Anti-proliferative effects of progesterone antagonists in the primate endometrium: a potential role for the androgen receptor

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In women and non-human primates, treatment with anti-progestins suppresses oestrogen-dependent mitotic activity in the endometrial glands. This anti-proliferative effect is paradoxical, because anti-progestins do not bind to the oestrogen receptor. Although this phenomenon has been termed a 'non-competitive anti-oestrogenic effect', it does not occur in all species or in other regions of the primate reproductive tract, so is best referred to as an 'endometrial anti-proliferative effect'. The abundance of androgen receptors is greatly increased by anti-progestin treatment, especially in the glandular epithelium in non-human primates and women. Such an increase could lead to an enhancement of androgen action in the endometrium. As androgens suppress oestrogen-dependent endometrial proliferation, the increased abundance of androgen receptors could mediate the anti-proliferative effects of anti-progestin treatment. This brief review evaluates the implications of these findings.

This review focuses specifically on the endometrial anti-proliferative effects of progesterone antagonists. More extensive reviews of the chemistry and physiological effects of progesterone antagonists have been published by Spitz and Robbins (1998) and Chwalisz *et al.* (2000). The first effective anti-progestin was mifepristone (RU486), and the literature on this compound has been reviewed several times (Baulieu *et al.*, 1987; Baulieu, 1991). Several other anti-progestins have been developed, including onapristone, ZK 137 316 and ZK 230 211 (Schering AG, Berlin), which have greater potency and higher specificity for the progesterone receptor (PR). In addition, mixed agonist-antagonists such as J1042 and J867 (Schering AG), known as either mesoproggestins or progesterone receptor modulators (PRMs), have been developed (Chwalisz *et al.*, 2000). PRMs can block progesterone action, but in the absence of progesterone can act as agonists. Both PRMs and anti-progestins have many potentially important clinical uses, and chronic low dose treatment with anti-progestins has been proposed for management of aberrant endometrial bleeding, endometriosis, mammary cancer and contraception (Cadepond *et al.*, 1997; Baird, 2000). However, concern has been expressed that anti-progestins might block the ability of progesterone to oppose oestrogen action in the endometrium, resulting in hyperplasia.

As a counter to this concern, several studies have indicated that anti-progestins have the unexpected ability to block endometrial proliferation, even during oestrogen-only treatment, although the mechanism of this action has

not been fully explained. Wolf *et al.* (1989) were the first to report that high doses of RU486 had an anti-endometrial effect in ovariectomized, oestrogen-treated macaques. As RU486 does not bind to the oestrogen receptor, these authors called this action a 'non-competitive anti-oestrogenic effect'.

Endometrial anti-proliferative effects of anti-progestins in the primate uterus

The effects of various regimens of RU486 and some Schering anti-progestins, such as ZK 137 316, on the endometrium of rhesus macaques has been reviewed by Chwalisz *et al.* (2000). All these anti-progestins induced increases in endometrial oestrogen receptor (ER) and PR, a classical effect of unopposed oestradiol, but they inhibited endometrial mitotic activity, wet mass and thickness. In addition, anti-progestin treatment led to stromal cell atrophy, stromal compaction and hyalinizing degeneration of the spiral arteries, all in the presence of high serum concentrations of oestradiol and increased ER. Similar paradoxical effects were found after long-term treatment of intact cyclic macaques with low doses of RU486 for 8 years (Grow *et al.*, 1998). In studies with ZK 137 316 in ovary intact, cyclic animals, the lowest doses allowed normal ovarian cycles, secretion of follicular phase concentrations of oestradiol and normal oviductal differentiation, but an overall suppression of endometrial growth, including a decrease in glandular cell proliferation, assessed on the basis of both mitotic counts and DNA synthesis. These data indicate that the endometrium is much more sensitive to the negative effects of anti-progestins than are other tissues that

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contain PR. Low doses of RU486 administered chronically to women also inhibited glandular mitosis and induced stromal compaction in the endometrium (Cameron *et al.*, 1996).

Anti-proliferative and apoptotic effects of anti-progestins have also been observed *in vitro* in various breast cancer cell lines, and these effects have been referred to as receptor-mediated 'cytostatic and cytotoxic' effects. Conceivably, RU486 might prove a valuable drug for treatment of breast cancer because of such direct anti-proliferative effects (Horwitz, 1992). Mifepristone has anti-oxidant properties and it has been proposed that the anti-proliferative action of RU486 may involve anti-oxidants blocking oxidative changes in low density lipoproteins that are important to the growth of human endometrium (Murphy *et al.*, 2000). However, the failure of known anti-oxidants such as vitamin E to have similar anti-proliferative effects (Murphy *et al.*, 2000) raises doubts about the generality of this hypothesis. Moreover, in macaques, anti-progestins do not appear to cause any anti-proliferative or anti-differentiative effects in the oviductal or vaginal epithelium, and do not reduce oestrogen-dependent bone mineral density in either women or macaques. In addition, in rodents treated with anti-progestin, oestradiol action in the endometrium is enhanced rather than suppressed, as indicated by increases in cell height and mitotic activity of the uterine luminal epithelium (Chwalisz *et al.*, 1998). In summary, the non-competitive anti-oestrogenic effects of chronic low dose anti-progestin therapy appear to be tissue- and species-specific, are most evident in the primate endometrium, and are best referred to as endometrial anti-proliferative effects. A full understanding of the mechanisms underlying these paradoxical effects has not yet been reached.

Is the androgen receptor involved in the endometrial anti-proliferative effect?

There is substantial evidence that exogenous androgens can inhibit the endometrium. Okon *et al.* (1998) suggested that in women with recurrent miscarriages, high androgen concentrations may specifically antagonize oestrogen action directly in the endometrium. Testosterone and danazol directly inhibit human endometrial cell proliferation in tissue culture in which the medium contains phenol red in amounts adequate to provide an oestrogenic stimulus (Rose *et al.*, 1988). Other studies show that androstenedione inhibits human endometrial cell growth and secretory activity *in vitro* and that androgen receptors are present in these cultured endometrial epithelial cells. Moreover, when cyproterone acetate (an androgen receptor antagonist) is added to the cultures, the effects of the androgens are blocked (Tuckerman *et al.*, 2000). Therapeutic progestogens that are highly androgenic result in endometrial atrophy. For example, marked endometrial atrophy is a feature of the endometrial response among women using a levonorgestrel-releasing intrauterine system (Critchley *et al.*, 1998). As the

suppressive effects of androgens on endometrial growth resemble the atrophic effects induced by anti-progestins, the possibility that the androgen receptor (AR) plays a role in anti-progestin action in human and non-human primate endometrium was evaluated using *in situ* hybridization, immunocytochemistry and ligand binding assays to assess changes in the endometrial AR during anti-progestin treatment (Slayden *et al.*, 2001).

Human endometrial samples from three groups of patients were available from three previously published studies. The first group consisted of fertile women who had been sampled during the proliferative, early mid- and late secretory phases of the normal menstrual cycle for studies of oestrogen receptor isoform expression (Critchley *et al.*, 2001).

The second group consisted of women with regular menstrual cycles treated either with placebo capsules or capsules of 2 mg mifepristone each day for 30 days. Such chronic, low dose treatment suppresses endometrial maturation and makes successful implantation unlikely (Batista *et al.*, 1992; Cameron *et al.*, 1996). The third group consisted of women who were treated with 200 mg oral mifepristone (or vehicle) 2 days after the ovulatory surge of LH and sampled 4–6 days later. Acute administration of a high dose of mifepristone (200 mg) in the early luteal phase also retards development of a secretory endometrium (Cameron *et al.*, 1997). All human endometrial samples were obtained with a Pipelle endometrial sampler.

Ovariectomized rhesus macaques were treated with implants of oestradiol for 2 weeks and then for 2 further weeks with either oestradiol alone, oestradiol plus RU486, oestradiol plus progesterone, or oestradiol plus progesterone plus RU486. Untreated, ovariectomized animals served as controls. Reproductive tract tissues were collected by mid-ventral laparotomy at the end of treatment. Some animals were also treated with oestradiol and various doses of ZK 137 316 (Schering AG).

All work with macaques received ethical approval from the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center.

Effects of anti-progestins in rhesus macaques

In macaques, oestradiol significantly increased AR binding capacity (assessed with a radioactive androgen) above untreated controls, and oestradiol plus RU486 treatment increased binding significantly further (Fig. 1). Oestradiol plus progesterone decreased AR binding, and oestradiol plus progesterone plus RU486 treatment caused an intermediate increase in AR binding. In macaques treated with oestradiol alone, all immunocytochemical staining for AR was localized to the stroma, and the glands were negative (Fig. 2). In macaques treated with oestradiol plus RU486, the stromal AR signal was enhanced and strong AR staining became evident in the glandular epithelium (Fig. 2). *In situ* hybridization for mRNA encoding AR showed similar patterns: after treatment with oestradiol alone, hybridization

Fig. 1. Endometrial total androgen receptor (AR) concentrations (mean $\pm$ SE) measured by [³H]R1881 binding assay in untreated (spay) macaques, and macaques treated with oestradiol (E₂), oestradiol plus RU486 (RU), oestradiol plus progesterone (P), and oestradiol plus progesterone plus RU486. ^{a-d}Bars with different letters are significantly different ($P < 0.05$). (Adapted from Slayden *et al.*, 2001.)

signals were evident only in the stroma, but after treatment with oestradiol plus RU486, the hybridization signal increased in the stroma and became very strong over the glandular epithelium (Slayden *et al.*, 2001). No evidence for AR expression was obtained by either immunocytochemistry or *in situ* hybridization in the vascular endothelium or smooth muscle of the spiral arteries under normal cyclic conditions or after anti-progestin treatment. The effects with ZK 137 316 were similar to those obtained using RU486.

Effects of anti-progestins in women

In women, during the natural menstrual cycle, immunocytochemical staining for AR also predominated in the stroma. The signal was greatest in the proliferative, early and mid-secretory phases and weakest in the late secretory phase. As in macaques, mifepristone treatment resulted in increased staining in the stroma and a marked increase in staining for AR in the glandular epithelium (Fig. 2). The same anti-AR antibody (F39.4; Biogenex, San Ramon, CA) was used to evaluate both human and macaque tissues. The increase in AR staining induced by mifepristone was evident after either treatment with 2 mg per day for 30 days or after a single oral dose of 200 mg. In summary, endometrial AR was highest in the stroma during the human proliferative phase (or during oestradiol treatment in macaques), and lowest during the late secretory phase in

women (or after oestradiol plus progesterone treatment in macaques). In both species, RU486 markedly increased AR expression in the glands and enhanced its expression in the stroma.

These data show that during the normal menstrual cycle, in both women and macaques, endometrial AR is primarily stromal in distribution. Similar findings have been reported previously in the human (Mertens *et al.*, 1996) and rhesus macaque endometrium (Adesanya *et al.*, 1999). Apparao *et al.* (2002) reported that, in endometrial biopsies from women with polycystic ovary syndrome (PCOS), the glandular and luminal epithelia had much stronger immunocytochemical staining for AR than they did in similar specimens from women without PCOS. These authors suggested that the combination of increased androgens and glandular AR in the endometrium of women with PCOS could play a major role in the infertility associated with this syndrome.

Working hypothesis for the endometrial anti-proliferative effect

In summary, during the natural menstrual cycle in normal women, any effects of androgens on the glands or vascular elements in the endometrium would be mediated indirectly through stromal AR. However, after treatment with anti-progestins (and perhaps in women with PCOS), androgens may have direct effects on the glands through the greatly increased AR in glandular cells. Any effects of oestrogen that are mediated by ER in the glands might then be counteracted by direct effects of androgens mediated by AR in the same cells. In addition, a large body of evidence indicates that oestrogen-dependent growth factors that are made by the stroma are essential for mitosis in the glands (Cooke *et al.*, 1997). Anti-progestins, by increasing AR in stromal cells, might lead to suppression of such factors by androgens. Whether these are the effects of testosterone, dihydrotestosterone, androstenedione or some other androgen, and whether the androgen source is the endometrial tissue or the systemic circulation is not known. It is possible that AR is activated through cross-talk by cellular factors that enhance its phosphorylation state (Ikonen *et al.*, 1994), but it is not known whether this occurs in the primate endometrium. All of these considerations demand further research.

The precise mechanism through which anti-progestin treatment increases AR during treatment with oestradiol is not clear, but our previous studies and those of others showed that anti-progestin treatment also increases ER and PR. Our current view is similar to that expressed by Chwalisz *et al.* (1991), who found an increase in ER after treatment with the anti-progestin, onapristone, in the endometrium of ovariectomized rabbits. These authors stated that: 'The increase in ER and ER mRNA concentrations after onapristone treatment in ovariectomized, oestradiol-treated animals suggests that this anti-gestagen abolished an inhibitory action of the unoccupied PR on ER biosynthesis'.

Fig. 2. Androgen receptor (AR) immunostaining in the endometrium of (a,b) macaques and (c,d) women. (a) In macaques treated with oestradiol alone, only the stromal compartment (S) shows definite AR staining; the glands (GI) are negative for AR. (b) Treatment with oestradiol plus RU486 resulted in a striking upregulation of AR in the glands and further increased stromal AR staining compared with that observed after treatment with oestradiol alone. (c) Human endometrium control sample, stained for AR. Only the stroma is positively stained. (d) Human endometrium after treatment with 200 mg mifepristone (RU486). Both glands and stroma are positively stained for AR. Scale bar represents 50 μ m. (Adapted from Slayden *et al.*, 2001.)

This view implies that unoccupied PR (in the absence of progesterone) has an inhibitory or 'braking' effect on ER synthesis. When this brake is removed by an anti-progestin, in the presence of oestradiol, the amounts of ER, PR and AR increase, as oestradiol can increase the expression of all these molecules. Androgens may also play a role in increasing AR after anti-progestin treatment as androgens enhance the biosynthesis of endometrial AR (Adesanya *et al.*, 1999; Apparao *et al.*, 2002).

How these increases in ER, PR and AR at the molecular level lead to mitotic inhibition and endometrial atrophy at the cellular and organ levels remains unclear. One of the keys to understanding these effects lies in the increased glandular AR, because AR is normally not expressed (or only minimally expressed) in the glandular epithelium. Stated simply, our hypothesis is that increases in endometrial AR, induced by anti-progestin treatment, allow androgens to suppress oestrogen-dependent glandular mitosis.

Conclusion

Paradoxically, anti-progestins block oestradiol action on glandular proliferation and suppress the overall growth of the primate endometrium. The mechanism involved in this endometrial anti-proliferative effect is not known, but the data reviewed above indicate that androgens play a previously unsuspected role.

As the androgen receptor is normally expressed primarily in the stroma, an increase in intracellular androgens bound to the glandular epithelium would represent a highly abnormal state that could suppress the metabolic rate and interfere with the mitotic machinery of the glandular cells. In addition, epithelial AR can directly mediate anti-proliferative effects of androgens, at least *in vitro* (Tuckerman *et al.*, 2000), and, because stromal AR is also increased, an enhanced sensitivity of the stroma to androgens could block any oestrogen-dependent, stromal-epithelial interactions involved in glandular mitosis. The

Fig. 3. Hypothetical and known effects of oestradiol administered either (a) alone or (b) combined with an anti-progestin (AP) on the primate endometrium. Growth factor means any stimulatory factor, including cytokines and chemokines. Anti-progestin treatment induces stromal compaction in the endometrium and this is symbolized by the darker background colour in the right panel. (a) In stromal cells, oestradiol (E₂) acts through oestrogen receptor (ER) to induce growth factors that have two effects: stimulation of epithelial mitosis and growth of the spiral arteries (which grow in the follicular phase under oestradiol dominance, and grow further in the luteal phase under progesterone dominance). The androgen receptor (AR) is present at low concentrations and can bind androgens but its effects are unknown. In epithelial cells, oestradiol acts through ER to enhance metabolic activity, as evidenced by the tall, hypertrophied, columnar cells, and presumably to facilitate mitosis. AR is absent or present in very low concentrations in epithelial cells. The diagram refers only to oestradiol-dependent arterial growth; the paradoxical effect of anti-progestins is that they block oestradiol-dependent as well as progesterone-dependent growth. (b) Addition of anti-progestins to oestradiol treatment leads to a great increase in AR, as well as ER and progesterone receptor (PR), in both epithelial and stromal cells. In stromal cells, the increases in androgen (T) action may overcome oestradiol action, inhibit growth factor production and result in two effects: suppression of epithelial mitosis and blockade of oestradiol-dependent spiral artery growth. In epithelial cells, the increases in androgen action may overcome oestradiol action and reduce metabolic activity, as evidenced by the short, atrophied cuboidal cells; this inhibition would favour mitotic suppression.

lack of AR expression in the vascular endothelium and smooth muscle walls also indicates that the atrophy and degenerative changes induced in the spiral arteries by anti-progestin treatment are indirectly mediated. Androgens may play a role in this atrophy and degeneration by suppressing biosynthesis of any oestrogen-dependent, stromal growth factors necessary for arterial growth (Fig. 3).

A cautionary note on this hypothesis is necessary. In addition to the blockade of glandular cell mitosis, anti-

progestins also induce severe compaction of the endometrial stroma, an increase in epithelial apoptosis and severe degenerative changes in the spiral arteries. Arterial atrophy and degeneration may greatly reduce endometrial blood flow, and this alone, through simple nutritional deprivation, could contribute to the endometrial atrophy that occurs in macaques during anti-progestin treatment. The matter is certainly complex and it is not known whether increased androgen action through increased AR can

induce all the endometrial changes induced by anti-progestins. Fortunately, a variety of androgens and anti-androgens is available as tools to conduct experimental studies, so the role of androgens in the paradoxical effects of anti-progestins in the endometrium should eventually be more thoroughly understood. We hope that the novel hypothesis put forward in this brief review will stimulate research along these lines. The anti-progestins and PRMs have tremendous potential for treatment of various diseases, and their use in clinical medicine is increasing. A more complete understanding of their mode of action should greatly benefit women's health.

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