Levonorgestrel, Medroxyprogesterone and Progesterone Cause a Concentration-dependent Reduction in Endometrial Cancer (Ishikawa) Cell Density, and High Concentrations of Progesterone and Mifepristone Act in Synergy

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Abstract. Endometrial hyperplasia is a precursor lesion of endometrial carcinoma. Clinical studies of endometrial hyperplasia have shown that levonorgestrel (LNG) is more therapeutically effective than medroxyprogesterone acetate (MPA). The present pharmacological in vitro study was performed to compare progestin effects on human endometrial cancer (Ishikawa) cells. Supraphysiological concentrations of progesterone (PG) and high concentrations of LNG and MPA were employed to determine the order of potency in reducing cell density. The order of potency was LNG>MPA>PG with respective 50% inhibitory concentrations (IC50) of 3.9±0.4, 30.4±3.4 and 45.3±2.7 μM. Mifepristone (MF) is a potent antiprogestin, but was unable to antagonize the PG-induced cell density reduction. For MF concentrations from 0.2 to 70 μM alone, a PG-mimetic effect was observed with an IC50 value of 19.0±1.7 μM. When PG and MF were combined, a marked reinforcement of the effect was seen. These observations indicate that extranuclear initiated signaling pathways are involved in the reduction of endometrial cancer cells exposed to high concentrations of PG and MF.

All progestins have in common the ability to regulate endometrial proliferation by suppressing estrogen-induced mitosis in the uterine glands and stroma. In a clinical study of endometrial hyperplasia, a precursor lesion of endometrial carcinoma, the effect of a routine therapeutic regimen, oral medroxyprogesterone acetate (MPA), was compared to intrauterine administration (IUD) of levonorgestrel (LNG). After three months, the IUD with LNG gave complete regression, whereas, oral administration of MPA caused regression in only 45% of the patients (1). The dissimilar administration route will necessarily contribute to this effect since higher local concentrations are achieved with IUD (2). However, the present study also questioned whether LNG was in fact more potent as compared to MPA.

Mifepristone (MF) is an antiprogestin that binds with high affinity to progesterone receptors (PR), 2- to 10-fold that of progesterone (PG) (3), but can act as a partial agonist in the absence of PG (4). This may explain why MF, similar to PG, has an antiproliferative effect on various cell types, including normal and transformed endometrial cells (5-10).

In the present work, we have characterized the pharmacological effects of supraphysiological PG concentrations and corresponding high concentrations of the two progestins LNG and MPA, and the antiprogestin MF on endometrial cancer (Ishikawa) cells.

Materials and Methods

Chemicals. PG, MPA, LNG and MF were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Cell culture. Ishikawa cells, a human cell line of transformed endometrial cells (11), were obtained from the European Collection of Cell Cultures (Salisbury, UK). Monolayer cultures were established in RPMI 1640 medium (Sigma-Aldrich Chemical Company) with either 10% (v/v) fetal calf serum (FCS), 10% (v/v) dialyzed FCS (dFCS) or 10% (v/v) stripped FCS (sFCS) obtained from PAA-laboratories (Pasching, Austria) without phenol red to avoid potential estrogenic effects (12) and without antibiotics. Cells were seeded at a density of approximately 1x10^5 cells/ml in 25 cm² culture flasks in humidified atmosphere (5% CO2) at 37°C with daily changes of serum supplemented medium. Medium with or without progestin and/or MF was renewed daily for the next five days. Medium and non-adherent cells were aspirated and adherent cells were detached by trypsinization and activity was terminated by adding serum (10% v/v) containing medium. The Ishikawa cell
densities were determined in a cytometer and viability was assayed by trypan blue dye exclusion.

**Significance of ethanol as solvent.** The progestins and MF were dissolved in ethanol. The final ethanol concentration in the culture medium was 0.2 % (v/v). Pilot experiments showed that cell densities with and without ethanol were linearly correlated, in plain FCS (slope=0.971 and intercept=0, r=0.999), in dFCS (slope=1.05 and intercept=0, r=0.993) and in sFCS (slope=1.14 and intercept=0, r=0.964). When PG and MF were combined, the final concentration of ethanol was 0.4 % (v/v), but this concentration did not affect Ishikawa cell growth (slope=0.940 and intercept=0, r=0.996).

**Significance of serum preparation.** Since removable serum factors may modulate the pharmacological effect of PG, the growth of Ishikawa cells and the effect of PG were tested with FCS, dFCS and sFCS. Table I shows that 95 μM PG had a cytotoxic effect, with a cell density reduction of 76.1±0.7% with FCS (n=2), 84.3±0.2% with dFCS (n=3) and 83.2±1.2% with sFCS (n=3) added. The 50% inhibiting concentrations (IC50) were 61.5±8.2 μM, 53.8±6.7 μM and 50.6±1.0 μM in the presence of FCS, dFCS and sFCS, respectively. These observations indicate that serum factors which are removable by dialysis or charcoal treatment have no or only minor impact on effect maximum or sensitivity to supra-physiological PG concentrations.

**Expression of PR.** The presence of progesterone receptors (PR) in Ishikawa cells was verified with immunocytochemistry (data not shown) and assayed as described elsewhere (13).

**IC50 values.** The IC50 values, defined as the concentrations causing half maximal growth inhibition after 5 days exposure to the active agents, were calculated according to Chou (14).

**Descriptive statistics.** The results are presented as mean±SEM (if not stated otherwise).

**Results**

**Effect of PG.** In the absence of PG the cell densities were 13.4±0.4 × 10^5 at the start of the experimental period and 39.6±0.8 × 10^5 cells/ml after 5 days. Figure 1 shows that a concentration-dependent effect was evident as early as 24 h after the addition of PG. During the defined experimental period, the concentration-dependent reduction in cell density showed a time-dependent change in the relative effect with a maximum effect after 4-5 days. The highest PG (70 μM) concentration caused a cytotoxic effect as early as 24 h after addition, with a reduction in cell density to 12.1±0.3 × 10^5 cells/ml. After five days, a further reduction to 5.7±0.4 × 10^5 cells/ml was seen.

**Sensitivity to PG, LNG and MPA.** The ability of LNG and MPA to reduce Ishikawa cell density was explored. In the presence of dFCS (n=3), the effect of the progestins showed an order of inhibition of LNG>MPA>PG (Figure 2). The respective IC50 values were 3.9±0.4 μM, 30.4±3.4 μM and 45.3±2.7 μM. Virtually identical results were obtained for cells grown in the presence of FCS (n=5). The order of potency among the progestins was again LNG>MPA>PG. The respective IC50 values were 4.0±0.3 μM, 30.0±0.1 μM and 50.2±1.3 μM.

<table>
<thead>
<tr>
<th>Cell density (10^5 cells/ml)</th>
<th>FCS (n=2)</th>
<th>dFCS (n=3)</th>
<th>sFCS (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.4±0.28</td>
<td>13.4±0.6</td>
<td>11.6±1.1</td>
</tr>
<tr>
<td>At the end of period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31.4±0.1</td>
<td>39.6±1.6</td>
<td>41.7±0.5</td>
</tr>
<tr>
<td>PG (95 μM)</td>
<td>7.3±0.2</td>
<td>5.7±0.6</td>
<td>6.7±0.7</td>
</tr>
</tbody>
</table>

Table I. Ishikawa cell density at the start and end of the experimental period (5 days) with and without PG are presented as mean value±SEM. Cells were seeded at a density of approximately 1×10^5 cells/ml.
Development of MF effect. In the absence of MF, the cell densities were 12.4±4.0 × 10^5 cells/ml and 30.2±0.7 × 10^5 cells/ml at the start of the experimental period and after 5 days, respectively. Figure 3 shows that a concentration-dependent effect was evident as early as 24 h after the first exposure to MF. The concentration-dependent reduction in cell density was present for the rest of the experimental period. The lowest concentration, 2.3 μM MF, had no or only a minor effect on cell density the first two days, but thereafter a 25 - 30% reduction was observed. After five days a 70% reduction was achieved with 23 μM, whereas, no living cells were left upon harvesting the fourth and fifth day after exposure to 70 μM. In separate experiments, the concentration response relationship was analyzed after five days. An IC50 value of 19.0±1.7 μM for MF was obtained.

Effect of PG and MF in combination. Since MF is classified as one of the most potent PG antagonists (15), the effect of PG (32 μM), MF (23 μM) and their combination was studied. In the absence of PG and/or MF the cell densities were 13.4±0.4 × 10^5 and 39.6±0.8 × 10^5 cells/ml at the start of the experimental period and after 5 days, respectively. Instead of antagonism, the two substances showed a reinforced effect on cell density reduction, evident throughout the exposure period (Figure 4). After five days the cell densities were reduced to 15.7±0.7, 9.3±0.3 and 6.2±0.5 × 10^5 cells/ml with PG, MF and their combination, respectively.

In an attempt to reverse the observed PG-induced reduction in Ishikawa cell density, various MF concentrations (0.23-23 μM) were combined with one supraphysiological concentration of PG (32 μM). After five days’ exposure, 23 μM MF increased the effect of 32 μM PG, whereas 0.23 μM and 2.3 μM MF had no marked influence on the effect of PG (Figure 5).

Discussion

In the present study we found that PG in pharmacological concentrations caused concentration-dependent reduction in Ishikawa cell density. These cells possess PR (16), but the present IC50 values (50-60 μM) of PG were approximately 1000-fold higher than the reported Kd values for PR (17). In previous studies on a transformed human cell line, C-4I cells which lack PR, we also found a concentration-dependent inhibition of cell density by progestins in high concentrations, but with somewhat lower IC50 values (13, 18). In addition, the failure of MF to antagonize the effect
of PG suggests that the observed effects are initiated by molecular targets other than PR.

The inhibition of estrogen-mediated proliferation of the uterine epithelium has been ascribed to nuclear PR (19). However, a more complex picture has emerged during the last decade. Progestin effects have been shown to be mediated through crosstalk between the classical and new molecular targets (20-23). The present results may indicate such a crosstalk between alternative pathways or may solely represent extranuclear initiated signaling (24). A recent study using microarray analysis, showed that supraphysiological PG concentrations modulated the expression of 247 different genes in Ishikawa cells (25), among these, several genes related to biological processes like cell cycle, cell proliferation and differentiation, and including genes involved in extranuclear signaling pathways.

The observed order of potency of LNG>MPA>PG also points to the higher effectiveness of IUD LNG than per orally administered MPA (1). In addition, the local administration (IUD) of LNG causes higher tissue concentrations (2). The observation that endometrial PR are almost completely down-regulated with a maintained clinical antiproliferative effect after three months with IUD LNG (26) also questions the contribution of PR to the normalization of endometrial hyperplasias.

Recent reviews characterize MF as a potent antagonist to PG, but also show it to have agonist characteristics (4, 27). This may explain the antiproliferative effect of MF on non-human and human endometrium (5, 7, 8, 10), the benign endometrial cell line EM42 (6, 9) and the human endometrial carcinoma cell line RL95-2 (6). However, the MF concentrations employed in the present study are far above those needed for saturation of PR. A recent study showed that MF inhibited growth of two endometrial cancer cell lines, HEC-1-A and Ishikawa, with IC50 values of 40-45 μM (28), in close agreement with the reported value (19 μM) in the present work.

As far as we know, it has not previously been reported that PG and MF act in synergy to reduce the density of growing endometrial cancer cells. Whether PG and MF have a
common primary extranuclear target is not known, but an inhibition of ABC-transporters by PG and MF has been reported, including that of P-glycoprotein (29-34) and multidrug resistance proteins (MRPs) (34-36). Retention of intracellular molecules by progestins or MF, such as cGMP (35), can inhibit growth and induce apoptosis.

In conclusion, the present study shows that high concentrations of PG and MF reduced Ishikawa cell density in a concentration-dependent manner, and the highest concentrations of both PG and MF were cytotoxic. Since PG and MF reinforced the effect of each other, the possibility exists that they have a common extranuclear molecular target which initiates signaling.

Acknowledgements

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