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Activity of Progesterone and Anti-Progestins in a Rat Mammary Primary Cell Culture System

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A primary culture system of virgin rat mammary epithelial cells, grown in a serum-free medium, was developed as a means of assaying the efficacy of compounds with known anti-progestational properties. Cells were grown in 24-well plates on hydrated collagen gels and could be cultured for at least seven days. Experiments were routinely stopped three days after overnight attachment of cells using fibronectin (4 μg/ml). DNA synthesis, measured by thymidine incorporation, was significantly increased by the addition of ovine prolactin (43 nM; $P < 0.01$) or progesterone (0.15 μM; $P < 0.05$) or both ($P < 0.01$) to the basal medium. When added to medium containing progesterone plus prolactin (complete medium), RU486 (mifepristone) and ZK98734 (lilopristone) significantly depressed DNA synthesis in a dose-dependent manner using doses ranging from 0.015 μM to 15 μM. Maximum inhibition was achieved at 15 μM for both compounds. DNA synthesis was 24.5 ± 2.6% (mean ± SEM, n = 4) and 32.0 ± 2.2% ($n = 3$) of that in complete medium for RU486 and ZK98734, respectively (both $P < 0.001$). There was no inhibitory effect of either compound in basal medium or basal medium plus prolactin, indicating the absence of toxicity and that the inhibitory effect is specific for a progesterone-mediated process. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Normal mammary growth *in vivo* is dependent upon both ovarian steroids and pituitary hormones [1]. Generally, progesterone is required for lobuloalveolar development and proliferation of mammary epithelium although, in the rat, prolactin is more important for alveolar development [2]. It has been demonstrated that one of the effects of estrogen *in vivo* is to increase progesterone receptor (PR) levels and this mechanism may account for at least some of the anti-tumour effects of anti-estrogens [3]. Moreover, progestin agonists have been shown to reverse the anti-tumour effects of the anti-estrogen, tamoxifen.

RU486 was first described by Philibert *et al.* [4] as a potent anti-glucocorticoid and was only later demonstrated to bind to both PR and the glucocorticoid receptor (GR) with high affinities [5]. ZK98734 was developed and does not to exhibit crossreactivity with GR [6]. It is still unclear what effect GR activity has on the efficacy of such drugs although unwanted side-effects can occur when RU486 is given prophylactically due to its anti-glucocorticoid activity. Anti-progestins are used in the treatment of breast cancer (see [7] for recent review). Bakker *et al.* [8] have reported a tumour inhibition to 90% in rats, which had tumours induced by DMBA treatment, with a single dose of RU486. When given in conjunction with an LHRH-agonist 75% inhibition was achieved, equivalent to the effect of tamoxifen treatment in the same study. Some success was also reported in the treatment of postmenopausal breast cancer patients. Using data from a range of hormone-dependent tumour models, Michna *et al.* [9] have reported that anti-progestins increase the number of tumour cells in the G_0/G_1 phase of the cell cycle. This is significant since the number of tumour cells in S phase is a known predictor of (node-negative) diploid tumours. They postulated that the anti-tumour properties of anti-progestins may be due to an ability to release the block in initiation of terminal differentiation found in mammary cancers *i.e.* restoring the normal apoptosis found in non-tumourogenic mammary cells.

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A rapid method of screening anti-progestational agents for their effects on mammary growth would be useful in drug discovery and development. We describe here a simple primary culture system using virgin rat mammary epithelial cells grown in a serum-free medium on attached hydrated gels of rat tail collagen. DNA synthesis is stimulated in this system by progesterone in the presence of prolactin. It has been used to study the effects of two known anti-progestins, RU486 and ZK98734, in inhibiting the effects of progesterone.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from Sigma (Poole, Dorset, UK) unless stated otherwise. Collagenase A, from *Clostridium histolyticum* (clostridiopeptidase A, activity 0.65 U/mg) was from Boehringer Mannheim U.K. (Lewes, Sussex). [methyl-^{3}H]Thymidine (185 GBq/mmol) was from Amersham International PLC (Little Chalfont, Bucks). RU486 (mifepristone) and ZK98734 (lilopristone) were gifts from Roussel-Uclaf (Romainville, France) and Schering AG Pharmaceutical Research (Berlin, Germany), respectively. Both compounds were dissolved in absolute ethanol before being diluted in basal medium. Care was taken to ensure final ethanol concentrations were constant at 0.1% for all treatments. Ovine prolactin (NIH-P-S-12) was dissolved in 0.01 M NaOH before being diluted in basal medium to a final concentration of 43 nM (1 mg/l). Water-soluble progesterone (82 mg progesterone/g solid) was dissolved into basal medium directly at 0.15 μM. Filters were cut from graded nylon mesh (Lockertex, Warrington, U.K.). Emulsifier Scintillator Plus (scintillation cocktail) was from Packard (Pangbourne, Berks).

Primary cell culture

Each experiment used mammary tissue from 3 virgin female Wistar rats, 80–100 days of age. Pooled mammary tissue was washed with phenol red-free Medium 199 containing 5 g/l bovine serum albumin (BSA, fraction V) and 25 mmol/l Hepes (BAH199, [10]) then minced finely before being digested at 37°C for 3 h in BAH199 containing 20 mg collagenase A and 50 mg hyaluronidase. The digest was sieved through a 1 mm filter. The filtrate was then passed through a 400 μm mesh and subsequently washed with BAH199, to remove collagenase/hyaluronidase, and stored on ice. The remaining, partially digested, retentate from the 1 mm mesh was rinsed back into the digestion flask using BAH199 containing fresh collagenase/hyaluronidase mixture. This was then digested for a further 2 h before being filtered, washed and pooled with the previous digest. The cells were sieved through a 210 μm mesh before being harvested from a 30 μm mesh [10]. Resultant acini, of a 30–90 μm size, were incubated at room temperature with BAH199 containing 0.05% pronase for 20 min. They were then washed with phenol red-free DMEM/Ham's F12, containing L-glutamine and 15 mM Hepes to which were added: kanamycin monosulphate, 0.128 g/l; gentamycin sulphate, 0.01 g/l; sodium bicarbonate, 1.2 g/l; BSA, 2.5 g/l; insulin (from bovine pancreas, 27 IU/mg), 1 mg/l; and transferrin (siderophilin, iron saturated), 10 mg/l. Fibronectin (4 μg fibronectin/ml medium) was added before cells were seeded onto 24-well plates, in 0.5 ml aliquots, into wells containing 0.5 ml polymerized rat tail collagen. Plates were subsequently incubated in air overnight at 37°C to allow cells to adhere. Medium was replaced with 0.5 ml fresh preparation, without fibronectin, and 50 μl of 10-fold concentrated test solutions were added as necessary. Concentrations of progesterone and prolactin used were based on previously published results [12]. All treatments were replicated four times in each experiment. Cultures were stopped after three to four days with medium changed daily. Gels were removed from wells and washed twice with 0.9% saline before being stored at −20°C.

Measurement of DNA synthesis

Synthesis of DNA was measured by incorporation of [methyl-^{3}H]thymidine (1 μCi or ×37 kBq/well) for the last 24 hours of culture [11]. Gels were dissolved in 2 M acetic acid and DNA precipitated by addition of 50 g/l trichloroacetic acid. After incubation at 70°C for 45 minutes with 8% diaminobenzoic acid, the reaction was stopped with 1 M hydrochloric acid. DNA concentrations were determined by fluorescence, at an excitation wavelength of 420 nm and an emission wavelength of 500 nm, using 0, 5 and 10 μg/ml calf thymus DNA standards as reference. Subsequently, a 10-fold volume of a scintillant cocktail was added and the samples were counted on a liquid scintillation analyser. Results were calculated as dpm/μg DNA.

Statistical analysis

To reduce inter-assay variation (see Results) values were normalized by expressing them as percent medium only within each assay. After normalization, results were compared with medium alone by ANOVA using a Genstat V program (Digital DEC) and by subsequently analysing standard errors of differences between sample means.

RESULTS

Primary culture assay development

Figure 1 shows a comparison of the DNA synthesis of cultures where cell clumps were grown within collagen gels or on top of the gels once they had set. There were no significant differences between the

culture methods. Subsequent cultures were all carried out 'on-gel'.

Time course studies showed a significant ($P < 0.01$) response to prolactin (1 mg/l; 43 nM) compared with the basal medium within two days of culture. Experiments thereafter were routinely terminated three to four days after additions of test compounds. If treatments were replicated four times, three rats routinely yielded enough cells for at least 40 treatments (160 wells). Using data from four separate assays the intra-assay coefficient of variation was 12.9%. The inter-assay coefficient of variation was 40.3% but this could be substantially reduced, to 23.7%, by expressing results as a percentage of medium only within each assay, so reducing animal variation.

Effects of prolactin, progesterone and anti-progestins on DNA synthesis

There was a significant increase in DNA synthesis when either ovine prolactin (43 nM) or progesterone (0.15 μM) was added to basal medium (Fig. 2; $P < 0.01$ and $P < 0.05$; $n = 4$). When progesterone was added together with prolactin there was a significant increase above the level for prolactin alone ($P < 0.05$; $n = 4$).

Addition of the anti-progestins RU486 or ZK98734 to basal medium containing prolactin (43 nM) + progesterone (0.15 μM) (complete medium) produced a dose-dependent, significant inhibition of DNA synthesis (Fig. 3). The effect of both compounds at the highest dose used (15 μM) was to reduce the three-fold increase in DNA synthesis caused by prolactin + progesterone to levels not significantly different from the basal medium. At this dose RU486 was slightly more inhibitory than ZK98734 but the difference was not significant.

To test whether RU486 or ZK98734 were toxic, or possess any progesterone agonist effects, they were added at the same concentrations to basal medium or to basal medium + prolactin (43 nM). There was no

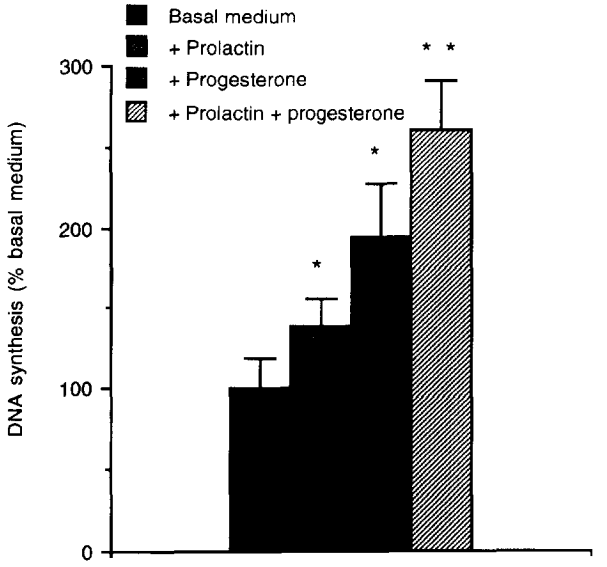

Fig. 2. Effects of basal medium, prolactin (43 nM), progesterone (0.15 μM) or prolactin + progesterone on DNA synthesis by rat mammary epithelial cells on collagen gels. Results are from four separate experiments, expressed as percentage of basal medium within each experiment. Significance levels are: *$P < 0.05$ and **$P < 0.01$ of each treatment compared with basal medium (control).

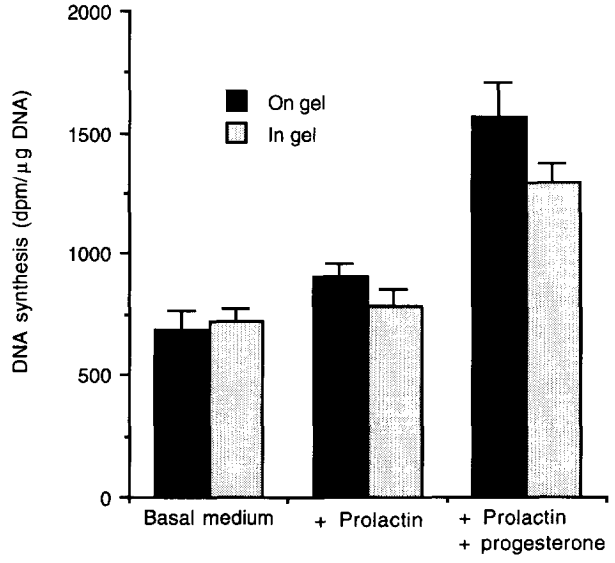

Fig. 1. Effects on DNA synthesis after 5 days of culture 'within gel' or 'on-gel' of virgin rat mammary epithelial cells grown in either serum-free basal medium, basal medium + prolactin (43 nM), or prolactin (43 nM) + progesterone (0.15 μM). Results are given as means + SEM of four replicates within a single experiment.

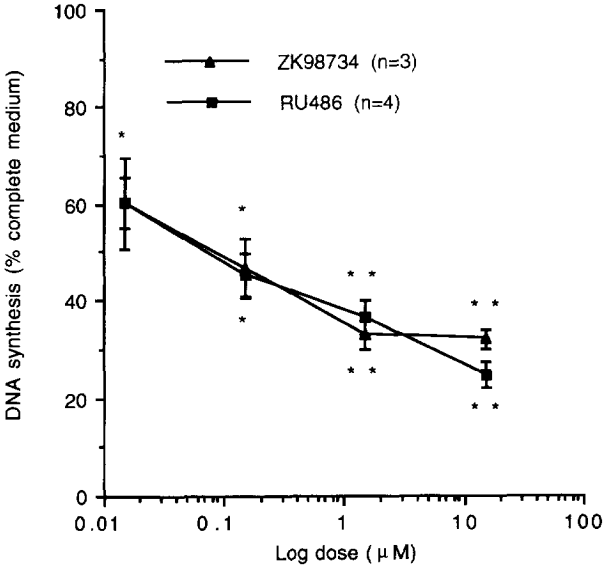

Fig. 3. Effects of anti-progestins RU486 or ZK98734 (0.015–15 μM) on DNA synthesis by rat mammary epithelial cells grown in medium containing prolactin (43 nM) + progesterone (0.15 μM) (complete medium). Results are from four or three separate experiments (respectively) and are expressed as percentage of complete medium within each experiment. Significance levels are: *$P < 0.05$ and **$P < 0.01$ of each dose compared with complete medium (control).

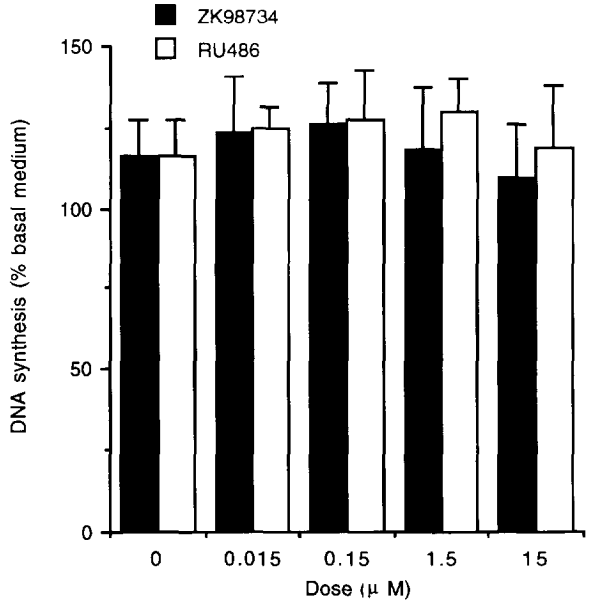

Fig. 4. Effects of prolactin (43 nM) + RU486 or ZK98734 (0.015–15 μM) on DNA synthesis by rat mammary epithelial cells. Results are from two separate experiments and are expressed as percentage of basal medium + prolactin within each experiment.

inhibitory effect of either compound in basal medium (results not shown) nor did either compound significantly increase the response to prolactin (Fig. 4).

DISCUSSION

McGrath *et al.* [12] described a rat mammary epithelial cell primary culture system in which cells were added to solutions of unpolymerized rat tail type 1 collagen which were subsequently polymerized. Cell suspensions were, therefore, created within the collagen gels and developed in three dimensions. Growth was assessed by measuring increase in cell number. Winder *et al.* [10], however, used sheep mammary epithelial cells to develop a primary culture system in which cells were cultured on polymerized hydrated collagen gels. Mitogenic effects were assessed by measuring DNA synthesis. This is a simpler and quicker method as gels can be pre-made and stored in batches, at 4°C, until required. Both methods were tried in the present study, using DNA synthesis as an end point, and gave similar results so subsequent experiments were all carried out 'on-gel'. In the method of McGrath *et al.* [12] the mammary digest is subjected to density gradient centrifugation using Percoll, following initial filtration, in order to separate out epithelial cell clumps. This was also tried in the present study and compared to the above described method of using a series of graded nylon filters. Again results were similar so density gradient centrifugation was not adopted.

The effects of prolactin and progesterone on DNA synthesis in this system are similar to the results of McGrath *et al.* [12] using 'in-gel' cultures. They found significant increases in cell number when prolactin, progesterone or prolactin + progesterone were added to a basal serum-free medium. They also demonstrated, using thioesterase II staining, that the cell type obtained with collagenase digestion and grown under these hormonal conditions was of luminal epithelial origin. Winder *et al.* [10] used antibodies to cytokeratin to demonstrate that the cells obtained in their sheep mammary primary collagen cultures were also epithelial, consisting mainly of luminal cells with a subpopulation of myoepithelial cells. Adapting essentially the latter method in the present experiment and, in addition, including an incubation with pronase in order to reduce myoepithelial cell attachment [13] the cultured cells showed the characteristic polygonal shape of luminal epithelial cells with no other cell types evident.

We have further demonstrated that mammary epithelial cells from virgin rats, cultured in a serum-free primary culture system on collagen gels, can be used to assay anti-progestins. The mammary cells show an increase in DNA synthesis when grown in a medium containing prolactin + progesterone. In this medium they respond to two well-characterized anti-progestins, with a decrease in DNA synthesis that is dose-dependent and reproducible over a wide dose range. There is no evidence for a toxic effect of RU486 or ZK98734 in cells grown in basal medium. The lack of effect when each was added together with prolactin indicates a lack of progesterone agonist activity and shows that the inhibitory effects are dependent on the presence of progesterone. The assay cannot, however, demonstrate at which stage the action of progesterone is opposed. This would require further studies, such as receptor binding of hitherto uncharacterized molecules.

There has been no satisfactory *in vitro* mammary gland assay system, to date, to assess the actions of compounds which modulate progesterone activity. A review of the *in vivo* bioassays available for anti-progestins was published by Bigsby [14]. He proposed an *in vitro* system using rabbit uterine stromal cells grown in serum-free medium. Progestational activity was indicated by secretion of a 42 kD protein, measured by SDS-polyacrylamide gel electrophoresis, fluorography and densitometry. RU486 and ZK98734 were compared in the absence or presence of progesterone (0.01 μM) at doses between 0.01 μM and 1 μM. Both compounds reduced the stimulatory effect of progesterone on the cells to or below basal levels, respectively. The role of progesterone in the uterus, however, is different from that in the mammary gland. In the uterus, estradiol is a proliferative hormone and progesterone a differentiating (as opposed to proliferative) one [3]. Hence, the progestins may exert a role in antagonising the tumourogenic actions of estrogen in the uterus. By contrast, in the mammary gland, progesterone has proliferative effects and is inhibitory to functional activities [15]. For this reason it is preferable

to test the efficacy of anti-progestins in a mammary cell primary culture system if the results are to be related to mammary tumourogenesis. Our assay thus provides a novel means to study the effects of progesterone modulators in mammary epithelial cells. This assay is reproducible and predictive of intrinsic properties of a given compound (*i.e.* as an agonist or antagonist) and has the potential for determining tissue selectivity.

Anti-progestins are increasingly being used, either alone or in tandem with anti-estrogens, in the treatment of breast cancer. New anti-progestins are being developed which, hopefully, confer all the potency of established compounds like RU486 without any of the unwanted side-effects with respect to steroidogenesis. We have demonstrated an animal primary cell culture model, based upon mammary epithelial cells, which offers a useful tool in the study of such drugs.

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