Prolactin Release-inhibitory Effects of Progesterone, Megestrol Acetate, and Mifepristone (RU 38486) by Cultured Rat Pituitary Tumor Cells

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ABSTRACT

The prolactin (PRL) release-inhibitory effects of progesterone, dexamethasone, medroxyprogesterone, and mifepristone (RU 38486) were studied in cultured pituitary tumor cells prepared from the 731Sa and 731Sb tumor. Both tumors contain similar numbers of estrogen and progesterone receptors, while only the 731Sa tumor also has glucocorticoid receptors. PRL release by the 731Sa tumor was stimulated by low concentrations of dexamethasone (10^{-10}-10^{-8} M) and was inhibited in a dose-dependent manner by higher concentrations (~86% by 10^{-7} M). In contrast only 10^{-8} and 10^{-6} M dexamethasone inhibited PRL release by the 731Sb cells by 14 and 24%, respectively. Progesterone caused a dose-dependent inhibition of PRL release, which was similar in the 731Sa and 731Sb tumors. Progesterone (10^{-6} M) inhibited PRL release by 62% and this inhibition was completely prevented by 100 nM estradiol, which was stimulatory to itself (+48%). Mifepristone inhibited PRL release by both tumors in a dose-dependent manner, but more powerfully in the 731Sa tumor. Both tumors contain similar numbers of estrogen and progesterone receptors, while only the 731Sa tumor also has glucocorticoid receptors. Megestrol acetate inhibited PRL release in both tumors in a dose-dependent manner, but more powerfully in the 731Sa tumor; a 10^{-6} M concentration of the compound inhibited PRL release by 52% in the 731Sa and by 26% in the 731Sb tumor cells. Megestrol acetate inhibited PRL release in both tumors in a dose-dependent manner, but more powerfully in the 731Sa tumor; a 10^{-6} M megestrol acetate even stimulated PRL release, suggesting a dexamethasone-like glucocorticoid effect of the drug on this tumor. Thereafter the interaction of mifepristone and megestrol acetate on PRL release was investigated. In the 731Sa tumor cells different combinations of both drugs neutralized each other's inhibitory effects on PRL release, while both drugs had additional inhibitory effects on PRL release by 731Sb tumor cells. Changes in PRL release by the cultured pituitary tumor cells were in all instances closely correlated with changes in the PRL content, the protein content, and the DNA content of the tumor cells. This suggests that the inhibitory effect of the compounds studied on PRL release is paralleled by an inhibitory effect on the number of pituitary tumor cells.

These studies show the importance of the presence of glucocorticoid receptors in the effectiveness and mechanism of action of the antitumor effects of megestrol acetate and mifepristone. In the presence of glucocorticoid receptors (731Sa tumor) the effects of a combination of both compounds are neutralized, while in the absence of these receptors (731Sb tumor) the antitumor effects of both drugs are additive, presumably via an effect on the progesterone receptor. These observations might be of importance in clinical studies involving combinations and/or sequential use of megestrol acetate, mifepristone, and dexamethasone in the treatment of endocrine-dependent cancer in humans.

INTRODUCTION

Synthetic progestins like megestrol acetate and medroxyprogesterone are effective in the treatment of patients with advanced breast cancer (1, 2). The mechanisms through which the progestins exert their antitumor effects are uncertain, however. They have been shown to exert progestin-like, antiestrogenic, antigonadotropic, androgenic, and glucocorticoid-like effects (3-5). Recently mifepristone (RU 38486) was introduced. It is a compound with a powerful progesterone and glucocorticoid receptor-blocking activity without agonist effects on these receptors, while it was also shown to have weak antiandrogenic activities (6-10).

In earlier studies we showed that both megestrol acetate and mifepristone exert a powerful inhibitory effect on the growth of the transplantable PRL/ACTH-secreting rat pituitary tumor 731Sa (11, 12). In the present study we investigated further the mechanism of action of the direct PRL release-inhibitory action of megestrol acetate and mifepristone on cultured 731Sa tumor cells. The 731Sa tumor cells contain estrogen, progesterone, and glucocorticoid receptors. We further developed a derivative of this tumor (731Sb), which contains a similar number of estrogen and progesterone receptors but lacks glucocorticoid receptors. The effects of progesterone, megestrol acetate, and mifepristone on PRL release by cultured 731Sa and 731Sb tumor cells were studied.

MATERIALS AND METHODS

Tumors. The 731Sa tumor was originally obtained from Dr. R. M. MacLeod (Charlottesville, VA) and produces PRL, as well as ACTH. This tumor induces after implantation in Buffalo rats very high circulating PRL and ACTH levels. The biological effect of ACTH can be recognized by an increase in adrenal weights of the tumor-bearing animals from 20-24 to 180-270 mg.

Primary cultures of 731Sa tumor cells released both ACTH and PRL, but after 10 serial passages no radioimmunologically measurable ACTH was detected. Reimplantation of these 731Sa tumor cells into Buffalo rats resulted in hyperprolactinemia and normal circulating ACTH levels, while the adrenal gland weights of the tumor-bearing animals remained normal.

The 731Sb tumor originated from an in vivo dedifferentiation of the 731Sa tumor. It lost its ACTH-secreting capacity spontaneously, as evidenced by normal circulating ACTH levels and a normal adrenal gland weight of the tumor-bearing rats.

Hormone receptor determinations showed 534 fmol/mg protein estrogen receptors, 70 fmol/mg protein progesterone receptors, and 31 fmol/mg protein glucocorticoid receptors in the 731Sa tumor and 315 fmol/mg protein estrogen receptors, 53 fmol/mg protein progesterone receptors, and 0 fmol/mg protein glucocorticoid receptor in the 731Sb tumor (13).

In Vitro Studies. Primary cultures of tumor cells were prepared by mincing pieces of tumor in Hanks' balanced salt solution with 1% bovine serum albumin. The mixture of blood elements and mechanically easily dissociable vital and dead tumor cells was layered on Ficoll-Isoaque (density, 1.077 g/cm³; prepared by University Hospital Dijkzigt Pharmacy) and centrifuged at 1000 x g for 20 min. The interphase fraction containing only the viable tumor cells was washed twice with Hanks' balanced salt solution. The viability of the resulting tumor cell suspension was greater than 90%. These tumor cells were resuspended in Eagle's minimal essential medium with Earle's salts containing 10% FCS, fetal calf serum: FCSα, dextran-coated charcoal-treated FCS without estradiol.
FCS (Gibco Biocult, Glasgow, Scotland), 2 mM 1-glutamine, 1% Eagle’s minimal essential medium with Earle’s salts and nonessential amino acids (Gibco), and 1 mM sodium pyruvate. Further 100 units/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml Fungizone were added to the incubation medium.

The cells were plated at 10^5 cells/dish in Falcon Petri dishes in 1 ml culture medium at 37°C in humidified air with 5% CO2. Cells and drugs were added together on day 1 and the media were harvested at day 7, except if stated otherwise.

Megestrol acetate (Novo Industries, Bagsvaerd, Denmark), mifepristone (RU 38486; Roussel-Uclaf, Paris, France), tamoxifen (ICI, Maclesfield, England), estradiol, dexamethasone, dihydrotestosterone, and progesterone (Sigma Chemical Co., St. Louis, MO) were dissolved first in absolute ethanol and further diluted in culture serum. The final ethanol concentration was less than 0.1% and this percentage was also added to control dishes not containing these drugs.

In some experiments charcoal-treated estrogen-stripped fetal calf serum was used. The 17β-estradiol content of the original fetal calf serum used in these studies amounted to 80-100 pm (final concentration on the dishes, about 10 pm), while 17β-estradiol concentrations had become undetectable after treatment for 30 min at room temperature with 0.5% (w/v) Norit and 0.05% (w/v) dextran T-70. Charcoal was removed by centrifugation for 30 min at 10,000 x g. The supernatant is referred to as dextran-coated charcoal-treated FCS (FCSABS).

At the end of an experiment the media were removed and centrifuged at 1000 x g and the supernatants were stored at -20°C. In addition the PRL content, the protein content and the DNA content of the attached tumor cells were investigated. The cells were scrubbed with a rubber policeman, harvested and measured according to methods described before (14). The protein content of the tumor cells was estimated using the reagent kit from Bio-Rad (Richmond, CA) with bovine serum albumin (Kabi, Stockholm, Sweden) as a standard.

Radioimmunoassay. Rat PRL concentrations of the culture media were measured by double antibody radioimmunoassay procedures using materials and protocols supplied by the hormone distribution office of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. All samples were assayed in duplicate. Statistical analysis was done by analysis of variance, followed by Duncan’s test for determining differences between control and experimental groups.

RESULTS

In Fig. 1 an example is given of the sensitivity of PRL release by the 7315a tumor cells to estradiol and tamoxifen. PRL release by the cultured pituitary tumor cells cultured in 10% FCSABS was followed for 14 days. 1 nM estradiol significantly stimulated PRL release at all time points (P < 0.01 versus control), while 100 nM tamoxifen inhibited PRL release significantly (P < 0.01 versus control). In addition it is shown that 1 nM estradiol significantly overcame the inhibitory effect of 100 nM tamoxifen on PRL release (P < 0.01 versus 100 nM tamoxifen). The sensitivities of PRL release by the 7315a and 7315b tumor cells to estradiol and tamoxifen were similar (data not shown). In the experiments presented in this study tumor cells were studied for 7 days and the effects of different hormones and drugs on PRL release are expressed as percentage of control PRL release.

First the sensitivity of PRL release by the 7315a and 7315b tumor cells to dexamethasone was studied (Fig. 2). In the 7315a cells there was a biphasic effect of dexamethasone; 10^{-10} and 10^{-9} M dexamethasone stimulated PRL release significantly by 33 ± 6% (67 ± 4%, respectively (P < 0.01 versus control), while 10^{-8} M, 10^{-7} M, and higher concentrations of dexamethasone inhibited PRL release significantly in a dose-dependent manner (Fig. 2). The sensitivity of PRL release to dexamethasone by the 7315b tumor cells, however, differed considerably from that observed in the 7315a cells. Only 10^{-4} and 10^{-3} M dexamethasone inhibited PRL release significantly by -14 ± 3% (P < 0.05 versus control) and by -24 ± 4% (P < 0.01 versus control), respectively. These differences between the sensitivities to dexamethasone of the 7315a and 7315b tumor cells reflects the presence of glucocorticoid receptors on the 7315a tumor and the absence of these receptors on the 7315b tumor.

PRL release by the 7315a and 7315b tumor cells was highly sensitive to progesterone. Fig. 3 shows the dose-response curves of progesterone on PRL release by 7315b tumor cells grown in medium plus 10% FCS, 10% FCSABS plus 100 nM estradiol, respectively. In the absence of estradiol (FCSABS), 10^{-13} M progesterone already inhibits PRL release significantly (~38%; P < 0.01 versus control), while the tumor cells cultured in untreated FCS were significantly less sensitive to progesterone at low concentrations (P < 0.01 in all instances: 10^{-13}, 10^{-11},
A further decrease in the sensitivity to progesterone was noticed if the tumor cells had been cultured in 100 nM estradiol. Progesterone (10⁻¹¹ M) did not inhibit the stimulatory effect of 100 nM estradiol on PRL release, but both 10⁻¹¹ and 10⁻⁹ M progesterone completely overcame this stimulation. The sensitivities to progesterone of PRL release by the 7315a and 7315b tumor cells were similar (data not shown).

The effects of different concentrations of mifepristone (RU 38486) and megestrol acetate on PRL release by the 7315a and 7315b tumor cells are shown in Fig. 4. In Fig. 4 (left) it is evident that mifepristone causes a dose-dependent inhibition of PRL release in both tumor cell suspensions. PRL release by the 7315a tumor cells, however, was significantly more inhibited by 10⁻⁶ M mifepristone than that by the 7315b tumor cells (−51 ± 3% versus −25 ± 3%, respectively; P < 0.01). The experiments shown in Fig. 4 were carried out with tumor cells cultured in 10% FCS. In Table 1 it is shown that the sensitivity of PRL release to mifepristone was significantly higher when the cells were cultured in FCS alone.

The sensitivity of PRL release by the 7315a and b tumor cells to megestrol acetate is shown in Fig. 4 (right). In contrast to the effects of mifepristone, megestrol acetate at low concentrations of 10⁻¹⁰-10⁻⁸ M inhibited PRL release by 7315b tumor cells significantly more than that by 7315a cells (P < 0.01 versus control and versus 7315a cells in all instances). In the 7315a tumor cells a biphasic curve was observed which was similar to that observed with dexamethasone; 10⁻⁹ M megestrol acetate stimulated PRL release by the 7315a tumor cells by 12 ± 2% (P < 0.05 versus control), while 10⁻⁸-10⁻⁵ M concentrations of the compound inhibited PRL release in a dose-dependent manner (P < 0.01 versus control for each concentration). Interestingly, 10⁻⁷-10⁻⁴ M megestrol acetate inhibited PRL release by the 7315a tumor cells more than that by 7315b cells (P < 0.01 for 10⁻⁷ and 10⁻⁶ M).

Thereafter the interaction of mifepristone and megestrol acetate on PRL release was investigated. In the 7315a tumor cells 10⁻⁷ M mifepristone significantly attenuated the inhibitory effect of 10⁻⁸ and 10⁻⁷ M megestrol acetate on PRL release while 10⁻⁴ M megestrol acetate completely overcame the inhibitory effect of 10⁻⁷ M mifepristone (Fig. 5). The combination of 10⁻³ M megestrol acetate and 10⁻⁷ M megestrol acetate did not inhibit PRL release (−7 ± 5%), while both drugs separately inhibited PRL release significantly (−20 ± 3% in response to 10⁻³ M megestrol acetate [P < 0.01 versus control] and −15 ± 3% in response to 10⁻⁷ M megestrol acetate [P < 0.05 versus control]). It was noted that the powerful inhibitory action of 10⁻⁷ M megestrol acetate (−76 ± 3%) was completely neutralized by 10⁻⁷ M mifepristone (−16 ± 4 versus −15 ± 3% for mifepristone alone). In the 7315b cell line, however, a different pattern of the interrelations between both drugs evolved; for example 5 × 10⁻⁴ M mifepristone inhibited PRL release by 52 ± 4% and 10⁻⁸ M megestrol acetate inhibited hormone release by 44 ± 3%. The combination of these concentrations of both drugs exerted in these cells an additive inhibitory action on PRL release (−84 ± 5%; P < 0.01 and P < 0.01 versus mifepristone and megestrol acetate alone).

Finally it was asked whether the changes in the release of PRL by the cultured pituitary tumor cells, as induced by dexamethasone, progesterone, mifepristone, and megestrol acetate, might reflect a change in the mitotic activity of the tumor cells. There was in all instances a close correlation between the release of PRL into the medium, the PRL content of the tumor cells, and the protein and DNA content of the tumor cells (P < 0.001 in all instances). About 9 times more PRL was found in the medium than intracellularly (versus Fig. 6). As an example the relationship in individual dishes is shown between the protein content of the tumor cells and PRL release into the medium [Fig. 6 (top)], and the tumor cell protein content and PRL content of the cells [Fig. 6 (bottom)] are shown of different concentrations of progesterone (control, 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M). Similar observations were done by measuring the DNA content of the tumor cells, and also with regard to the effects of dexamethasone, mifepristone, and megestrol acetate on protein content and DNA content of the cultured pituitary tumor cells.

DISCUSSION

In this study we used two PRL-secreting pituitary tumors, which originated from the estrogen-induced PRL/ACTH-secreting pituitary tumor 7315a. Both tumors contain similar amounts of estrogen and progesterone receptors, but the 7315a tumor contains also glucocorticoid receptors, which are absent in the 7315b tumor. The significance of this difference is underlined by the observation that 10⁻⁸ M dexamethasone inhibited PRL release by the 7315a cells by nearly 50%, while only very high concentrations (10⁻⁶ and 10⁻⁵ M) of this glucocorticoid significantly inhibited hormone release by the 7315b cells.

Our studies give some insights in the mechanism of action and the interrelationships of progestins (like megestrol acetate)
Table 1: Effect of different fetal calf serum preparations on the sensitivity of PRL release by 7315a tumor cells to mifepristone

<table>
<thead>
<tr>
<th>Serum Preparation</th>
<th>Cells cultured in 10% FCS</th>
<th>Cells cultured in 10% FCSABT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Mifepristone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^-10 M</td>
<td>93 ± 3*</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>10^-9 M</td>
<td>88 ± 2*</td>
<td>87 ± 3*</td>
</tr>
<tr>
<td>10^-8 M</td>
<td>80 ± 2*</td>
<td>68 ± 2*</td>
</tr>
<tr>
<td>10^-7 M</td>
<td>74 ± 4*</td>
<td>50 ± 2*</td>
</tr>
<tr>
<td>10^-6 M</td>
<td>49 ± 3*</td>
<td>38 ± 3*</td>
</tr>
</tbody>
</table>

* P < 0.01 versus control.
* P < 0.01 versus cells cultured with the same concentration of mifepristone in FCS.
* P < 0.05 versus cells cultured with 10^-4 M mifepristone in FCS.

Fig. 5. Effect of 100 nM mifepristone on megestrol acetate-induced changes in PRL release by 7315a tumor cells cultured in 10% FCS. PRL release after 7 days is expressed as percentage of control (points, mean; bars, SE; N = 4/group).

and the new antiprogestational drug mifepristone (RU 38486). It is well known that the progestins not only bind to the progesterone receptor but have also antiestrogenic, androgenic, and glucocorticoid-like effects (4, 5, 15). Mifepristone was characterized as a powerful progesterone receptor-blocking drug and a glucocorticoid receptor blocker without agonist activity, while it also seems to have some antiandrogenic activities (6–11).

The 7315a and b tumor cells in culture react with regard to PRL release in a manner similar to that of several tumor cell lines like the MCF7-cell line with regard to their duplication/DNA content; they are stimulated by estradiol and inhibited by tamoxifen and progesterone. The inhibitory effect of progesterone on PRL release is antagonized by estradiol, as has been described before in other cell systems (16). It has not been shown that this points to a specific antiestrogenic effect of progesterone; its effects might be mediated via an unrelated mechanism of action. The same applies for example to the difference in sensitivity to mifepristone in FCS- and FCSABT-cultured tumor cells. The picture evolving from our studies with mifepristone and megestrol acetate suggests the importance of glucocorticoid receptors on these tumors. In the presence of such receptors in 7315a cells, mifepristone is more active in its PRL release-inhibitory effects than in their absence (7315b cells). This probably is caused by the antiguocorticoid action of the drug. In accordance with this, in the 7315a tumor cells higher concentrations of megestrol acetate are needed to inhibit PRL release. In the absence of glucocorticoid receptors (7315b cells), however, PRL release is more sensitive to megestrol acetate. The slight stimulation of PRL release in response to 10^-7 M megestrol acetate in the 7315a cells probably points to a cytoprotective effect as was also observed with low concentrations of dexamethasone. In the presence of glucocorticoid receptors, the PRL release-inhibitory effect of high concentrations of megestrol acetate seems at least partly mediated via these receptors, because mifepristone is able to virtually completely overcome this effect. In contrast, in the absence of glucocorticoid receptors, the inhibitory effects on PRL release of both drugs are additive. These observations might be of clinical importance as part of the tumor growth-inhibitory effect of a combination of both drugs on endocrine-dependent cancers in humans which contain glucocorticoid receptors might well be neutralized. Caution in the simultaneous use in clinical trials of progestins like megestrol acetate, mifepristone, and glucocorticoids is suggested.

Our studies suggest that the actions of progesterone, progestins (megestrol acetate), and a progesterone receptor antagonist (mifepristone) on PRL-secreting pituitary tumor cells cannot be explained simply by agonist, mixed agonist-antagonist, and antagonistic effects on the progesterone receptor. In fact, progesterone exerted a more powerful inhibitory action on PRL release.

Fig. 6. Relation between PRL release (ng/dish) and the protein content (top) and between the intracellular PRL content and the protein content (bottom) of 7315a tumor cells cultured for 14 days in the presence of different concentrations of progesterone (10^-3–10^-4 M); 4–5 dishes/group (cf. Fig. 2).
release than its receptor antagonist mifepristone. Several groups of investigators have studied this question extensively in human breast cancer cell lines. Bardon et al. (17) showed that a 100 nM concentration of synthetic progesterin R5020 and 100 nM mifepristone separately exert a similar inhibitory effect on the growth of the progesterone receptor-positive human breast cancer cell line T47D. A low concentration of 1 nM R5020 protected these cells slightly against the antimitotic effect of mifepristone. Since higher concentrations of R5020 are also growth inhibitory, the cells were not to be rescued from the effect of higher concentrations of mifepristone. Horwitz (18) also showed that mifepristone acts in the T47D tumor cell line as a progestin in terms of growth inhibition; 1 nM and 10 nM of R5020 inhibited the cell number after 10 days by 50 and 60%, respectively, while 1 nM mifepristone inhibited growth by 55%. Combining R5020 and mifepristone, however, had no significant effect and cell growth remained uninhibited. Mullick and Katzenellenbogen (19) further investigated the possible mechanism of action of mifepristone in MCF-7 and T47D human breast cancer cell lines. They found that mifepristone-labeled progesterone receptor complexes exist in a heavier form (6S), which can be converted to a small 4S form by disaggregation with urea. These authors hypothesized that the antagonsitic action of drugs like mifepristone may result from their ability to engage the receptor in a biologically nonproductive interaction, which they detected as a larger sedimenting species.

We showed evidence that the changes in PRL release by the cultured pituitary tumor cells as induced by dexamethasone, progesterone, mifepristone, and megestrol acetate reflect changes in the number of tumor cells. Although the actual number of tumor cells was not measured, parallel changes in PRL release and the protein and DNA content of the tumor cells support such a close correlation between hormone secretion and mitotic activity. Unrelated changes, for example, in the number of fibroblasts seem to be excluded inasmuch as the protein and DNA content of the tumor cells also closely correlated with the PRL content of the tumor cells.

In conclusion, the present study shows that progesterone, megestrol acetate, and mifepristone inhibit PRL release by and probably the mitotic activity of cultured estrogen-progesterone receptor-positive pituitary tumor cells (7315b) by a mechanism which probably involves only the progesterone receptor. In the model of the estrogen-, progesterone-, and glucocorticoid receptor-positive tumor 7315a, a different mechanism of action of both drugs is suggested, in which the glucocorticoid-like (megestrol acetate) and antiglucocorticoid (mifepristone) activities prevail and in which both drugs (partly) neutralize each other’s antitumor effects.

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