Molecular and Cellular Endocrinology, 95 (1993) 23–29

MCE 03040

Sequential appearance of relaxin, prolactin and IGFBP-1 during growth and differentiation of the human endometrium

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(Received 19 February 1993; accepted 19 April 1993)

Key words: Relaxin; Prolactin; IGFBP-1; Endometrium (human)

Summary

Relaxin (RLX) is a product of the human corpus luteum, pregnancy decidua and placenta, prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) are products of the cyclic endometrium and of the pregnancy decidua. All three proteins are thought to function interdependently in endometrium/decidua as local factors within the uterus without reaching the systemic circulation. In this study, the avidin-biotin immunoperoxidase method for immunolocalization with monoclonal or polyclonal antibodies has been applied to serial sections of endometria obtained from patients at different stages of the menstrual cycle and in early and late gestation. This allowed the cellular localization of the three proteins to be followed simultaneously through the reproductive stages from cyclic endometrium to term gestational decidua. The production, as opposed to sequestration of RLX from an ovarian source was demonstrated by the application in parallel of an antibody to the processed hormone and its connecting peptide. RLX was shown localized to the glandular and luminal epithelia in the proliferative and secretory phases. The decidualized stromal cells also immunostained for RLX in the late secretory phase and in early and late pregnancy. PRL was localized first to the glandular epithelium and then stroma, appearing after RLX, IGFBP-1 appeared later in the secretory phase and predominantly in the decidualized stromal cells confirming previous studies. In contrast, all three proteins were immunostained in early pregnancy and increased to term gestation. The sequential appearance of the three proteins suggests that they are regulated by different mechanisms in the early part of the menstrual cycle but the maintenance and increase in the late secretory phase and in early pregnancy suggests coordination by a common mechanism or stimulus.

Introduction

RLX, PRL and IGFBP-1 mRNAs are all expressed in human decidual tissue, suggesting a special function for these peptides in the uterus during pregnancy (Sakbun et al., 1990; Clements et al., 1983; Julkunen et al., 1988; Huang et al., 1987). PRL and IGFBP-1 mRNAs are also present in non-pregnant endometrium in the secretory phase of the menstrual cycle (Julkunen et al., 1988; Huang et al., 1987), the hybridization signals for both being detected in stromal cells (Huang et al., 1987; Julkunen et al., 1990). PRL and IGFBP-1 have also been shown to be secreted by the endometrium at this phase of the menstrual cycle (Maslar and Riddick, 1979; Rutanen et al., 1986). There is a growing body of evidence to suggest that the IGFBPs are important modulators of the biological effects of IGFs which, in turn, stimulate cell proliferation and differentiation, as well as having insulin-like metabolic effects on several cell types (Baxter and Martin, 1989). There is no information on RLX gene expression in the human non-pregnant endometrium, although early work using a porcine RLX antibody suggested that RLX was a product of this tissue (Yki-

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Jarvinen et al., 1983). It has recently been shown that RLX is a product of the term decidua (Sakbun et al., 1990) and that both RLX genes (H1 and H2) are expressed in this tissue (Hansell et al., 1991). RLX added to long-term endometrial cell cultures stimulates the production of PRL (Huang et al., 1987) and IGFBP-1 (Thrailkill et al., 1990; Bell et al., 1991). The temporal and spatial relationships of these three factors during the reproductive cycle in vivo are unknown. Accordingly we have studied the concomitant cellular localization of RLX, PRL and IGFBP-1 in serial sections of the same tissue specimens from endometria at different phases of the menstrual cycle as well as in decidua parietalis with adherent fetal membranes from early and term pregnancies.

Materials and methods

Tissue specimens

Cyclic endometrial specimens were collected from 15 women undergoing hysterectomy for uterine leiomyomata, and four samples were obtained by curettage performed for dysfunctional bleeding at the Jorvi Hospital, Espoo, Finland. A piece of each specimen was stained with hematoxylin and eosin for histological examination and dating of the cyclic endometria. Nine specimens represented the proliferative phase, two the early secretory phase (days 15 to 19), four were from the mid-secretory phase (days 20 to 24) and four were from the late secretory phase (days 25 to 28). Decidual tissue was collected from seven women undergoing early pregnancy termination for social reasons (gestational age 9 weeks 6 days to 11 weeks 6 days), and from three women having dilatation and curettage for a blighted ovum as diagnosed by routine early pregnancy ultrasound (gestational age 9 weeks 5 days to 12 weeks). Placenta with attached fetal membranes were collected from four women after elective Cesarean section at term who had not undergone labor, and from four women after spontaneous onset of labor with no augmentation and normal delivery at term at Kapiolani Women's and Children's Medical Center (Honolulu, HI). All tissue samples were collected after obtaining informed consent from the patient and permission of the local ethical committees. Pieces (1 cm^2) of the fetal membranes with adherent decidua were dissected and rolled. All tissues were immediately taken to the laboratory on ice and processed within 1 h after removal. The sampled tissues were immediately fixed in Bouin's solution (16–24 h at 25°C) and then processed for paraplast embedding. Five to seven-micron sections were cut and mounted on glass slides. Pieces of fixed tissue as well as sectioned specimens were sent for immunocytochemistry to the other laboratory involved in this study. This eliminated any potential differences of immunolocalization due to tissue processing.

Antibodies

An affinity-purified polyclonal antiserum made in a goat (Lot 5800-78) to chemically synthesized H2 RLX was kindly supplied by Genentech (San Francisco, CA), and used at a 1:150 dilution. An equivalent concentration of goat IgG (Sigma, St. Louis, MO) was used as its control. In addition, a monoclonal antibody against amino acids 34–58 of the C chain of prorelaxin (1Hb, Genentech, San Francisco) was used at 25 μg/ml and a control monoclonal antibody identically prepared to human immunodeficiency virus protein (HIV; 10F6 8974-44) at the same concentration.

A polyclonal rabbit antiserum to hPRL was a gift from the National Hormone and Pituitary Agency NIDDK (hPRL-3, AFP-/C11580). This antibody was used at a final dilution of 1:500 and its specificity for these tissues previously described (Ali et al., 1991). A mouse monoclonal antibody No. 6303 against purified IGFBP-1 and a control antibody against sex-hormone binding globulin were obtained from Medix Biochemica, Kauniainen, Finland. The specificity of monoclonal antibody (Mab) 6303 has been described earlier (Rutanen et al., 1988; Rutanen et al., 1991a and b). Mab 6303 and the control were used at a 1:500 dilution.

Immunocytochemical staining

Deparaffinized and rehydrated sections were immunostained for RLX, PRL and IGFBP-1 as described previously (Sakbun et al., 1990; Ali et al., 1991; Rutanen et al., 1991a,b). Peroxidase activity was demonstrated cytochemically by incubation with a peroxidase substrate containing either diaminobenzidine (for PRL and RLX) 0.5 mg/ml with 0.01% hydrogen peroxide in Tris-buffer 0.1 M, pH 7.2, or 3-amino-9-ethyl-carbazol in acetate buffer, pH 5, (for IGFBP-1). The sections were rinsed in water and counterstained with hematoxylin and mounted for bright field microscopy.

The specificity of the staining reactions was tested by substitution of the monoclonal antibodies to human RLX and IGFBP-1 with monoclonal antibodies identically produced to HIV and sex-binding globulin respectively, see above, or by substituting normal rabbit serum at the same concentration as the polyclonal antibody to PRL, or goat IgG at the equivalent concentration as the polyclonal antibody to human RLX. An additional test of specificity for human RLX was the use of two antibodies produced by different methods one of which was directed to the C-peptide. This antibody would detect prorelaxin and/or C-peptide but not processed RLX and theoretically demonstrates a cell of synthesis as opposed to a cell sequestering processed RLX. Preabsorption studies were conducted by incubation of the primary antibody at the concentration used for immunocytochemistry in a range of concentrations of antigen (1–2000 ng/ml), for 48 h at 4°C. Reduced

staining was obtained with the polyclonal antibody to human RLX when it was preabsorbed over the concentration range 10–50 ng/ml.

Results

RLX immunostaining was detected and predominated in the epithelial cells lining the glands and the uterine lumen at all stages of the menstrual cycle with both antibodies to RLX. The staining intensity in these cells increased from the proliferative phase to the mid-secretory phase shown semi-quantitatively in Table 1. The intensity of stain varied from gland to gland in both phases of the cycle and in early pregnancy, with some staining more darkly whilst others were very lightly stained. An example of epithelial cell staining in the glands of a proliferative phase specimen with the polyclonal antibody is shown in Fig. 1A. The control goat IgG showed the complete lack of staining, shown on a serial section in Fig. 1B. The same specimen is shown in Fig. 1C, immunostained with the antibody to RLX C-peptide, and shows identical glandular epithelial cell staining as in Fig. 1A. The control for this antibody showed no background stain, Fig. 1D.

In the proliferative phase, some areas of the stroma immunostained weakly, but by the late secretory phase (d.25) all four specimens of decidualized stroma cells stained intensely with both RLX antibodies. The epithelial cells of the glands also immunostained very positively on d.25 as seen in the example in Fig. 1E. A section from a late secretory phase specimen which was immunostained with the antibody to RLX which had been preabsorbed with 10 ng recombinant H2 RLX shows reduced staining in the glands and the stroma, Fig. 1F.

Neither PRL nor IGFBP-1 immunostaining are shown in Fig. 1 because both have been previously reported. They were repeated in the same tissues as RLX in order to link the three proteins temporally and spacially.

PRL showed a different pattern of staining in comparison to RLX in the cyclic endometria (Table 1). It localized weakly or not at all to the epithelial cells of the glands and uterine lumen and weakly to the stromal cells in all the proliferative phase specimens (not shown). However, by the late secretory phase the staining intensity increased markedly in both the glandular and luminal epithelia as well as in the stroma. The glandular cell staining for PRL was very much more uniform between different glands than seen for RLX. The stroma cells near the luminal epithelial surface immunostained for both RLX and PRL more intensely than the rest of the stroma.

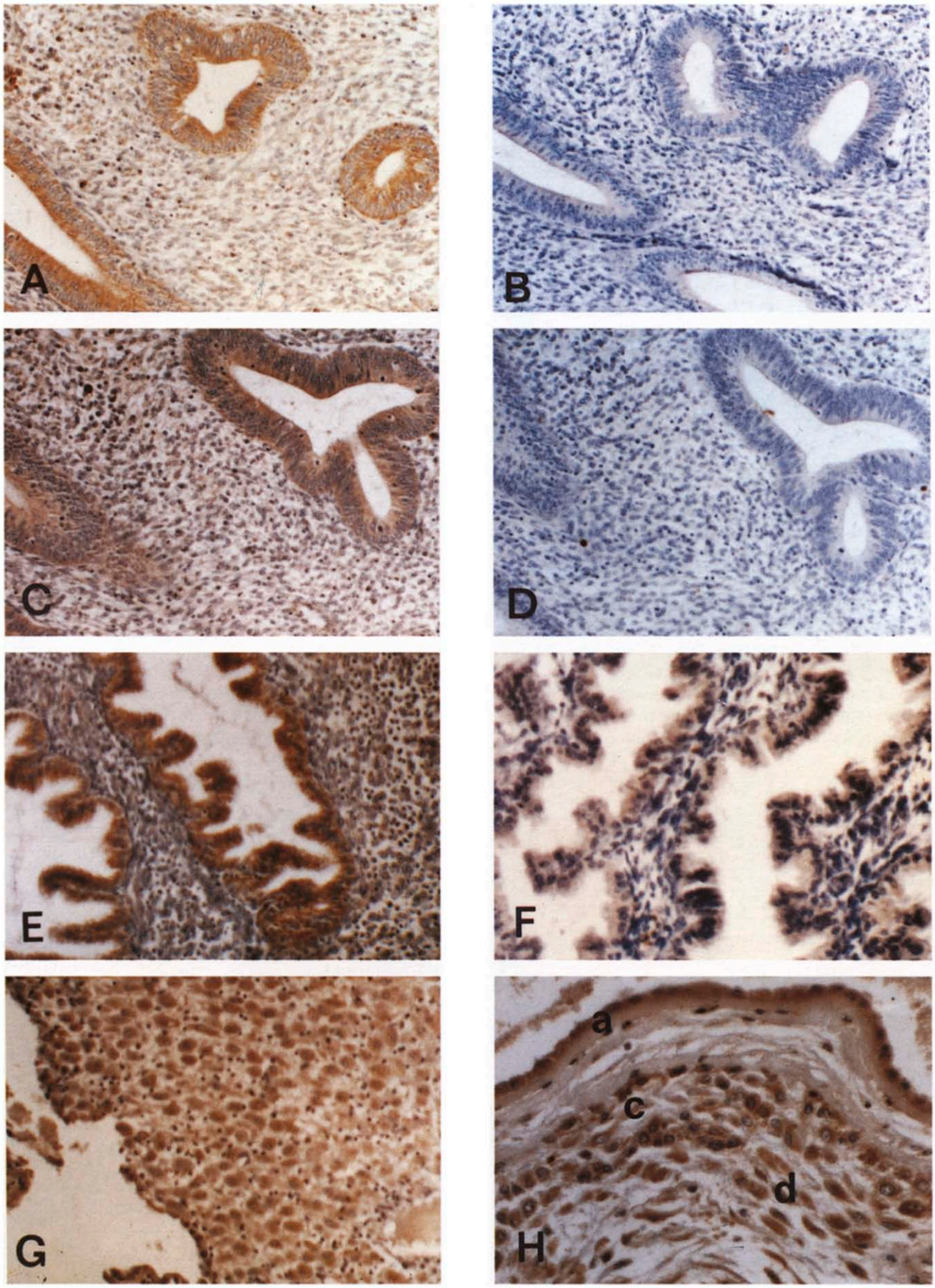
IGFBP-1 staining was absent from all proliferative phase specimens as previously demonstrated (Rutanen et al., 1991b), and was present in only two out of the four specimens collected in the mid secretory phase, when it localized to some epithelial cells only in one specimen and strongly to some stromal cells only in the other (Table 1). However, IGFBP-1 stained strongly but non-uniformly in all four specimens obtained in the late secretory phase, and was localized almost exclusively to the stromal cells, being strongest in the predecidualized stromal cells beneath the luminal epithelium. The control monoclonal antibody at the same concentration showed complete absence of stain.

TABLE 1

SUMMARY OF THE LOCALIZATION PATTERN AND SEMI-QUANTITATIVE INTENSITY OF STAIN FOR RELAXIN, PRL AND IGFBP-1 IN THE HUMAN ENDOMETRIUM, DECIDUA AND FETAL MEMBRANES

Reproductive state	Cell type	Relaxin *	Prolactin *	IGFBP-1 *
Proliferative	Epithelia (glandular and luminal)	+ +	±	–
($n = 9$)	Stroma	±	±	–
Early secretory	Epithelia	+ +	+	–
day 15–19 ($n = 2$)	Stroma	±	+	–
Mid secretory	Epithelia	+ + +	+ +	±
day 20–24 ($n = 4$)	Stroma	+ +	+ +	±
Late secretory	Epithelia	+ + +	+ +	+
day 25–28 ($n = 3$)	Stroma	+ +	+ +	+ + +
Early pregnancy ($n = 10$)	Epithelia	+ + +	+ +	+ +
	Decidualized stroma:			
	(zona compacta)	+ + +	+ + +	+ + +
	(zona spongiosa)	–	–	–
Late pregnancy	Amniotic epithelia	+	+	–
Term ($n = 8$)	Chorionic cytotrophoblast	+ +	–	–
	Decidua (parietal)	+ + +	+ + +	+ + +

* Staining intensity on an arbitrary scale.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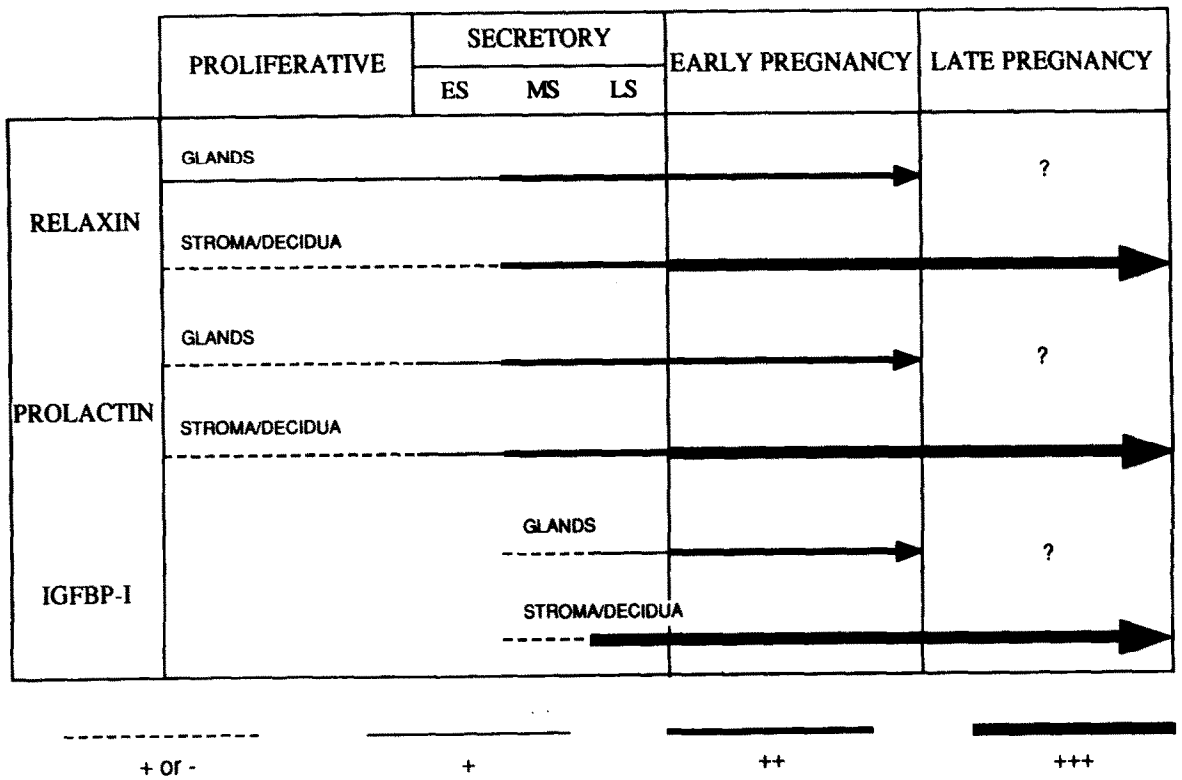

Fig. 2. Diagrammatic representation of the intensity of immunostaining in the glandular epithelium and stroma/decidua for RLX, PRL and IGFBP-1 through the menstrual cycle into early and late pregnancy. Samples of glandular tissue were unobtainable in late pregnancy and shown as ? The thickness of the line relates to the intensity of stain and the dashed line shows where staining was either negative or only weakly positive.

In the early pregnancy decidua, the antibodies to RLX, PRL and IGFBP-1 gave similar patterns of localization (Table 1). All three proteins were localized in the decidualized stromal cells of the zona compacta region, whereas the non-decidualized stromal cells in the zona spongiosa remained unstained. The intensity of the staining varied from weak to strong in different tissues and different areas of the same sample, but were similar for all three peptides in each specific area of each specimen. In seven out of 10 decidual samples, RLX, PRL and IGFBP-1 were also localized in some of the glandular epithelial cells in addition to decidualized stromal cells. The glands were either negative for all three proteins or they were positive for all of them. An example of an early pregnancy tissue is shown immunostained for RLX (Fig. 1G), showing the well stained decidual cells. The staining pattern for all three peptides was the same in specimens obtained from patients with a blighted ovum (not shown) as well as those from early pregnancy termination.

At term pregnancy, the decidual cells in the parietal decidua attached to the chorion laeve stained for all three proteins as previously demonstrated (Sakbun et al., 1990; Ali et al., 1991; Rutanen et al., 1991b). The cytoplasmic staining of the decidua and chorionic cytotrophoblast for RLX is shown in Fig. 1H. The chorionic cytotrophoblast of the chorion laeve showed no staining with the antibodies to either PRL or IGFBP-1,

Fig. 1. Immunolocalization of human RLX to the endometrium/decidua as a function of the menstrual cycle and pregnancy. All sections were counterstained with hematoxylin. (A) Proliferative phase endometrium: predominant staining in the epithelial cells lining the glands and very light stromal cell staining is shown with the polyclonal antibody to processed human RLX. ×150. (B) Shows a control serial section of A stained with the same concentration of goat IgG in place of the primary antiserum. ×150. (C) A section of the same block used in A and B has been immunostained with a monoclonal antibody to the C-peptide of human RLX. The same cellular pattern of staining as in A is shown. ×150. (D) Shows a serial section of C stained with the same concentration of a control monoclonal antibody and shows no staining. ×150. (E) Late secretory phase endometrium: increased staining in the epithelial gland cells and the epithelium of the uterine lumen (not shown), the decidualized stroma also shows increased staining in this cross-section stained with the polyclonal RLX antibody. ×250. (F) Reduced staining is evident when a serial section of E was stained with the polyclonal antibody to human RLX which had been preabsorbed with 10 ng of H2 RLX. ×250. (G) Early pregnancy decidua: the stroma in this section stained with the polyclonal antibody to RLX and shows the cytoplasmic staining in the cells of the zona compacta region. ×150. (H) Late pregnancy amnion, chorion and decidual roll: the cytoplasm of the amnion (a), chorionic cytotrophoblast (c) and decidual (d) cells show dense staining for RLX with the polyclonal antibody to RLX. ×220.

but stained with both antibodies to RLX as previously reported (Sakbun et al., 1990). There were also differences in the staining of the amniotic epithelium, which stained for RLX and PRL, but not with the antibody to IGFBP-1. There were no differences in the staining pattern between tissues collected before and after labor and delivery. An attempt to summarize the intensity and timing of appearance of immunostaining for RLX, PRL and IGFBP-1 in the glandular epithelium and stroma/decidua through the cycle into early pregnancy and at term gestation is shown in Fig. 2. This shows the sequential appearance of RLX in the glandular and luminal epithelium, followed by PRL in the glands and stroma and IFGBP-1 almost exclusively in the decidualized stroma.

Discussion

This is the first study to show that RLX is probably produced in specific sites in the endometrium/decidua of the menstrual cycle, and in these sites in early and late pregnancy. PRL and IGFBP-1 have been separately immunolocalized previously in these tissues (Ali et al., 1991; Rutanen et al., 1988, 1991a,b). However, in this study, these proteins were immunolocalized simultaneously and their appearance correlated with that of RLX. The results are important because they provide an in vivo basis for several studies conducted on separated endometrial cells in long term culture, in which functional relationships between RLX, PRL, IFGBP-1 have been sought (Huang et al., 1987; Thrailkill et al., 1990; Bell et al., 1991; Tseng et al., 1992).

The immunolocalization of RLX to the glandular and luminal epithelium in the proliferative phase of the cycle precedes and may induce the appearance of PRL and IGFBP-1. It has recently been shown that treatment of endometrial cells in vitro with RLX alone stimulated the production of both PRL and IGFBP-1 mRNAs by these cells (Tseng et al., 1992). The source of RLX mediating such effects in vivo has not been addressed in these studies. Because the cyclic corpus luteum expresses the H2 RLX gene (Hansell et al., 1991) which is translated into detectable protein (Stoelk et al., 1991) and because RLX levels in the systemic circulation are detectable by the midluteal phase (Stewart et al., 1990), it has been assumed that this luteal relaxin targets the endometrium. However, the results presented here show an additional local source of RLX in the cycle. Its production is suggested by co-localization of its C-peptide, since this is not a part of the processed hormone and should not be localized to cells sequestering or binding RLX. The identical results obtained with this antibody and one to the processed hormone indicate production as opposed to its sequestration from the luteal source. It is not known whether conversely the endometrial RLX contributes to the levels detected in serum in the menstrual cycle (Stewart et al., 1990); neither is it known whether both the RLX genes are expressed and translated in this tissue, as the antibodies currently available are unable to distinguish these proteins.

Towards the middle of the secretory phase of the cycle, as ovarian progesterone levels rise, the synergistic effects of RLX and progesterone may further stimulate stromal cell differentiation. This in turn, would induce additional local RLX from the differentiated stromal cells, and together with the steroids could bring about incremental increases in the secretion of RLX, PRL and IGFBP-1. In the event of pregnancy, this would continue. Indeed, studies in vitro have shown a synergistic action of RLX and progesterone to stimulate PRL and IGFBP-1 gene expression in endometrial stromal cells (Tseng et al., 1992). It was noted in these studies, that the actions of RLX and progesterone appeared different, that progesterone caused cell growth and RLX enhanced its ability to increase cell mass and differentiate the stromal cells (Tseng et al., 1992).

Thus, the sequential appearance demonstrated here in vivo, of RLX, PRL and IGFBP-1 in endometrium suggests differential regulation for each protein in the proliferative phase of the cycle. This appears to become coordinated by the mid secretory phase, coincident with the differentiation of areas of stroma seen as areas of cells which immunostained for all three proteins. This degree of coordination was intensified in early pregnancy as the steroid milieu changed.

This study attempts to link the in vivo status of endometrial tissue for three locally produced proteins with contemporary in vitro findings and has been limited to time points in the reproductive cycle when human endometrium is available. The results suggest that applications of a variety of techniques to this tissue may allow the complex interrelationships between autocrine/paracrine and endocrine messages to be further defined. This will lead to a better understanding of the role of the endometrium/decidua in implantation as well as in parturition.

Acknowledgements

This work was supported by Sigrid Juselius Foundation and the Cancer Society of Finland, and NIH Grant HD-24314, a grant to the University of Hawaii (G12-RR-03061) under the Research Centers in Minority Institutions Program of the NIH, and TKC is a MARC undergraduate scholar supported by a grant GM07684.

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