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A Baboon (*Papio anubis*) simulated-pregnant model: cell specific expression of insulin-like growth factor binding protein-1 (IGFBP-1), type I IGF receptor (IGF-1 R) and retinol binding protein (RBP) in the uterus*

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In order to test the hypothesis that the baboon conceptus/placenta regulates the synthesis of specific proteins in the endometrium, we developed a simulated-pregnant baboon model. Baboons ($n = 2-6$/group) were treated with increasing amounts of human chorionic gonadotrophin (hCG) for 10 or 12 days beginning on day 6 or 7 PO. Uterine tissues were obtained at day 18 PO following 12 days of hCG treatment. Animals in the day 25 and 32 PO group were treated for 10 days with hCG. Following the hCG treatment, estradiol (E) and progesterone (P) implants were inserted subcutaneously. Control groups consisted of E and P treatment only (day 25 PO), or ovariectomy on day 6 or 7 PO followed by hCG plus E and P treatment (days 18 and 25 PO). Serum samples were obtained daily or once every 2 days and analysed for E and P by radioimmunoassay. hCG activity in serum was determined by a Leydig cell bioassay. Portions of the endometrial tissue were either subjected to organ explant culture, analysed by immunocytochemistry or extracted for RNA. Peripheral serum levels of hCG, E and P in the experimental groups fell within the 95% confidence interval limits of hormone concentrations achieved during pregnancy. The morphology of the endometrium in the hCG treated baboons and pregnant baboons was similar i.e., distended convoluted glands, many spiral artery beds, a loose vacuolized stroma, and increased collagen staining. However, in the absence of hCG (E + P treatment only) the glands tended to be straight rather than corkscrew-shaped, and decreased stromal vacuolization and collagen staining was evident. ^{35}S-methionine labeled proteins in explant culture conditioned media (TCM) were analysed by two-dimensional SDS–PAGE and fluorography. A comparable pattern of protein synthesis was apparent in all treatment groups except for a low molecular weight (27 000–30 000 daltons) group of polypeptides which only was evident in TCM from the hCG treated baboons. A similar group of proteins are also secreted by the baboon endometrium during pregnancy. The immunocytochemical localization of estrogen (ER) and progesterone receptors (PR) was comparable to that observed in pregnant baboons. IGFBP-1 localization was confined to the glandular epithelium in the hCG treated groups (intact and ovariectomized) and was virtually undetectable in the E and P treated group. The intensity of IGFBP-1 staining was variable within each of the hCG treatment groups on days 18, 25 and 32 PO. This variability was also apparent by Western blot analysis, immunoassay of proteins in TCM and on Northern blots of total RNA from the same animals. In contrast, IGF-I R immunostaining was evident in both glandular and surface epithelium of all treatment groups. Expression of RBP was confined to the basal glands. The characteristic upregulation of RBP synthesis in the functionalis observed during early pregnancy was not apparent in any of the treatment groups. In summary, these studies indicate that exogenous hCG in conjunction with E and P, can induce the general morphological and biosynthetic changes the baboon endometrium undergoes during early pregnancy. In addition, this hormonal treatment is also capable of maintaining the epithelial expression of IGFBP-1, IGF-1 and RBP. However, other factors from the conceptus appear to be necessary to induce the cell specific changes in the expression of these three proteins that are observed during pregnancy.

Keywords: baboon; simulated-pregnancy; uterus; morphology; proteins; insulin-like growth factors

Introduction

Complex interactions between the developing embryo and the maternal endometrium occur during implantation. The morphological changes that take place during the establishment of pregnancy in the human have been described to a limited extent in the classic studies of Hertig & Rock (1949) and more extensively in the rhesus monkey and baboon (Enders & Schlafke 1986; Enders, 1993). In order to correlate morphological changes in the primate endometrium with specific secretory changes, we have focussed our efforts on characterizing the proteins synthesized by the baboon uterus during the menstrual cycle and early pregnancy.

Our *in vivo* studies on the baboon endometrium indicate that there are overall increases in protein biosynthesis (Fazleabas *et al.*, 1993a), cell-specific changes in IGFBP-1 expression (Fazleabas *et al.*, 1989; Tarantino *et al.*, 1992), up-regulation of RBP synthesis (Fazleabas *et al.*, 1994), and alterations in estrogen and progesterone receptor localization and IGF-I R expression during early pregnancy (Hild-Petito *et al.*, 1992, 1994). Of all these changes, only IGFBP-1, IGF-I R and RBP localization and expression are markedly different between the implantation site and non-implantation site, suggesting that the baboon conceptus/placenta regulates the cellular expression of these proteins within the uterus. These changes parallel two distinct periods of placental development in the baboon (Hendrickx, 1971). Between days 18 and 25 of pregnancy (8–15 days post-implantation), transformation of stromal cells to decidual cells and trophoblast penetration is associated with IGFBP-1 synthesis at the implantation site and subsequently at adjacent sites (Tarantino *et al.*, 1992; Fazleabas *et al.*, 1993b). Establishment of a definitive placenta and completion of organogenesis in the fetus, which occurs between days 25 and 40 of pregnancy (Hendrickx, 1971), coincides with the peak of RBP synthesis (Fazleabas *et al.*, 1994).

Estradiol and progesterone, are involved in endometrial development during the menstrual cycle (Brenner & Slayden,

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1994) and are essential for maintenance of pregnancy (Tullner, 1984; Ravindranath & Mougdal, 1987, 1990). In addition, chorionic gonadotropin (CG) from the trophoblast may act directly on the endometrium to regulate pregnancy-induced endometrial changes in the primate (Arias-Stella, 1954).

However, the potential interactions of steroid and trophoblast hormones on the differentiation of the primate endometrium during pregnancy have not be investigated. Thus, the objectives of this study were: (1) to design a baboon model to stimulate the hormonal environment of early pregnancy in order to determine the role of estradiol, progesterone, CG and other potential trophoblast or ovarian factor(s) in regulating the development of the endometrium of pregnancy, and (2) to elucidate the role of conceptus factors (i.e., CG or other factors) in regulating the cell specific expression of IGFBP-1, IGF-I R and RBP at the site of implantation.

Results

Serum hormone levels

Figure 1 depicts the hormonal treatments used in the simulation model. The peripheral serum levels of hCG, estradiol, and progesterone in baboons during simulated pregnancy are depicted in Figure 2 and fall within the 95% confidence intervals of pregnancy values. Serum levels of hCG increased during the treatment interval and were not different from baboon (b) CG levels during pregnancy. Estradiol levels increased, peaking on day 18 PO of simulated pregnancy. A similar pattern was observed during pregnancy although estradiol levels continued to rise after day 25 PO in pregnant baboons. Progesterone levels remained elevated during simulated pregnancy and were comparable to levels measured in pregnant baboons up to day 25 PO. The dramatic increased in estradiol and progesterone levels after day 25 PO in pregnant baboons represents the contribution of increasing placental steroidogenesis. The serum levels of estradiol and progesterone in steroid treated baboons were also within the 95% confidence intervals for pregnant baboons (data not shown).

Morphology

During pregnancy, the endometrium demonstrated a characteristic appearance based on Gimori trichrome staining of paraffin sections. The morphological changes observed in the four major treatment groups (pregnancy, simulated pregnancy, ovariectomized simulated pregnancy and steroid treat-

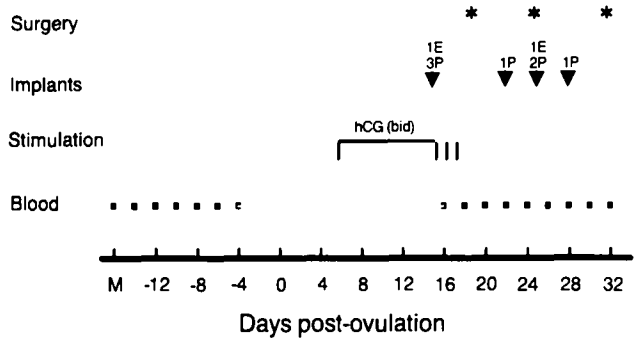

Figure 1 Experimental Design. Nonpregnant baboons were bled from menses until surgery as indicated. The day of ovulation (day 0) was designated at 48 h after the estradiol surge. Baboons received increasing doses of hCG for 10 days beginning on day 6 or 7 PO. Tissues were removed on day 18 PO from hCG treated baboons that received two additional days of hCG. The other hCG-treated (10 days) baboons received estradiol (E, 6 cm) or progesterone (P, 6 cm) implants on the indicated days (number indicates number of implants inserted). Surgery was performed on days 18, 25 or 32 PO. Steroid only treated baboons received no exogenous hCG and implants were inserted beginning on day 6 PO

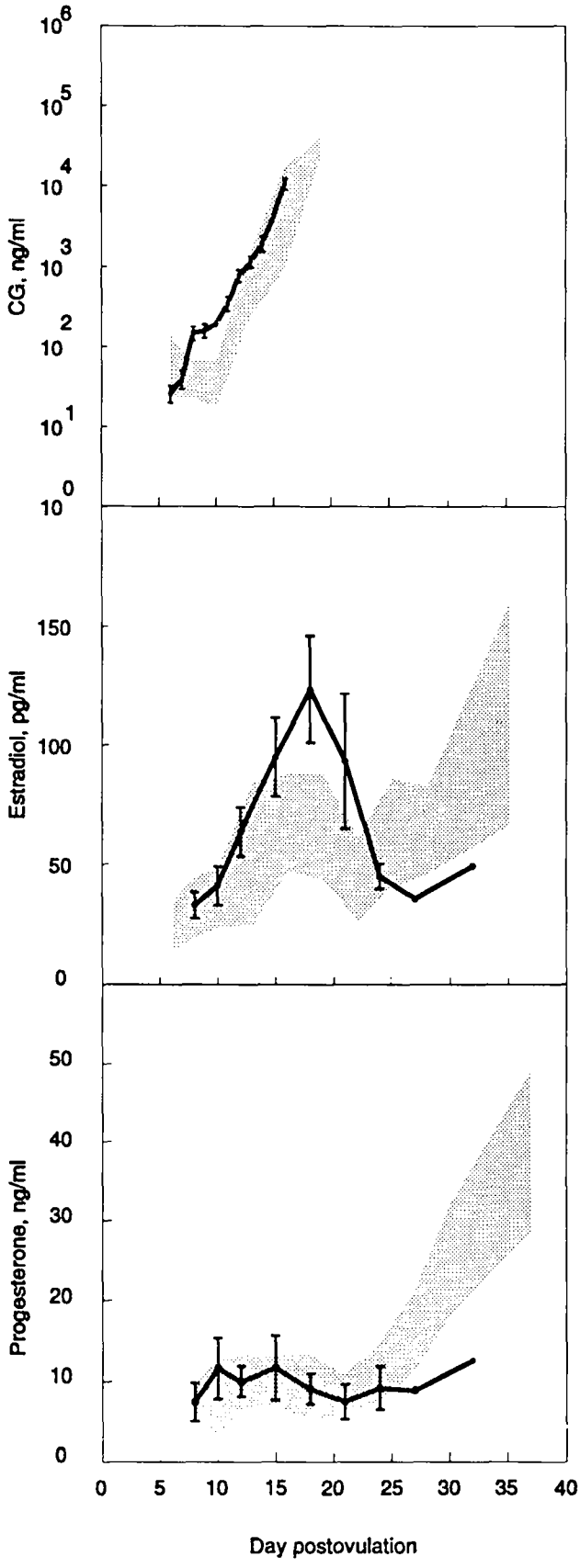

Figure 2 Peripheral serum levels of CG (ng/ml), estradiol (pg/ml) and progesterone (ng/ml) in baboons during simulated early pregnancy. Points represent X ± SEM ($n = 3-8$). Shaded area represents the 95% confidence interval for pregnant baboons ($n = 10$). Note that the increase in estradiol and progesterone after day 25 PO in pregnant baboons represents placental biosynthesis

Table I Morphological characteristics of the endometrium from pregnant baboons and hormone treated baboons

Region of the Endometrium	*Morphological Characteristics*	*day 18–32 pregnancy*	*day 18–32 simulated pregnancy*	*day 18–25 ovariectomized, simulated pregnancy*	*day 25 steroid treatment only*
Upper Functionalis	glands	distended, convoluted	distended, convoluted	distended, convoluted	smaller, distended, straight
	epithelium	cuboidal	cuboidal	cuboidal	taller cuboidal
	stroma	loose	loose	loose	compact
	vacuolization	abundant	abundant	abundant	abundant
	'pre-decidual' or decidual cells	pre-decidual cells present, some decidual cells by day 25 PO	pre-decidual cells present	pre-decidual cells present	pre-decidual cells present
	collagen/ECM	abundant	abundant	slightly less abundant	some
	sprial arteries	some beds of spiral arteries	some beds of spiral arteries	some beds of spiral arteries	some beds of spiral arteries
Lower Functionalis	glands	distended, convoluted	distended, convoluted	distended, convoluted	distended, straight
	epithelium	cuboidal	cuboidal	cuboidal	cuboidal
	stroma	loose	loose	loose	loose
	vacuolization	abundant	abundant	slightly less abundant	slightly less abundant
	'pre-decidual' or decidual cells	few pre-decidual and decidual cells around spiral arteries	few pre-decidual around spiral arteries	few pre-decidual around spiral arteries	few pre-decidual around spiral arteries
	collagen/ECM	abundant	abundant	slightly less abundant	abundant
Basalis	glands	distended, convoluted	distended, convoluted	distended, convoluted	distended, convoluted
	epithelium	columnar	columnar	columnar	columnar
	stroma	loose	loose	loose	loose
	vacuolization	abundant	abundant	abundant	abundant
	'pre-decidual' or decidual cells	none present	none present	none present	none present
	collagen/ECM	present	present	present	present
	spiral arteries	abundant	abundant	abundant	abundant

ment only) are summarized in Table 1. Throughout the endometrium, large deposits of extracellular matrix (ECM), particularly collagen, were observed, and the glands were distended and convoluted (Table 1 and Figure 3A). In the functionalis the glandular epithelium was low cuboidal whereas glands of the basalis tended to be columnar. The stroma was generally loose with evidence of vacuolization and contained many beds of spiral arteries. Decidualized stromal cells (large spherical cells with a large nucleus) were present by day 25 of pregnancy at the implantation site and around spiral arteries. The endometrium from hCG treated baboons (simulated pregnancy) demonstrated similar morphological changes (Table 1 and Figure 3C) except that decidualized stromal cells were not present. In contrast, the endometrium from the steroid only treated baboons demonstrated some, but not all of these morphological characteristics (Table 1 and Figure 3E). The upper third of the endometrium had a more compact stroma with very little increase in extracellular matrix and little evidence of vacuolization. In addition, the glands were small and rounded with few convolutions. However, the lower two-thirds of the endometrium contained a loose stroma with increased collagen and vacuolization. The glands were distended, but tended to be straight rather than corkscrew shaped in appearance. The endometrium from ovariectomized, hCG treated baboons was similar to intact hCG treated baboons except for a slight decrease in vacuolization and extracellular matrix formation in the functionalis (Table 1).

Endometrial protein synthesis

The incorporation of [^{35}S]methionine into synthetic polypeptides was similar in all treatment groups (Figure 3; B, D and

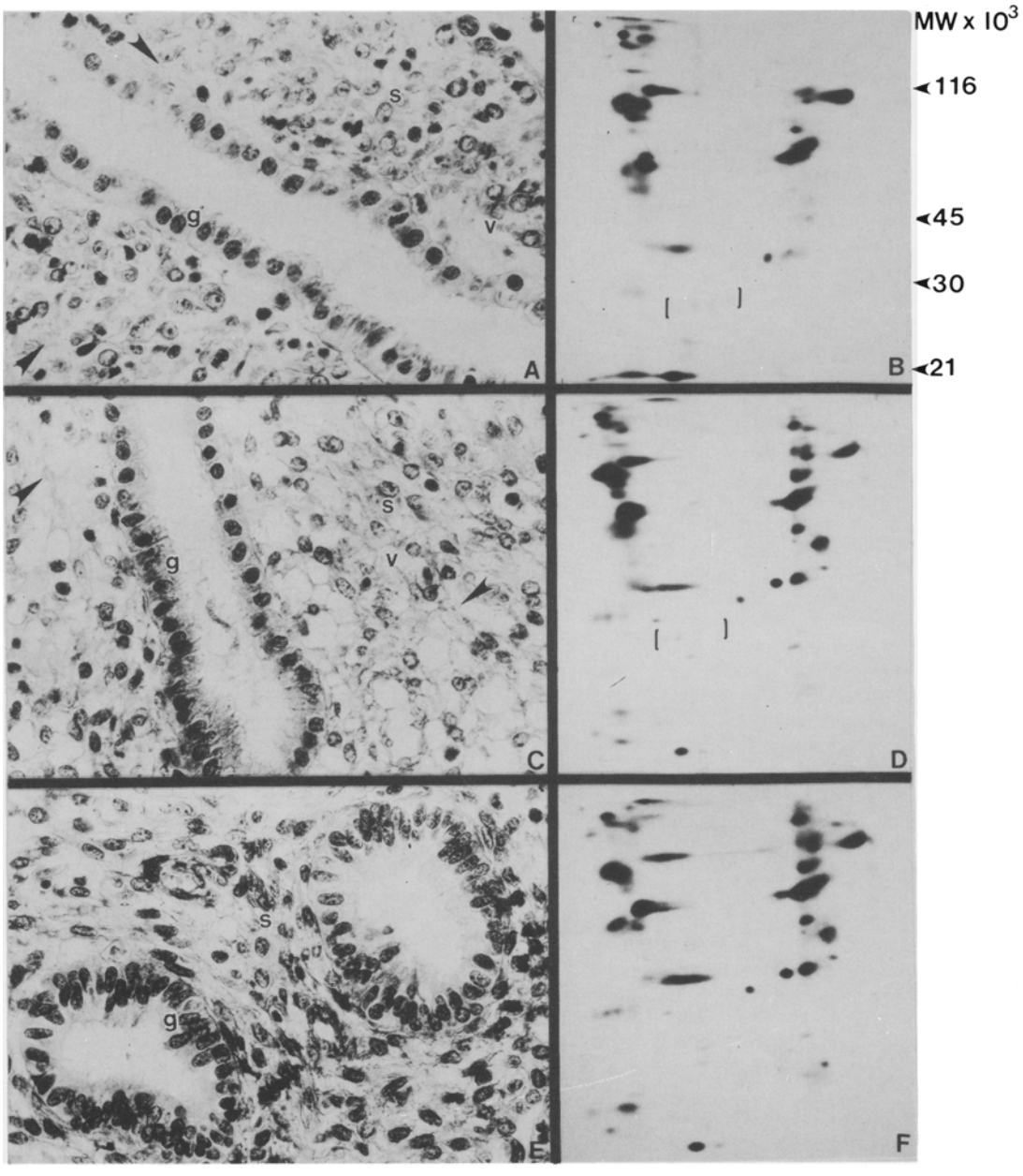

Figure 3 Endometrial morphology and protein synthetic profile on day 25 PO from pregnant (**A** and **B**) hCG treated (**C** and **D**) and steroid only treated (**E** and **F**) baboons. During pregnancy the glands (g) and stroma (s) had a characteristic appearance (**A**) that was mimicked by hCG and steroid treatment (**C**). In contrast, endometrium from steroid only treated baboons (**E**) was similar in appearance to endometrium of the luteal phase. The pattern of endometrial synthetic polypeptides was similar in all animal groups except for the appearance of a group of proteins (bracketed) at a molecular weight of 27 000–30 000 daltons during pregnancy (**D**) and following hCG treatment (**E**). v = vacuolization, extracellular matrix deposits are indicated by arrowheads. **A**, **C** and **D**. Mag × 400

F). The pattern of protein synthesis of all treatment groups was comparable to mid luteal stage tissue and pregnancy (Fazleabas & Verhage, 1987; Fazleabas *et al.*, 1993a). However, a group of low molecular weight (27 000–30 000 daltons), isoelectric variant polypeptides synthesized by the endometrium during pregnancy (Figure 3B, bracketed) were also evident in ^{35}S-methionine labeled tissue culture media (TCM) from hCG treated baboons (Figure 3D, bracketed), but not following steroid treatment alone (Figure 3F). Endometrium from ovariectomized, hCG treated baboons also synthesized this group of low molecular weight proteins (data not shown).

Steroid receptors

Localization of estrogen and progestin receptors (ER and PR, respectively) in baboon endometrium during simulated pregnancy is shown in Figure 4 and summarized in Table 2. ER were not detected in glands and stroma of any region of the endometrium following hCG treatment on days 18, 25 or 32 PO (Figure 4A and Table 2). A similar absence of staining for ER was also observed in steroid treated baboon endometrium (day 25 PO), except for occasional staining in the glandular epithelium (Figure 4C and Table 2). ER was also absent in the glands and stroma of endometrium from pregnant baboons at all time points examined (Table 2, see Hild-Petito *et al.*, 1992). In contrast, PR was present in many stromal cells throughout the endometrium during simulated pregnancy on days 18–32 PO, whereas staining was absent in the glands (Figure 4B and Table 2). Steroid treatment alone (day 25 PO) resulted in a similar staining pattern for PR, except for occasional staining in glandular epithelium (Figure 4D and Table 2). PR staining in stroma, but not glandular

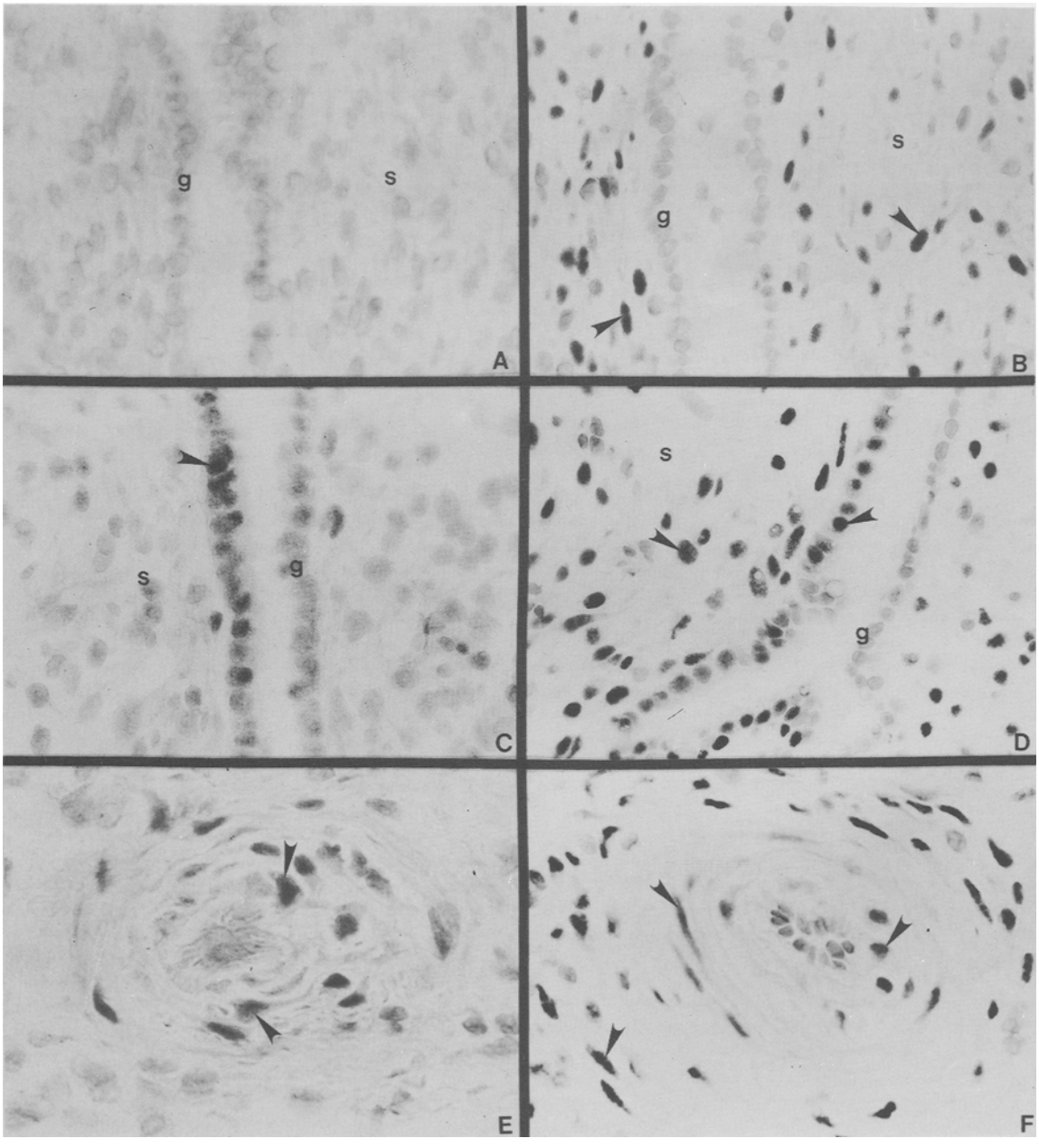

Figure 4 Immunocytochemical localization of ER and PR in hCG treated and steroid-only treated baboons. ER was not detected in glandular epithelium (g) or stroma (s) during simulated pregnancy (**A**). ER was also absent in most glands during steroid treatment, however, a few cells demonstrated positive (arrowheads) nuclear stain (**C**). In contrast, PR was present in the nuclei of many stromal cells (arrowheads), but not glandular epithelium, during hCG treatment (**B**). Likewise. PR was detected in many stromal cells (arrowheads) and a few glandular epithelium (arrowheads) during steroid treatment (**D**). ER were detected in the nuclei (arrowheads, **E**) of smooth muscle cells of spiral arteries during simulated pregnancy and PR were associated with spiral arteries and stromal cells surrounding the spiral arteries (arrowheads, **F**). **A** and **B** day 25 PO hCG and steroid treatment; **C** and **D** steroid treatment only; **E** and **F** day 32 PO hCG and steroid treatment. **A–D** Mag 300 ×, **E** and **F** Mag × 480

epithelium, was observed throughout pregnancy (Table 2, see Hild-Petito *et al.*, 1992). Both ER and PR staining was associated with spiral arteries during simulated pregnancy (Figure 4E and F and Table 2), steroid treatment (Table 2), and pregnancy (Table 2, see Hild-Petito *et al.*, 1992). ER (Figure 4E) appeared to be localized to the smooth muscle

Table 2 Localization of estrogen (ER) and progestin (PR) receptors in the endometrium of pregnant and hormone treated baboons

	day 18 simulated pregnancy[1]		*day 25 simulated pregnancy*[1]		*day 32 simulated pregnancy*		*day 25 steroid treatment only*		*day 18–32 pregnancy*[2]	
Cell type	*ER*	*PR*	*ER*	*PR*	*ER*	*PR*	*ER*	*PR*	*ER*	*PR*
Glands	0	0	0	0	0	0	±	±	0	0
Stroma	0	+	0	+	0	+	0	+	0	+
Spiral arteries[3]	+	+	+	+	+	+	+	+	+	+

[1]Ovariectomy had no effect on ER and PR localization in baboons treated with hCG and steroid hormones. [2]No differences were detected on days 18–32 of pregnancy. Therefore, these groups were combined, see Hild-Petito *et al.* (1992) for details. [3]ER staining was present in SMC, whereas PR staining was associated with fibroblasts. 0 = no specific staining. + = positive staining. ± = occasional staining

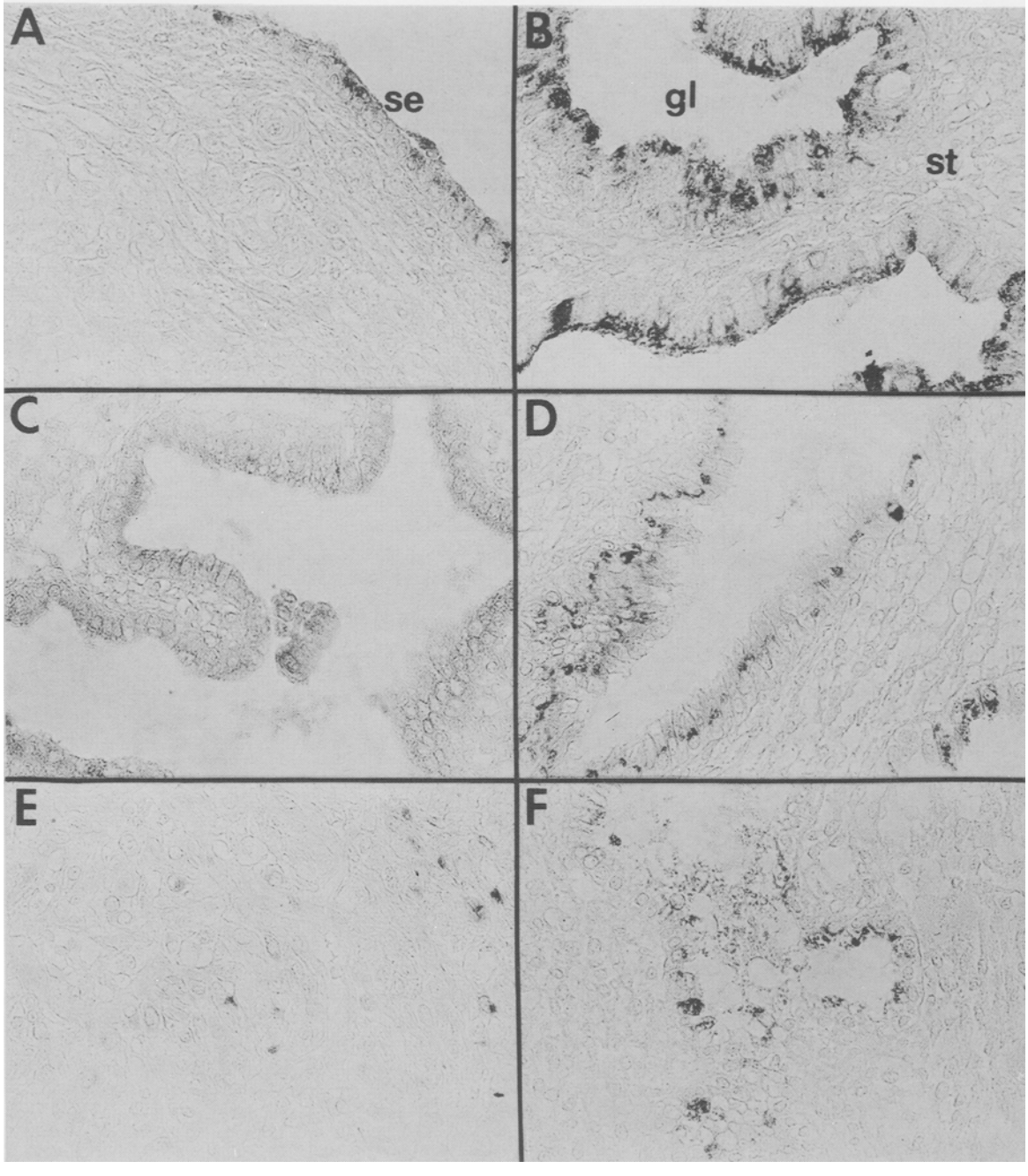

Figure 5 Immunocytochemical localization of IGFBP-1 in endometrial tissues from hCG (**A**, **B**, **D**, **E** and **F**) and steroid only treated (**C**) baboons. (**A** and **B**) surface epithelium and glandular epithelium, day 18 PO, hCG only; (**C** and **D**) glandular epithelium, day 25 PO; estradiol and progesterone treated (**C**), and hCG and steroid treated (**D**); (**E** and **F**) surface epithelium and glandular epithelium, day 32 PO, hCG and steroid treated. Note the presence of immunoreactive IGFBP-1 in the glandular epithelium of only the hCG and estradiol plus progesterone treated baboons and the absence of stromal staining at the luminal surface or around spiral arteries (**A** and **E**). se = surface epithelium; gl = glands; st = stroma. Mag × 290

cells of the spiral arteries, whereas PR (Figure 4F) was associated with fibroblasts of the spiral artery or endometrial stromal cells.

Insulin-like growth factor binding protein-1

Figure 5 shows the immunocytochemical localization of IGFBP-1 in the functionalis and basalis regions of the endometrium with and without hCG treatment. Apical staining of the surface (Figure 5A) and glandular (Figure 5B) epithelium is observed at day 18 PO following hCG treatment, whereas there was limited induction of IGFBP-1 in the absence of hCG on day 25 PO (Figure 5C). In hCG-treated animals, IGFBP-1 immunoreactivity continued to be present in the glandular epithelium at days 25 and 32 PO (Figure 5D and F). In ovariectomized baboons treated with hCG, the staining pattern for IGFBP-1 was similar to that observed in other hCG treated animals at comparable time points (data not shown). However, in contrast to pregnant baboons, stromal staining for IGFBP-1 in the upper functionalis was not evident in any of the treatment groups. The limited and variable immunoreactivity for IGFBP-1 in the endometrial glands of non-pregnant hCG treated baboons was also reflected in the ligand and Western Blot analysis of explant culture media (Figure 6A and B). IGFBP-2 and 4 were the major binding proteins detectable on ligand blots in all treatment groups (Figure 6A), while IGFBP-1 synthesis, presumably of glandular origin, was only detectable by Western immunoblotting in some animals in the hCG treated groups on days 25 and 32 PO (lanes 2 and 4; Figure 6B). Long term steroid treatment (day 25 PO) in the absence of hCG failed to maintain IGFBP-1 synthesis (lanes 10 and 11, Figure 6B). Similar results were obtained when an immunoradiometric assay for IGFBP-1 was performed on explant culture media (Figure 7). Immunoreactive IGFBP-1 was readily detectable in the functionalis explants from hCG treated baboons. This region contains the majority of the glandular tissue. The steroid-only treated group showed minimal synthesis. In addition, the variability in glandular production of IGFBP-1 between animals seen on the Western blots was also reflected in this assay. Northern blot analysis of total RNA from the functionalis region of the endometrium (Figure 8) confirmed that the presence of IGFBP-1 mRNA was associated with the presence of the protein detected by Western blots and by immunoassay. IGFBP-1 mRNA expression was also variable, however there was a correlation in the mRNA expression and IGFBP-1 protein for each animal. IGFBP-2 mRNA expression in all treatment groups was similar and corresponded to the ligand blot analysis (data not shown).

Type 1 insulin-like growth factor receptor

The immunocytochemical localization of IGF-I R is shown in Figure 9 and summarized in Table 3. As was observed with IGFBP-1, immunoreactivity was limited to the glandular and surface epithelium of the endometrium throughout simulated-pregnancy (days 18–32 PO). However, unlike IGFBP-1 the epithelial staining pattern observed was similar in all hormone treated groups (Table 3) and appeared to be independent of hCG treatment. In pregnant baboons, epithelial staining was markedly reduced and stromal staining was apparent at the implantation site by day 18 PO (Table 3, see Hild-Petito *et al.*, 1994). This is in contrast to the simulated-pregnant baboons where glandular epithial staining was maintained throughout the treatment period and stromal staining was absent.

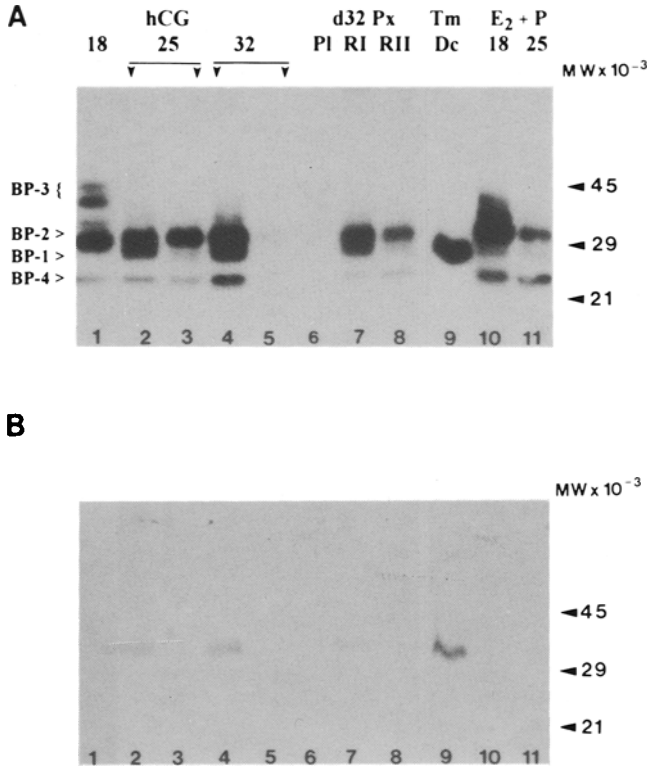

Figure 6 Ligand (**A**) and Western (**B**) blots of TCM proteins (25 μg). Lanes 1–5 are from hCG treated baboons, lanes 6–8 are placenta, implantation site and non-implantation site media from a day 32 pregnant baboon, lane 9 is 1 μg of term decidua media and lanes 10 and 11 are steroid only treated baboons. The same membrane was first probed with [^{125}I]IGF-I and then subjected to Western blot analysis using an IGFBP-1 monoclonal antibody. The IGFBPs on the ligand blots are identified based on their electrophoretic mobilities. Note that IGFBP-2 and 4 are the primary IGFBPs seen in ligand blots of all samples, except term decidua (lane 9). Immunoreactive IGFBP-1 was variable in hCG treated baboons (lanes 2 and 4; **B**) but present in implantation site (lane 7) and term decidua (lane 9) media. No immunoreactive IGFBP-1 was present in media from long term steroid only treated baboons (lanes 10 and 11)

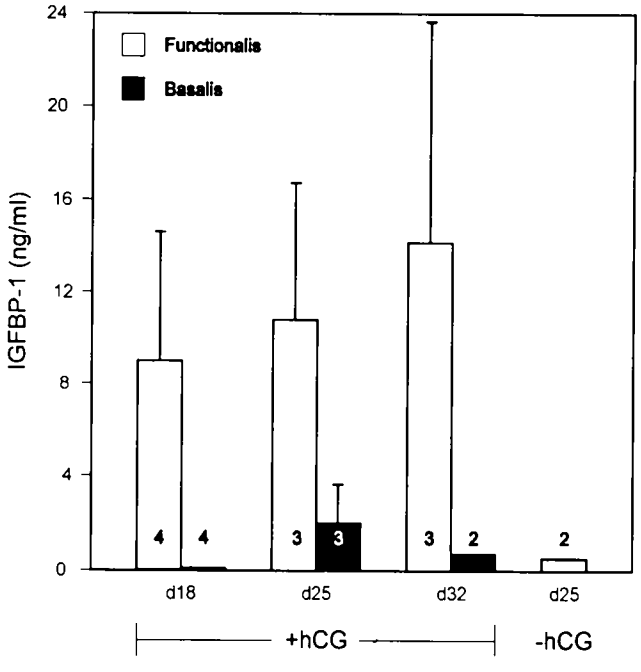

Figure 7 Immunoreactive IGFBP-1 (X ± SEM) in TCM of baboon endometrium ± hCG treatment. Note that the majority of immunoreactive IGFBP-1 in present in the functionalis media of hCG treated baboons. This region contains the majority of the glandular tissues present within the endometrium. The numbers within the bars indicate the number of animals assayed in each treatment group

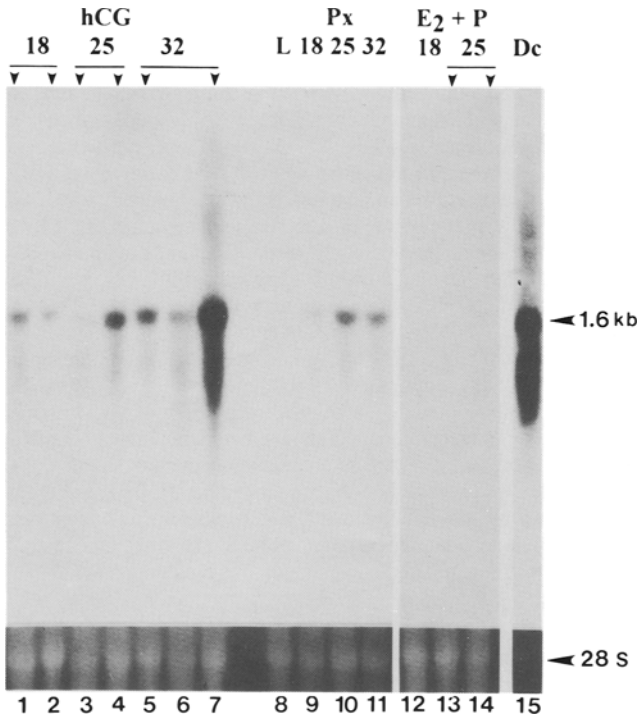

Figure 8 Northern blot of total endometrial RNA (20 μg) hybridized with a 1.2 kb human IGFBP-1 cDNA probe. Note the variable expression of mRNA in the hCG treated baboons (lanes 1–7) which was comparable to the immunoreactive data shown in Figures 5, 6B and 7. mRNA from steroid only treated baboons showed no hybridization (lanes 12–14). The increase during pregnancy (lanes 9–11) and the high expression in term decidua (lane 15; 1 μg total RNA) is readily apparent. Lane 8 is a late luteal sample

Retinol binding protein

In hCG treated, simulated-pregnant baboons basal expression of RBP in the endometrium was maintained in both the presence (Figure 10D and E) or absence of the ovaries (data not shown). However, the up-regulation in the functionalis region observed during pregnancy was not evident in the absence of a conceptus (Figure 10A and B). Endometrium from steroid treated baboons showed faint staining for RBP in the basal grands (Figure 10F).

The immunocytochemical data for RBP was further substantiated by Western and Northern Blot analysis. Figure 11 shows the limited secretion of RBP by the functionalis region (Figure 11A) compared to the basalis region (Figure 11B) of the endometrium throughout the treatment period in both the hCG plus steroid and steroid only treated groups. Northern Blot analysis of total RNA from the functionalis region demonstrated that the decrease in protein synthesis was associated with reduced mRNA expression (Figure 12).

Discussion

In previous studies we demonstrated that the baboon endometrium undergoes dynamic changes in secretory activity and protein expression during early pregnancy (ie: days 18–32 PO; Fazleabas *et al.*, 1993a). Changes associated with IGFBP-1, IGF-I R and RBP were specifically induced at the site of implantation (Tarintino *et al.*, 1992; Fazleabas *et al.*, 1994; Hild-Petito *et al.*, 1994), while changes in protein biosynthesis and steroid receptor localization appeared to be similar at both the implantation site and non-implantation sites (Hild-Petito *et al.*, 1992; Fazleabas *et al.*, 1993b). In order to more clearly define the role of the conceptus in modulating these changes, we developed a baboon simulated-pregnancy model which differentiates between CG and steroid hormone effects on the endometrium from other conceptus-induced changes. The results from this study suggest that exogenous hCG and steroid treatment can induce the morphological and general secretory changes observed during pregnancy. The presence of hCG appears to be a prerequisite since continuous treatment with estrogen and progesterone alone do not induce similar changes. The effects of CG appear to be directly on the primate endometrium since CG/LH receptors have been detected in the glandular epithelium during the luteal phase (Reshef *et al.*, 1990), and ovariectomy followed by hCG treatment had similar results. The effects of CG on endometrial function are presumably modulated by cAMP, which in turn can regulate steroid hormones during pregnancy (Hussa *et al.*, 1980, Feinman *et al.*, 1986, Benoit *et al.*, 1988).

The generalized effects of hCG on the morphology, secretory activity and steroid receptor localization of the non-pregnant baboon endometrium are comparable to those observed in pregnancy. However, the cell-specific changes in IGFBP-1, IGF-I R and RBP expression at the implantation site appear to require additional conceptus signals. Similar differences in expression at the implantation site compared to the non-implantation site have been reported for basic fibroblast-growth factor in the rat uterus (Carlone & Rider, 1994) and leukemia inhibitory factor in the rabbit uterus (Yang *et al.*, 1994). Furthermore, differences in uterine epidermal growth factor receptor gene expression between pregnant and ovariectomized, steroid-treated mice also suggest that the blastocyst influences cell-specific gene expression in the uterus (Das *et al.*, 1994a,b). However, as we have noted in our studies in the baboon (Fazleabas *et al.*, 1991, 1993b) and as with previous studies in the mouse (McMaster *et al.*, 1991), not all of the uterine proteins are directly influenced by the conceptus. Interestingly, the genes whose expression appears to be influenced by pregnancy in the primate (this study) and rodent are either growth factors, their receptors or binding proteins (Kapur *et al.*, 1992; Das *et al.*, 1992, 1994a,b). These data add support to the hypothesis that growth factors and their receptors are necessary for uterine cell proliferation and differentiation during the establishment of pregnancy (Pollard, 1990).

Decidualization, or uterine stromal cell differentiation, is an essential feature of pregnancy in rodents and primates. Decidualization of stromal fibroblasts is associated with cellular proliferation followed by differentiation. Our *in vivo* studies (Tarantino *et al.*, 1992; Fazleabas *et al.*, 1993b) suggested that the baboon conceptus plays and obligatory role in inducing decidualization. This study substantiates this hypothesis since hormonal treatments alone were insufficient to induce the cell specific changes observed during pregnancy. Following implantation, the stromal fibroblasts undergo extensive modifications to form decidual cells in baboons (Enders and Schlafke; 1986) and their transformation in the baboon is associated with the sequential expression of α smooth muscle actin, IGF-I R and IGFBP-1 (Tarantino *et al.*, 1992; Fazleabas *et al.*, 1993b; Christensen *et al.*, 1994; Hild-Petito *et al.*, 1994).

We postulated that IGFBP-1 complexed with IGFs at the implantation site may provide a mechanism to locally enhance the growth promoting effects of IGFs at the fetal/maternal interface in the baboon (Fazleabas *et al.*, 1991, 1993b). These effects are proposed to be mediated by the IGF-1 receptor whose expression is coincident with IGFBP-1 expression in the luminal and glandular epithelium and stromal cells in early pregnancy (Hild-Petito *et al.*, 1994). The question that remains to be resolved is the nature of the conceptus factor(s) that induces these cellular differentiations. One potential candidate could be IGF-II. Studies in the human by Ohlsson *et al.* (1989) demonstrated that high steady state expression of IGF-II mRNA by human trophoblast is a post-implantation event and is correlated with the

formation and/or expansion of the cytotrophoblastic shell and decidualization. The possible mechanism by which IGF-II may potentiate the cellular differentiation of stromal fibroblasts via the IGF-I R is by disrupting the IGFBP barrier at the cell surface (Conover *et al.*, 1994) and thereby increasing the ligand-receptor interaction. It is important to note that the IGF/IGF-I R/IGFBP complex could also synergize with other growth factors to induce stromal cell proliferation and differentiation. Epidermal growth factor, transforming growth factor-α and the epidermal growth factor receptor are expressed together with IGF-I R and IGFBP-1 at the implantation site in the baboon (Slowey *et al.*, 1994). In the mouse uterus epidermal growth factor and IGF-I regulate epithelial-stromal interactions in a paracrine manner and human trophoblast cell differentiation induced by IGF-I is potentiated by epidermal growth factor (Bhaumick *et al.*, 1992; Hana & Murphy, 1994).

Progesterone is essential for IGFBP-1 and RBP synthesis during the menstrual cycle (Fazleabas *et al.*, 1989, 1994). However, during pregnancy and simulated-pregnancy the production of these two proteins does not appear to be solely regulated by progesterone. Since progesterone receptor was absent in all glandular epithelial cells that stained for IGFBP-1 and RBP and long-term treatment with estrogen and progesterone was insufficient to maintain glandular synthesis, progesterone may serve a permissive role during pregnancy. Chorionic gonadotrophin, acting directly on endometrial receptors on the glandular epithelium (Reshef *et al.*, 1990) may enhance and/or maintain the synthesis of these two proteins in the short term. Following the termination of hCG injections, and in the absence of additional conceptus factors, glandular synthesis diminishes in spite of estrogen and progesterone being maintained at levels comparable to pregnancy.

In summary, using a simulated-pregnant baboon model we have demonstrated that the combination of hCG and steroid hormones induces morphological changes in the endometrium that are comparable to pregnancy. In addition CG can act directly on the endometrium to induce the synthesis of specific proteins (M_r 27–30 000), and maintain the glandular

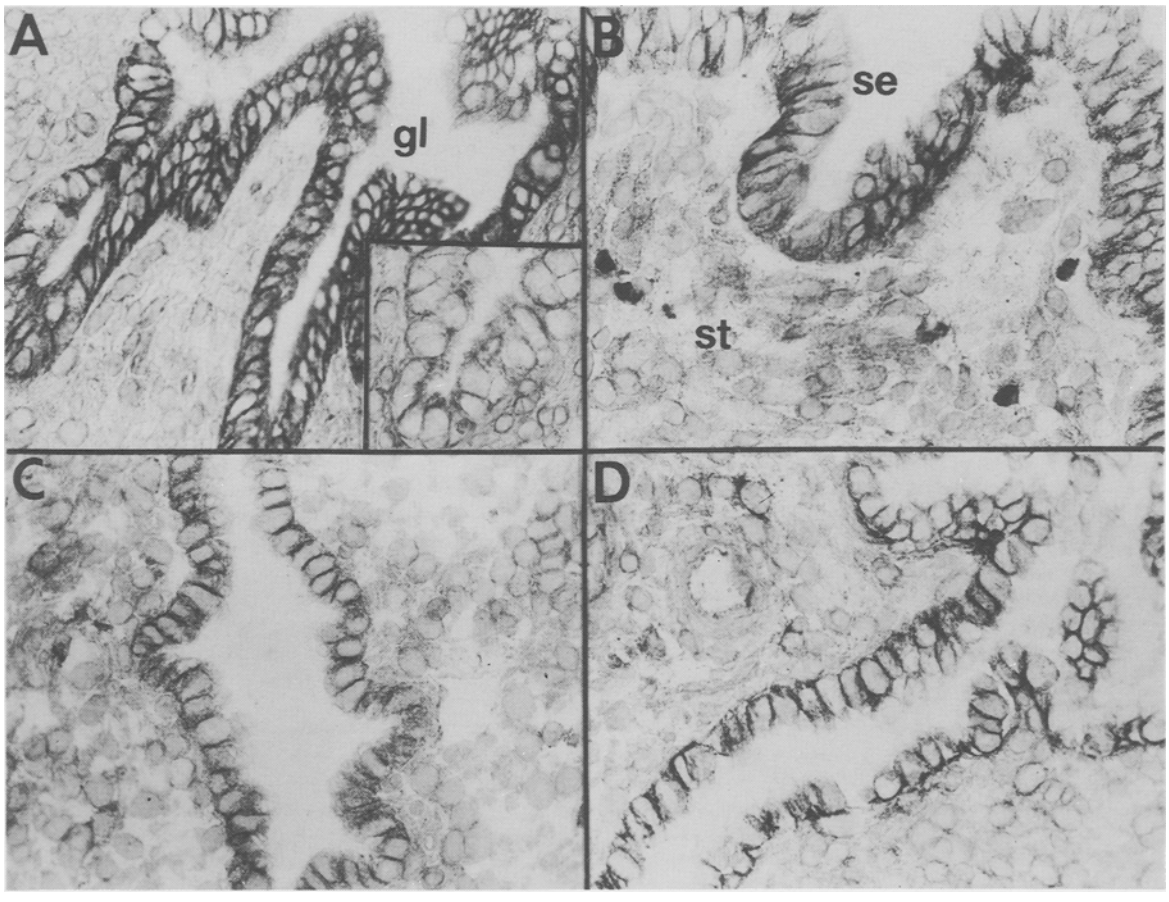

Figure 9 Immunocytochemical localization of IGF-I R in simulated-pregnant baboon endometrium. (**A**) basal glands, day 18 PO, hCG treated, (**B** and **C**) surface and basal glands, day 25 PO steroid treated (E + P only); (**D**) basal glands, day 32 PO hCG and steroid treated. Note the distinct membrane localization of IGF-I R in epithelial cells and the absence of any stromal staining. Unlike IGFBP-1 and RBP, no differences were noted between hCG and steroid only treated baboons. Inset, (**A**) pre-immune control. gl = glands; se = surface epithelium; st = stroma. Mag × 290

Table 3 Localization of IGF-I receptors in the endometrium of pregnant and hormone treated baboons

Cell type	*day 18 simulated pregnancy*[1]	*day 25 simulated pregnancy*[1]	*day 32 simulated pregnancy*[1]	*day 25 steroid treatment*	*day 18 pregnancy*[2] *IS*	*NIS*	*day 25 pregnancy*[2] *IS*	*NIS*	*day 32 pregnancy*[2] *IS*	*NIS*
Glands	+	+	+	+	±	±	±	±	±	±
Surface epithelium	+	+	+	+	NP	±	NP	±	NP	±
Stroma	0	0	0	0	±	0	+	0	+ +	+

[1]Ovariectomy had no effect on IGF-I receptor localization in baboons treated with hCG and steroid hormones. [2]Data summarized from Hild-Petito *et al.* (1994). + = positive staining. ± = some positive cells while other cells do not stain. + + = many positive stained cells. 0 = no staining. NP = Not present. IS = Implantation site. NIS = Non-implantation site

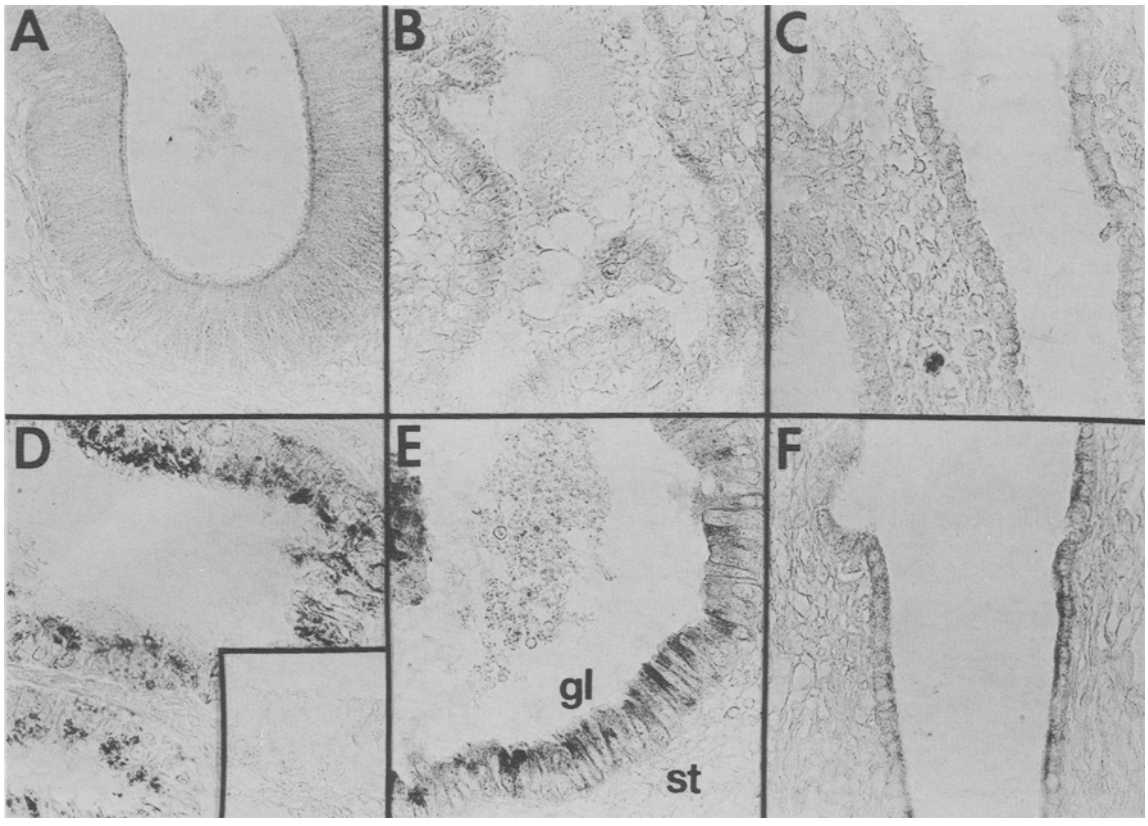

Figure 10 Immunocytochemical localization of RBP in simulated-pregnant baboon endometrium. (**A–C**) are glands in the mid-functionalis region on the endometrium from day 18 PO hCG treated (**A**); day 25 PO hCG and steroid treated (**B**); day 25 PO steroid only treated (**C**). (**D–F**) are the corresponding basal glands from the same tissue sections. Inset, (**D**) Preimmune control. Note that RBP staining is evident only in the basal glands of hCG treated baboons (**D** and **E**). gl = glands; st = stroma. Mag × 290

production of IGFBP-1, IGF-I R and RBP. However, additional conceptus factors appear to be necessary to initiate complete stromal cell differentiation (i.e., decidualization) and induce the changes in cell-specific expression of IGFBP-1 and IGF-I R that occur at the implantation site during pregnancy. Our current studies are focused on identifying the conceptus factor(s) necessary to induce this transformation.

Materials and methods

Materials

Tissue culture supplies were purchased from Gibco (Grand Island, NY). Kodak film (X-Omat AR) was a product of Eastman-Kodak (Rochester, NY). Acrylamide, *bis*-acrylamide, and other reagents for gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Molecular weight standards, SDS, and all other chemicals of reagent grade or better were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biology reagents were obtained from Fisher Biotech (Itasca, IL), and the random primer labeling kit was a product of Boehringer-Mannheim (Indianapolis, IN).

Monocloncal antibodies to IGFBP-1 (B2H10 and C4H11) were kindly provided by Dr Stephen C. Bell (University of Leicester, Leicester, UK). Monoclonal antibody against the IGF-I R (αIR3) was a product of Oncogene Science (Uniondale, NY), and a polyclonal antibody to human RBP was purchased from Dako Corp (Capenteria, CA). ^{125}I labelled IGF-I and enhanced chemiluminescence (ECL) substrates were purchased from Amersham International (Arlington Heights, IL) and enzyme conjugated second antibodies were products of Biorad Laboratories (Richmond, CA). The cDNA to human IGFBP-1 was cloned in our laboratory (Fazleabas *et al.*, 1989) and the cDNA to porcine conceptus RBP, which has a 91% nucleotide sequence identity with the coding region for human serum RBP, was a kind gift from Dr R Michael Roberts, University of Missouri, Columbia, MO (Trout *et al.*, 1991).

Tissue collection

All experimental procedures were approved by the Animal Care Committee of the University of Illinois. Uterine tissue was obtained at laparotomy from adult female baboons (*Papio anubis*) following a hCG and steroid treatment protocol (Figure 1) or following steroid treatment only to mimic pregnancy. Nonpregnant baboons were bled as indicated in Figure 1. The serum levels of estradiol and progesterone were determined using the appropriate coat-a-count RIA kits (Diagnostic Products Corp, Los Angeles, CA). Ovulation was assumed to occur 2 days after the estradiol surge based on previous studies (Knobil & Hotchkiss, 1988). In order to stimulate early pregnancy, nonpregnant baboons received increasing doses of hCG (30, 60, 90, 180, 360, 720, 1440, 2880, 5760, 11520 IU/dose; Profasi, Serono Laboratories Inc., Norwell, MA) twice daily for 10 days beginning on days 6–7 PO. This regimen was based on a protocol used to simulate pregnancy in the rhesus monkey (Ottobre & Stouffer, 1984). Tissues were obtained on day 18 PO from baboons receiving 2 additional days of hCG injections (12 000 IU/dose; CG only; $n = 6$). Additional hCG-treated baboons were treated subcutaneously with 6 cm silastic implants containing either 17 β estradiol or progesterone (Fazleabas *et al.*, 1988) on days 14, 21, 24 and 27 PO as indicated in Figure 1. Endometrium was obtained on day 25 ($n = 4$) and day 32 PO ($n = 3$).

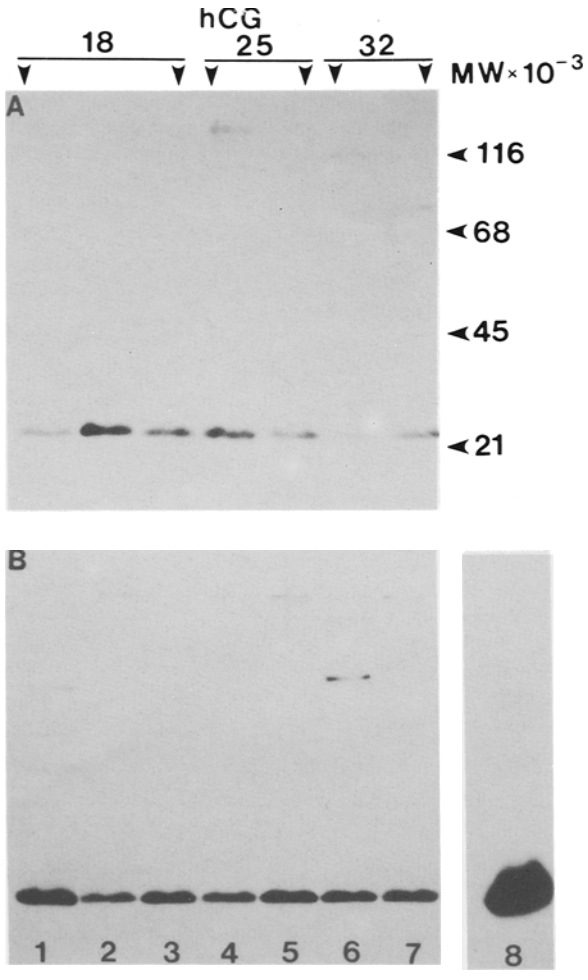

Figure 11 Western blot of functionalis (**A**) and basalis (**B**) explant culture media (25 μg protein) from hCG and steroid treated baboons immunoreacted with RBP antibody. Note the variable synthesis of RBP in the funtionalis (**A**) region (day 18 > 25 > 32) compared to the basalis region (**B**) in which RBP levels remained constant in all treatment groups. Lane 8 is a liver media control

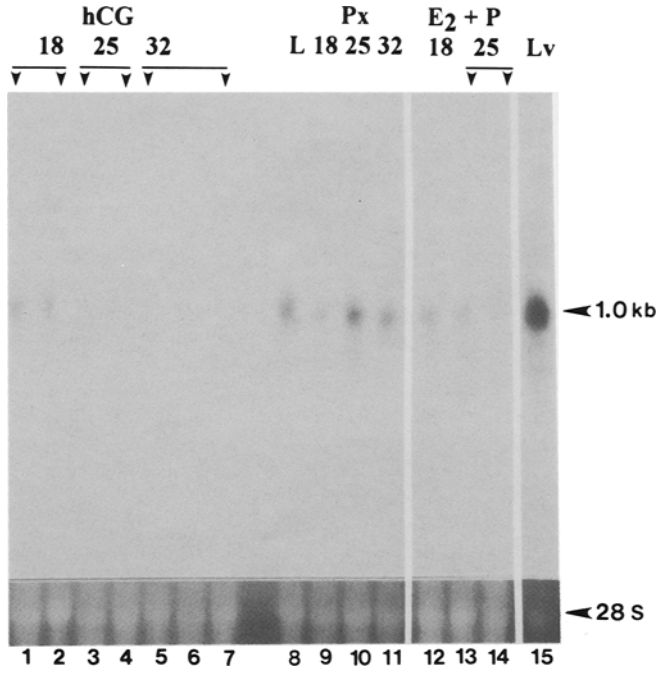

Figure 12 Northern blot of total endometrial RNA (20 μg) hybridized with a 590 bp porcine conceptus RBP cDNA probe. The same blot shown in Figure 8 was stripped and reprobed. Note that the functionalis region from hCG treated (lanes 1–7) and steroid only treated (lanes 12–14) contain very low amounts of mRNA which corresponds to the immunoreactive data shown in Figures 10 and 11. Funtionalis from pregnant baboon (lanes 9 11) and the liver control (lane 15) show strong hybridization. Lane 8 is a late luteal sample

An additional group of cycling baboons were treated with estradiol and progesterone only in order to mimic the steroid levels present during early pregnancy in the absence of hCG. In this group, one estradiol implant (3 cm) was placed subcutaneously on days 6 and 9 PO respectively and two additional implants were inserted on day 13 PO. Progesterone implants (6 cm, one on each day) were added on days 6, 9, 13 and 20 PO and the endometrium obtained on day 25 PO ($n = 4$).

In order to determine if CG affects the endometrium directly or requires the presence of the corpus luteum and/or ovary, baboons were bilaterally ovariectomized on days 6–7 PO and hCG treatment started immediately following surgery. In addition, one estradiol and three progesterone implants were placed subcutaneously at surgery and additional implants were added on the days indicated for the steroid only treatment group. Endometrial tissue was collected on days 18 ($n = 2$) or 25 ($n = 3$) PO.

For comparison, uterine tissue was collected from pregnant baboons as previously described (Hild-Petito *et al.*, 1992; Tarantino *et al.*, 1992). Mature cycling baboons were mated with fertile males during the periovulatory period, as determined by sex skin tumescence. Uterine samples were obtained on days 18 ($n = 3$), 25 ($n = 5$), 32 ($n = 3$) PO. The stage of pregnancy was verified by ultrasound, and circulating levels of baboon (b) CG, estradiol, and progesterone (Herring *et al.*, 1991; Fortman *et al.*, 1993).

Hormone assays

Circulating levels of hCG in the serum of treated baboons was determined by Leydig cell bioassay (Ellingwood *et al.*, 1980). This same assay was used to determine serum levels of bCG during early pregnancy as previously reported (Fortman *et al.*, 1993). The intra and interassay coefficients of variation were 11.2% and 18.7% respectively. The values for bCG from pregnant baboons were used to generate the 95% confidence interval with hCG stimulated baboons.

Serum levels of estradiol and progesterone were determined by coat-a-count RIA kits. Samples were tested in our validated RIA (Fazleabas *et al.*, 1988) and no statistical differences were noted in the absolute values between the assay systems. The intra and interassay coefficients of variation were 12.6% and 9.9% for estradiol and 6.1% and 16.1% for progesterone. Thus, previously reported estradiol and progesterone values from pregnant baboon serum (Fortman *et al.*, 1993) were used as a data base to generate a 95% confidence interval for comparison.

Morphology and protein synthesis

Pieces of uterine tissue were fixed in Bouin's fixative, embedded in paraffin and sectioned as previously described (Fazleabas *et al.*, 1993a). The tissue sections were deparaffinized, rehydrated and stained with Gimori's trichome stain (Sheehan, 1980), which stains nuclei red, cytoplasm pink and collagen in the extracellular matrix green. The sections from each group were analysed at the the light microscopic level to evaluate the morphological changes.

To determine the general protein synthetic capacity of the endometrial tissue, explant cultures were performed as described previously (Fazleabas & Verhage, 1987). Briefly, tissue pieces (2–3 mm^2) were incubated in serum free modified

minimum essential media in the presence of ^{35}S labeled methionine. The TCM (100 000 c.p.m./gel) was subjected to two dimensional SDS–PAGE as previously described (Fazleabas & Verhage, 1987). Fluorographs of TCM from hCG and steroid treated, steroid only treated and pregnant baboons were compared following identical exposure times. The percent incorporation of [^{35}S]methionine into proteins of greater than 6–8000 daltons were $13.3 \pm 3.6\%$, $2.71 \pm 0.53\%$, $4.1 \pm 0.67\%$ ($X \pm SEM$) following hCG and steroid treatment at days 18, 25 and 32 PO respectively. In the absence of hCG treatment, the incorporation was 7.6 ± 4.0 at day 25 PO. Endometrium from ovariectomized, hCG and steroid treated baboons incorporated $4.1 \pm 1.2\%$. There were no statistically significant differences between the treatment groups ($P > 0.05$).

Immunocytochemistry

Tissues were processed for indirect immunocytochemical localization of ER, PR and IGF-I R as previously described (Hild-Petito *et al.*, 1992, 1994). Briefly, frozen blocks were sectioned (4–6 μm) in a Reichert-Jung 2800 Frigocut N (Cambridge Instruments, Buffalo, NY) and thaw mounted onto slides. The sections were freeze substituted in acetone/calcium chloride, fixed at 4°C in 0.2% picric acid plus 2% paraformaldehyde followed by 85% ethanol. After blocking nonspecific binding with normal rabbit serum, the sections were incubated overnight at 4°C with specific monoclonal antibodies against either the human ER (H222, 10 μg/ml); Greene *et al.*, 1980), PR (JZB39, 2.5 μg/ml; Press & Greene, 1988) or IGF-I R (αIR3, 7.5 μg/ml). Nonspecific staining was determined in adjacent sections by substituting the receptor antibodies with purified rat immunoglobulin G for steroid receptors or mouse ascites fluid for IGF-I R at the same concentrations. The antigen-antibody complex was visualized using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) as the chromogen.

For IGFBP-1 and RBP localization, sections (5 μm) were cut on a rotary microtome from paraffin embedded tissue. The monoclonal antibodies to IGFBP-1 were used at a dilution of 1:750 and the RBP polyclonal antibody was used at a 1:300 dilution. The immunoreactive products were visualized using an ABC Vecstain kit (Vector Laboratories Inc., Burlingame, CA) as previously described (Tarantino *et al.*, 1992; Fazleabas *et al.*, 1994).

Immunoblotting and ligand blotting

TCM proteins (25 μg) from the functionalis and basalis regions of the endometrium were separated by one-dimensional SDS–PAGE under non-reducing (IGFBP) or reducing (RBP) conditions. The proteins were then transferred to nitrocellulose membranes as described by Towbin *et al.* (1979).

The non-reduced ligand blot was incubated overnight at 4°C with [^{125}I]IGF-I (2×10^5 c.p.m./ml) as described (Hossenlopp *et al.*, 1986; Fazleabas & Donnelly, 1992). The IGFBPs that bound the labeled ligand were visualized by autoradiography. The nitrocellulose membrane was then incubated overnight at room temperature with the IGFBP-1 monoclonal antibody (C4H11) at a 1:1000 dilution and the immunoreactive product visualized using alkaline phosphatase conjugated second antibody. Baboon decidual culture medium from late pregnancy, and implantation site and non-implantation site TCM from day 32 of pregnancy were run as positive controls.

The blots obtained following electrophoresis under reducing conditions were incubated overnight with the polyclonal antibody against RBP at 1:1000 dilution. Immunoreactive product was visualized using the ECL system. Baboon liver explant culture medium was run as a positive control.

Immunoassay for IGFBP-1

IGFBP-1 in TCM (100 μl; diluted 1:2) was assayed in duplicate using a commercial immunoradiometric assay for IGFBP-1 (Diagnostic Systems Laboratories, Webster, TX). The lower limit of detectability was 0.04 ng/ml and the intra and inter-assay coefficients of variation for high and low values were 3.8% and 3.4% respectively.

Northern blots

Total RNA (20 μg) from the functionalis region of each of the treatment groups was analysed by electrophoresis on a 1% agarose-formaldehyde gel and the RNA was transferred to nitrocellulose membranes (Sambrook *et al.*, 1989). Equal RNA loading was assessed by ethinium bromide staining of the gel prior to transfer. The blot was then baked for 2 h at 80°C in a vacuum oven before pre-hybridization for 4–6 h at 50°C in 6-strength Standard Sodium Phosphate ethylenediamine-tetraacetic acid Buffer (SSPE), 2 g dextran sulfate, 25% formamide, 5-strength Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out for 16 h at 50°C in the same buffer containing 1×10^6 c.p.m./ml of the cDNA probe. The membrane was first probed with a 1.2 kb EcoRI fragment of human IGFBP-1 cDNA (Fazleabas *et al.*, 1989), stripped, and reprobed with a 590 bp EcoRI fragement of porcine conceptus RBP cDNA (Trout *et al.*, 1991). Both probes were radiolabeled by random primer labeling with [$\alpha^{32}P$]dCTP (3000 Ci/mmol; Amersham International) to a specific activity of approximately 1×10^6 c.p.m./μg. After each hybridization, the filter was washed twice for 30 min/wash at 50°C in double-strength SSPE and 0.2% SDS followed by two more washes for 15 min each at 50°C in 0.1-strength SSPE followed by a 5 min wash at 60°C in 0.1-strength SSPE. After air-drying, the filter was exposed to Kodak X-Omat film with a Dupont Cronex Intensifying screen at −80°C.

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