Progesterone and oestrogen receptors in the decidualized mouse uterus and effects of different types of anti-progesterone treatment

Z. Parandoosh^{1*}, D. L. Crombie¹, T. A. Tetzke^{1*}, J. S. Hayes¹,
R. B. Heap^{2,3} and M-W. Wang^{1†}

¹Ligand Pharmaceuticals, Inc., 9393 Towne Centre Drive, San Diego, CA 92121, USA;

²The Babraham Institute, Babraham, Cambridge CB2 4AT, UK; and ³School of Clinical Medicine, University of Cambridge, Cambridge CB2 2SW, UK

Pseudopregnant mice were treated systemically with monoclonal anti-progesterone antibody (DB3) (model 1), or progesterone receptor antagonists RU486 or ZK98,299 (ZK299) (model 2) on day 3 *post coitum*. On day 4, sesame oil was administered intraluminally into one uterine horn to induce decidualization. On day 7, the average mass of the oil-injected horn was 335.2 ± 52.4 mg, eight times greater than that of the non-injected horn (40.8 ± 5.3 mg; $P < 0.001$). After treatment with DB3, RU486 or ZK299, the masses of the injected horns did not differ significantly from those of non-injected horns. In the control group, concentrations of progesterone receptors (ligand-binding assay) increased twofold in the decidualized (52.2 ± 7.4 fmol mg⁻¹) compared with the non-injected horn (26.0 ± 7.6 fmol mg⁻¹; $P < 0.05$), whereas oestrogen receptor content (ligand-exchange assay) decreased by 53% (104.9 ± 18.2 versus 224.3 ± 18.1 fmol mg⁻¹; $P < 0.001$). In model 1, antibody-treated animals showed a tenfold increase in the concentration of progesterone receptors (261.7 ± 81.1 fmol mg⁻¹; $P < 0.001$), but there was no differential distribution of progesterone or oestrogen receptors in the oil-injected versus non-injected uterine horns. In model 2, uterine progesterone and oestrogen receptors again showed no differential response between injected and non-injected horns regardless of the route of administration (systemic or intraluminal). Concentrations of progesterone receptors in RU486-treated (35.8 ± 9.4 fmol mg⁻¹) and ZK299-treated (32.0 ± 10.2 fmol mg⁻¹) mice were comparable to those in non-injected horns (35.3 ± 6.3 and 34.2 ± 5.1 fmol mg⁻¹, respectively) and were not significantly different from the control group (26.0 ± 7.6 fmol mg⁻¹). The results show that oil-induced decidualization is accompanied by increased concentrations of progesterone receptors and decreased concentrations of oestrogen receptors. When decidualization is blocked by anti-progesterone treatment (antibody against progesterone or progesterone receptor antagonist), there are differing effects on receptor responses with an increase in progesterone receptors and decrease in oestrogen receptors after passive immunization, and no change in progesterone receptors and a reduction in oestrogen receptors after anti-progestins. The anti-decidualization effect in the two models was therefore achieved via dissimilar uterine receptor responses.

Introduction

The primary dependence of uterine proliferation and differentiation on the co-ordinated effects of progesterone and oestradiol in early pregnancy is well established. The hormonal control of these events has been investigated in detail at the morphological, biochemical and molecular levels. Knowledge of these processes gives valuable insights into essential features of the successful establishment of pregnancy in mammals that

show a pronounced decidualization of the uterine stroma, as in rodents and humans (Psychoyos, 1973; Finn, 1977; Glasser, 1990; Abrahamson and Zorn, 1993; Tang *et al.*, 1994).

Decidualization, initiated by an embryonic or artificial stimulus, depends upon previous sensitization by a sequence of oestrogen and progesterone secretion. In mice, preovulatory ovarian oestrogen stimulates uterine epithelial cell proliferation on days 1 and 2 of pregnancy; progesterone from the newly formed corpus luteum initiates the proliferation of stromal cells potentiated by increased secretion of oestrogen during the preimplantation period. Decidualization can be artificially induced in pseudopregnant females on day 4. With the advent of anti-progestins, attention is currently focused on two topics:

*Present address: Prizm Pharmaceuticals, Inc., 11035 Roselle Street, San Diego, CA 92121, USA.

†Correspondence.

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first the molecular mechanisms by which anti-progestins interrupt decidualization and, thereby, prevent the establishment of pregnancy; and second the cellular mechanisms involved, since there is growing evidence that paracrine or autocrine, as well as endocrine, pathways are used.

Expression of several genes in the endometrium has been shown to be regulated by progesterone (for example, *c-myc*, genes encoding uteroglobin, uteroferrin and calcitonin). A potential molecular mechanism associated with the process of decidualization was reported by Wang *et al.* (1994) and Zhang *et al.* (1994), namely, the local synthesis of mRNA for heparin-binding epidermal growth factor (HB-EGF). In mice, the message for this growth factor is stimulated in epithelial cells by oestradiol and in stromal cells by progesterone and oestradiol (Wang *et al.*, 1994). In rats, HB-EGF is stimulated in stromal cells by progesterone treatment and by an artificial decidual stimulus. The time interval from artificial stimulus to raised mRNA encoding HB-EGF is 8–24 h and corresponds to the period of maximum stromal mitotic activity. The anti-progestin ZK98,299 (ZK299) blocks decidualization and progesterone-induced expression of mRNA encoding HB-EGF (Zhang *et al.*, 1994).

Steroid anti-progestins such as RU486 and ZK299, and antibodies against progesterone such as DB3 are highly effective as anti-implantation agents in laboratory models of implantation (Vinijsanun and Martin, 1990; Roblero and Croxatto, 1991; Wang and Heap, 1992). Anti-progestins interfere with receptor function either by inhibiting transcription factor activities (McDonnell *et al.*, 1993) or by preventing receptor binding to the specific hormone responsive elements (Truss *et al.*, 1994). Antibodies, however, act by ligand scavenging which significantly diminishes target organ responses (Rider *et al.*, 1986; Wang and Heap, 1992).

The aim of the present study was to determine whether the distinct anti-decidualization mechanisms of antibodies against progesterone and anti-progestins result in similar or contrasting effects on uterine concentrations of progesterone and oestrogen receptors. The hypothesis is that anti-decidualization effects can be achieved by more than one route of receptor disruption. Our goal was set within a well-established framework of knowledge that uterine progesterone receptors are rapidly upregulated by oestradiol (Manni *et al.*, 1981; Kraus and Katzenellenbogen, 1993), uterine oestrogen receptors are downregulated by oestrogen (Manni *et al.*, 1981; Shupnik *et al.*, 1989) and progesterone (Hsueh *et al.*, 1976; Pavlik and Coulson, 1976), and that progesterone will block oestradiol-induced progesterone receptor upregulation – a response that can be inhibited by simultaneous administration of the anti-progestin, RU486 (Kraus and Katzenellenbogen, 1993).

Materials and Methods

Reagents

Progesterone receptor antagonists RU486 (Mifepristone: Roussel-Uclaf, Romainville) and ZK299 (Onapristone: Schering AG, Berlin) were dissolved in sesame oil. Monoclonal antibody (IgG1) directed against progesterone (DB3) was prepared in BALB/c mice and purified as described by Ellis *et al.* (1988).

Animals

Mature, virgin BALB/c mice (10–12 weeks old; Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in a light, temperature-controlled room, with food and water *ad libitum* (Diet LM-485, Teklad, Madison, WI). Females were caged with vasectomized males of the same strain between 17:00 and 10:00 h. Pseudopregnancy was dated from the morning when a vaginal plug was detected (day 1; Bronson *et al.*, 1966).

Decidualization

Pseudopregnant mice were treated with 100 µg RU486 (*s.c.*), 100 µg ZK299 (*s.c.*), or 9.0 nmol (1.35 mg) DB3 (*i.p.*) at 10:00 h on day 3 (DB3 on day 2 for progesterone receptor measurements). Control animals received an equivalent volume of oil. On day 4 (16:00 h), 10 µl sesame oil was injected intraluminally into the right uterine horn (injected) and the left horn was left undisturbed (non-injected control). In another series of experiments, the same quantity of RU486 or ZK299 was injected intraluminally into the right uterine horn. At necropsy (day 7), the uterine horns were removed, trimmed, blotted and weighed. The gain in uterine wet mass was calculated by subtracting the mass of the non-injected horn from that of the injected horn.

Preparation of tissue extract for receptor binding

Whole cell receptor extract was prepared in 50 mmol Tris-HCl buffer l^{-1} (pH = 7.4) containing 30% (v/v) glycerol, 1.0 mmol EDTA l^{-1} , 12 mmol monothiolglycerol l^{-1} , 1.0 mmol phenylmethylsulfonyl (PMSF) fluoride-protease inhibitor l^{-1} and 0.5 mol potassium chloride l^{-1} (90–100 mg uterine tissue ml^{-1}). The preparation was incubated at 4°C for 60 min with resuspension every 10 min. The suspension was centrifuged (105 000 g for 45 min) and the supernatant frozen and stored at –70°C for subsequent binding studies as described below. An aliquot of the cell extract was taken for protein assay (Bradford, 1976).

Oestrogen receptor assay

After dilution (1.0 mg ml^{-1} protein) with buffer A (10 mmol Tris-HCl buffer l^{-1} , pH 7.4, containing 10% (v/v) glycerol, 1.0 mmol EDTA l^{-1} , 12 mmol thioglycerol l^{-1} and 1.0 mmol PMSF l^{-1}), the uterine cell extracts (250 µl) were incubated with 10 nmol [3H]oestradiol l^{-1} (specific activity: 136 Ci $mmol^{-1}$; Amersham International, Amersham, Bucks) in the presence or absence of excess (200-fold) diethylstilboestrol (Sigma Chemical Co., St Louis, MO) for 60 min at 37°C. The preparation was cooled to 4°C and an equal volume of hydroxylapatite (HAP) slurry (Ho *et al.*, 1985) was added. After incubation for 30 min, the mixture was centrifuged at 800 g and the supernatant discarded. The HAP pellet was washed with 2.0 ml HAP-washing buffer (10 mmol Tris-HCl l^{-1} , pH 7.4, 5.0 mmol dithiothreitol l^{-1} and 1.0% (v/v) Triton X-100) and transferred to vials containing 10 ml of scintillation fluid for radioactivity determination. The exchange condition measures the total oestrogen receptor content (occupied and

unoccupied binding sites) (Anderson *et al.*, 1972). The extent of binding was calculated based upon the specific activity of [^3H]oestradiol and protein concentration.

Progesterone receptor assay

Uterine cell extracts (1.0 mg ml^{-1} protein; $200\text{ }\mu\text{l}$) were incubated at 4°C for a minimum of 12 h with 10 nmol [^3H]progesterone l^{-1} (specific activity: 103 Ci mmol^{-1} ; Amersham) in the presence or absence of excess (200-fold) cold progesterone (Sigma). Bound [^3H]progesterone was determined using HAP as described above. Before the assay, samples from mice treated with DB3 were reacted with rabbit anti-mouse IgG at 4°C for 30 min. The amount of anti-mouse IgG ($0.1\text{ }\mu\text{g}$ per $1.0\text{ }\mu\text{g}$ extract) used was optimized to maximally precipitate DB3 from the system and minimize its interference with the binding assay. Radioactivity was determined with a liquid-scintillation counter. The specific activity of [^3H]progesterone and protein concentration were used for calculation of progesterone receptor content.

Statistical analysis

Pairwise statistical analyses were performed using two-tailed Student's *t* test. Unless stated otherwise, all results are reported as means $\pm$ SEM.

Results

Effects of anti-progesterone treatment upon decidualization

There was a marked increase in uterine mass in control females following a decidual stimulus (sesame oil) given on day 4 of pseudopregnancy (Table 1). Oil-injected horns displayed

an eightfold increase in wet mass compared with non-injected horns (335.2 ± 52.4 versus $40.8 \pm 5.3\text{ mg}$; $P < 0.001$) at day 7 of pseudopregnancy. Systemic injection (i.p. or s.c.) of DB3, RU486 or ZK299, or intraluminal administration of RU486 or ZK299, completely blocked decidualization ($P < 0.001$; Table 1).

Effects of anti-progesterone treatment on progesterone receptor concentration

The concentration of progesterone receptors increased two-fold after decidualization ($P < 0.05$; Fig. 1). In antibody-treated females, progesterone receptor concentration increased substantially compared with the control group in non-injected (tenfold, $P < 0.001$) and injected (fivefold, $P < 0.001$) uterine horns. The anti-progestins, RU486 and ZK299, had no effect on progesterone receptor concentrations in either horn when administered systemically (Fig. 1). Progesterone receptor concentration in both horns was similar following intraluminal injection of RU486 or ZK299 (data not shown).

Effects of anti-progesterone treatment on oestrogen receptor concentration

The concentration of oestrogen receptors in the control group decreased significantly (53%) in the decidualized uterine horn ($P < 0.001$; Fig. 2). This decrease occurred in conjunction with an eightfold increase in tissue mass. A similar reduction in oestrogen receptor concentration occurred in injected and non-injected horns ($P < 0.01$ to $P < 0.001$) after systemic treatment with DB3, RU486 or ZK299. Thus, even though the horns did not respond to a decidualizing stimulus after anti-progesterone treatment there was about a 50% reduction in their oestrogen receptor concentrations compared with the

Table 1. Effects of anti-progesterone antibody (DB3) and progesterone receptor antagonists (RU486 and ZK299) on oil-induced decidualization in mice

Treatment	Number of mice	Net gain in uterine mass (mg)	Percentage inhibition	Ovary mass (mg)
Control	8	294.5 ± 55.6	0	5.0 ± 0.3
Systemic treatment				
DB3	6	0.8 ± 2.7^a	99.8	4.8 ± 0.4
RU486	6	3.3 ± 2.8^a	98.9	5.5 ± 0.3
ZK299	5	6.0 ± 1.3^a	98.0	4.4 ± 0.4
Local treatment				
RU486	6	9.7 ± 3.8^a	96.7	4.7 ± 0.4
ZK299	6	3.2 ± 1.3^a	98.9	4.1 ± 0.5

Net gain in uterine mass was calculated by subtracting the mass of the control horn from that of the injected horn (wet masses of injected and non-injected horns were 335.2 ± 52.4 and $40.8 \pm 5.3\text{ mg}$, respectively). Percentage inhibition was estimated using the following formula:

$$\% \text{ Inhibition} = 100 - \frac{\text{Gain in mass in treated group}}{\text{Gain in mass in control group}} \times 100$$

Values are means $\pm$ SEM.

^aSignificantly different from the control group ($P < 0.001$).

Fig. 1. Effects of anti-progesterone treatment on the concentration of uterine progesterone receptors in mice. Virgin BALB/c mice were treated with either progesterone receptor antagonists (RU486 or ZK299; 0.1 mg per animal, s.c.) or a monoclonal antibody against progesterone (DB3; 1.35 mg per animal, i.p.) on day 3 of pseudopregnancy (day 2 for DB3). Control females received 0.1 ml oil. On day 4, 10 μ l of sesame oil was injected into the lumen of (■) the right uterine horn; (□) the left horn was left undisturbed. Uterine samples collected at necropsy on day 7 were homogenized and assayed for progesterone receptor (fmol mg⁻¹ protein). *, **Values (means $\pm$ SEM) are significantly different from respective controls (* P < 0.05; ** P < 0.01).

non-injected control horn (Fig. 2). The effects were similar whether RU486 or ZK299 was administered systemically or locally into the uterine lumen (data not shown).

Discussion

The two models of anti-decidualization illustrate that antagonism of the actions of progesterone either by anti-progesterone immunization or by steroid antagonists is achieved by contrasting effects upon progesterone and oestrogen receptors. Decidualization increases uterine concentrations of progesterone receptors about twofold by day 7 of pseudopregnancy. In the anti-progesterone antibody model, concentrations of progesterone receptors were increased between five- and tenfold in oil-injected and non-injected horns, respectively. No evidence of decidualization, however, was obtained. Thus, increases in concentrations of uterine progesterone receptors do not in themselves predicate decidualization.

Inhibition of decidualization in the antibody model prevents increases in capillary permeability, normally associated with deciduoma formation (Rider *et al.*, 1985). After antibody treatment at day 2 there is a rapid increase in the circulating concentration of progesterone, the majority of which is bound by antibody with high affinity. Simultaneously, there is a significant fall in uterine progesterone concentrations which

Fig. 2. Effects of anti-progesterone treatment on concentration of uterine oestrogen receptors in mice. Virgin BALB/c mice were treated with either progesterone receptor antagonists (RU486 or ZK299; 0.1 mg per animal, s.c.) or a monoclonal antibody against progesterone (DB3; 1.35 mg per animal, i.p.) on day 3 of pseudopregnancy. Control females received 0.1 ml oil. On day 4, 10 μ l of sesame oil was injected into the lumen of (■) the right uterine horn leaving (□) the left horn undisturbed. Uterine samples collected at necropsy on day 7 were homogenized and assayed for oestrogen receptor (fmol mg⁻¹ protein). **, *** Values (means $\pm$ SEM) are significantly different from respective controls (** P < 0.01; *** P < 0.001).

after day 3 return to normal (Nowak *et al.*, 1990), and a marked increase in the expression of progesterone receptor and its mRNA in the endometrial stromal cells as assessed by immunocytochemistry and *in situ* hybridization (Whyte *et al.*, 1992). Our present results provide further quantitative data that confirm this latter observation.

Increases in endometrial progesterone receptors after passive immunization implicate unopposed oestrogen action (Crombie *et al.*, 1994) or removal of progesterone inhibition (Clark and Markaverich, 1988) as a cause of stimulation. There is a transient increase in uterine epithelial mitoses, a decrease in stromal mitoses (Rider *et al.*, 1986), and a significant reduction in uterine progesterone concentrations at day 2 and day 3 (Nowak *et al.*, 1990), all consistent with the idea of initial unopposed oestrogen effects. In the antibody model, concentrations of uterine oestrogen receptors were not stimulated, but rather reduced to about 50% of the control value. An explanation may lie in the initial oestrogen-induced downregulation of the oestrogen receptor followed by the chronic effect of uterine progesterone as its concentration returns to normal values by day 4. Uterine oestrogen receptor values at day 7 were similar to those found after decidualization in the control group when comparable uterine progesterone concentrations would occur. It is noteworthy that after decidualization there was an eightfold increase in tissue mass and total uterine oestrogen receptor was estimated to be greater than 3000 fmol per oil-injected horn compared with about 700 fmol per non-injected horn. This dilution effect of oestrogen receptor

concentration by tissue growth after decidualization is well known (Villego *et al.*, 1981) and reflects the synergistic effects of oestrogen and progesterone on oestrogen receptor synthesis and uterine growth.

Anti-progestins RU486 and ZK299 prevent decidualization as reported previously (Crombie *et al.*, 1994; Zhang *et al.*, 1994). In this paper, we demonstrate that the anti-decidualization effect is not associated with changes in uterine progesterone receptor concentrations, which remained similar to control values. This result differs markedly from the effect of passive immunization. Oestrogen receptor concentrations decreased by approximately 50%, and values were comparable to those seen in the decidualized horn of control females. These findings contrast with reports that in early human pregnancy RU486 (200 mg) causes a marked increase in immunocytochemical staining of oestrogen receptors, and to a lesser degree in progesterone receptor staining in stromal cells (Perrot-Applanat *et al.*, 1994). Similarly, oestrogen receptors in decidua and myometrium increased after RU486 treatment in late pregnant rhesus macaques ($20 \text{ mg kg}^{-1} \text{ day}^{-1}$), presumably because of a decreased inhibition by progesterone on oestrogen receptors (Haluska *et al.*, 1990). However, the relative distribution of these receptors in different uterine compartments was not investigated in the present study. Other workers have found that RU486 (600 mg) in early human pregnancy causes an increase in oestrogen receptor and a decrease in progesterone receptor content of decidua, although results are influenced by dose and time of sampling (Zaytseva *et al.*, 1993). Responses of uterine progesterone and oestrogen receptors to anti-progestins, therefore, are complex, and are influenced by the time course, the species and the protocol adopted.

In summary, decidualization is associated with increased progesterone receptor and decreased oestrogen receptor concentrations. Decidualization can be prevented either by binding of progesterone by antibody or inhibition of the actions of progesterone by receptor antagonists (RU486 and ZK299). Each mechanism is associated with distinct and different effects upon progesterone receptor concentrations. Anti-progesterone antibody produces a significant increase in concentrations of uterine progesterone receptors and a significant decrease in concentrations of oestrogen receptors. These responses suggest that progesterone receptor upregulation is due to an unopposed oestrogen effect shortly after antibody injection. Downregulation of oestrogen receptors probably occurs with chronic progesterone stimulation as its concentration increases and returns to normal after passive immunization. The upregulation of the progesterone receptor in the antibody model occurs predominantly in stromal cells (Whyte *et al.*, 1992) but it fails to increase stromal cell sensitivity to a decidual stimulus. Other endometrial factors, such as the production of HB-EGF (Zhang *et al.*, 1994), angiogenic factors (Smith, 1994), local prostanoid production associated with increased capillary permeability (McRae and Heap, 1988) or leukaemia inhibitory factor (Stewart *et al.*, 1992) are probably missing or deficient. In the second model, steroid anti-progestins have no effect on concentrations of uterine progesterone receptors but reduce oestrogen receptors by more than 50% through a mechanism(s) that require further clarification. Therefore, lack of stromal cell sensitivity to a decidual stimulus appears to be related less to

abnormal uterine progesterone or oestrogen receptor concentrations than to the absence in these two anti-decidualization models of other elements of progesterone-induced receptor-mediated gene expression. The discovery that anti-decidualization effects can be achieved by very different, and counterintuitive receptor responses is important and highly relevant to our understanding of the failure of implantation and the development of new approaches to fertility regulation.

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