in the glands was less marked during early secretory phase, but expression of PRA decreased continuously during the secretory phase and there was no apparent rise coincident with the second oestrogen peak, as was observed for PRB. The discordance of progesterone down-regulation of PRA and PRB in the glandular epithelium suggests differential sensitivity of the two isoforms to the effects of progesterone during the secretory phase of the menstrual cycle. The maintenance of PRA expression during the mid-secretory phase was an unexpected observation, but is suggestive of a continued need for progesterone action, mediated by PRB, in glandular tissue at this time.

In the stroma, there was little or no decrease in PRA expression in early and mid-secretory phase: PRA was always the predominant isoform observed and there were minimal changes in the expression of this isoform throughout the early–mid-secretory phases. Expression of PRB protein, however, fluctuated similarly to the fluctuations in PRB expression in the glandular epithelium, but at a lower magnitude.

**Heterogeneity and homogeneity of PRA and PRB expression in the endometrium during the cycle**

Although the expression of PRA and PRB varied within the glands and stroma during the menstrual cycle, a striking feature of the expression of these proteins was the homogeneity of their relative expression within a cell compartment. Adjacent epithelial cells showed very similar expression of PRA and PRB and this suggested a concordance between cells in the mechanisms responsible for setting the relative PRA and PRB gene expression level. The mechanisms which determine relative cellular expression of PRA and PRB are poorly understood. Our previous studies show preferential up-regulation of PRB by oestrogen in human breast cancer cells (Graham et al., 1995); as is the case in human endometrial tissue (Mangal et al., 1997), in chicken spleen and lung (Pasanen et al., 1997), and in the freshwater turtle oviduct (Reese and Callard, 1989). There is also evidence that oestrogen increases PRA more than PRB protein concentrations in chicken oviduct (Syvala et al., 1997), suggesting that oestrogen stimulation of PRA and PRB is likely to be cell-, tissue- and species-specific. It is also likely that mechanisms other than stimulation by oestrogen influence PR expression, as PR is expressed in some ER negative breast tumours (Horwitz, 1981). PR expression can be regulated by growth factors (Katzenellenbogen and Norman, 1990) and ERα-null mice express low concentrations of PR mRNA (Shughrue et al., 1997). The mechanisms that underlie the expression of PRA and PRB in the human endometrium are not understood and further studies are ongoing in this area.

By contrast with the homogeneity observed between cells in glands and stroma, there were regional areas of broad heterogeneity within samples, particularly in the secretory phase of the cycle, with isolated glands in the functionalis expressing a relative level of PR isoforms within all its cells which was different to that expressed by neighbouring glands. When these heterogeneous
regions were examined for individual expression of PRA or PRB, it was evident that the heterogeneity was largely due to inter-gland fluctuations in PRA expression, at a time when progesterone-mediated PR down-regulation was taking place. This suggested that PRA expression decreased upon progesterone exposure more rapidly in some glands than in others, and that a combination of systemic and local influences may be responsible for PR isoform regulation. Alternatively, it is possible that the proximity of a gland to a spiral artery may influence the rapidity of progesterone-mediated effects, as progesterone-mediated predecidualization is first observed in stromal cells located in the immediate vicinity of a blood vessel.

In addition to the differences between glands in PRA expression, there was heterogeneity between the functionalis and basalis layers of the endometrium, often with little concordance between the relative expression of PRA and PRB seen in these two layers. This broad level of heterogeneity implied that there may be a tissue region-specific effect on relative expression of PRA and PRB, through mechanisms still to be explored. Differences in cyclical PR expression have been reported, by Noe and colleagues, between the endometrium and the myometrium, and also between the subendometrial and outer layers of the uterine wall, suggesting that the human uterus is comprised of functionally distinct regions with distinct PR regulation profiles (Noe et al., 1999).

The deeper layers of the endometrium are known to respond differently to the effects of progesterone during the secretory phase of the menstrual cycle compared to the upper regions (Robertson, 1981; Okulicz and Balsamo, 1993). Work on non-human primates has demonstrated that, in contrast to the functionalis, glandular ER and PR expression is maintained in the basalis, and that proliferation in this region is not inhibited despite high progesterone concentrations at this time (Okulicz and Balsamo, 1993). This suggests that PR expression is constitutive and that progesterone may act as a mitogen on basalis epithelial glands involved in preparation for subsequent tissue regeneration during menstrual reconstruction. The predominance of PRA observed in these glands implicates the involvement of this PR isoform with progesterone-mediated proliferation of the basalis. It is interesting that a predominance of the PRA isoform is evident in normal stromal tissue proliferation in response to progesterone during the latter half of the menstrual cycle and is also observed in highly proliferating malignant breast tumours (unpublished observations).

In summary, this study has explored the heterogeneity of expression of PRA and PRB in target cells of the human uterus throughout the menstrual cycle. There was remarkable homogeneity in the relative expression of PRA and PRB in adjacent cells within the same tissue compartment, suggesting that the mechanisms regulating relative PR isoform expression are similarly active in these cells. By contrast, heterogeneity between glands was observed in the secretory phase in the functionalis of the endometrium, and was also seen between the basalis and functionalis of the endometrium. Heterogeneity of PR isoform expression between glands in the functionalis in the secretory phase, when PR concentrations decrease under the influence of progesterone, suggests PR down-regulation to be an asynchronous event. Moreover, heterogeneity of relative PRA and PRB expression between glands in the functionalis and basalis layers of the endometrium shows clearly that the same cell types can respond differently to hormonal stimuli based on their location within the endometrium. This suggests that different areas of the endometrium have distinct hormonal responses and differential relative expression of PRA and PRB, and this may be associated with differential regulation of downstream targets of progesterone action.

Taken together, the results of this study support the view that heterogeneity of PR expression within different areas of the endometrium may contribute to specific progesterone responses in those areas. However, within areas, the relative expression of PRA and PRB is under tight control leading to adjacent cell homogeneity of PRA and PRB expression within each tissue compartment. Loss or alteration in relative PR isoform expression may be associated with endometrial pathology.

Acknowledgements

The authors would like to thank the Department of Anatomical Pathology, Westmead Hospital, Westmead,
NSW, Australia for the tissue samples used in this study. This work was supported by the National Health and Medical Research Council (NHMRC) of Australia, the Leo and Jenny Leukaemia and Cancer Foundation and the University of Sydney Cancer Research Fund. P.A.M. and E.M.M. are supported by NHMRC Dora Lush Biomedical Postgraduate Research Scholarships.

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