Mechanisms Involved in the Evolution of Progestin Resistance in Human Breast Cancer Cells

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ABSTRACT

The emergence of resistant cells reduces the efficacy of many forms of drug therapy in human breast cancer. In order to understand some of the possible mechanisms by which hormonally dependent human breast cancers develop resistance to progestin therapy we have developed a human breast cancer cell line (5-RP) which is resistant to the growth inhibitory effects of progestins in culture. These cells routinely grow in 10 μM medroxyprogesterone acetate (MPA). The cell line was developed from T-47D-5 human breast cancer cells by stepwise selection in increasing concentrations of MPA. The progestin-resistant phenotype was relatively stable as assessed by the removal of MPA from the medium for varying periods of time. 5-RP cells passaged in the absence of MPA were still essentially insensitive to the growth inhibitory effects of MPA for at least 22 passages. Even at 53 passages out of the drug the 5-RP line was still less sensitive than the original T-47D-5 parent line.

Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) receptor mRNA were both increased in the 5-RP line compared to the T-47D-5. Consistent with increased TGF-α expression, the EGF receptor measured by ligand binding was decreased. When the cells were removed from MPA, TGF-α expression declined gradually, but EGF-receptor mRNA levels increased, as did EGF-binding activity.

These cells remained estrogen and progesterone receptor positive. Although progestins did not downregulate estrogen receptor expression, they did downregulate progesterone receptor expression in the 5-RP line. The progesterone receptor level of the 5-RP line, in the absence of MPA, was approximately 58% of that found in T-47D-5 cells, even after MPA had been removed for long periods of time. This decrease in receptor level was reflected in decreased ability to respond to progestins as assessed by the decreased ability of MPA to activate expression of both an endogenous gene (EGF receptor) as well as a transfected progesterin-responsive gene (MVTM-TK-CAT).

Progestin resistance in the 5-RP cell line appears to be multifactorial, involving both increased growth factor expression and decreased receptor levels. It is likely, however, that these two aspects do not account entirely for the progestin-resistant phenotype and as yet other unidentified mechanisms may also be involved.

INTRODUCTION

Although antiestrogens remain the first choice of hormonal manipulative therapy for human breast cancer, progestin treatment of hormone-dependent breast cancer is receiving renewed attention. Tumors which have become resistant to antiestrogen treatment often may respond subsequently to other forms of endocrine therapy such as progestin treatment (1). These observations suggest that antiestrogens and progestins may act by different mechanisms to promote remission in breast cancer.

MATERIALS AND METHODS

Materials. MPA and estradiol 17β were purchased from Sigma Chemical Co. (St. Louis, MO). R 5020, [3H]R 5020 (87 Ci/mmol), [3H]estradiol 17β (37.9 Ci/mmol), [32P]dCTP were purchased from NEN (Lachine, Quebec, Canada). 4-Hydroxytamoxifen was a gift from ICI (Macclesfield, Cheshire, England). Murine epidermal growth factor was purchased from Collaborative Research (Bedford, MA). Dulbecco's minimal essential medium powder and fetal bovine serum were purchased from Gibco/BRL (Burlington, Ontario, Canada). All other cell culture medium ingredients were purchased from Flow Laboratories (Mississauga, Ontario, Canada). Leupeptin and pepstatin A were from Sigma, aprotonin (10,000 kallikrein inactivating units/ml) was from Miles Pharmaceuticals (Rexdale, Ontario, Canada), and recombinant human TGF-α was from UBI, Inc. (Lake Placid, NY).

Cells. The T-47D-5 line was kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia) in whose laboratory it was originally isolated (4). The cells were grown in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum, glutamine, glucose, and penicillin-streptomycin (3). Cells were harvested by scraping the cells with a rubber policeman. After centrifugation the cell pellet was frozen and stored at -70°C until RNA was isolated.

The 5-RP cell line was isolated from T-47D-5 cells by plating an aliquot of these cells in the above medium containing 1 μM MPA. Medium containing MPA was replenished every 3-4 days. When the surviving cells had grown to a high density but were still less than confluent, an aliquot of these cells was plated in the presence of a higher concentration of MPA. Sequentially, 1.0, 2.5, 5.0, 7.5, and 10 μM MPA was used.

For cell growth experiments, cells were plated at approximately 104 cells/30-mm dish. Two days later the medium was replaced with fresh medium containing varying concentrations of the drugs to be tested. Cells were harvested, in triplicate, 5 days later and cell numbers were counted using an electronic cell counter.

RNA Extraction and Northern Blot Analysis. RNA was isolated by the guanidinium thiocyanate/cesium chloride method (5). Poly(A)+ RNA was isolated by one cycle of oligo(dt)-cellulose chromatography. Ten to 15 μg of poly(A)+ RNA was denatured in 50% (v/v) formaldehie and 2.2 M formaldehyde, size separated by electrophoresis.

Received 2/23/90; accepted 2/4/91.

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1 This work was supported by grants from the National Cancer Institute (Canada), the Medical Research Council (Canada), and the Manitoba Health Research Council. L. C. M. is a Cancer Institute (Canada) Scientist, L. J. M. is a Medical Research Council (Canada) Scholar, M. S. J. W. holds a Medical Research Council (Canada) studentship award, and T. M. holds a Manitoba Health Research Council studentship award.

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2051
on 1% (w/v) agarose gels containing 2.2 m formaldehyde, and then blotted onto nitrocellulose (7). Filters were baked for 2 h at 80°C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized sequentially with the 1.9-kilobase pair human EGF cDNA insert from λ EGF15(c) (8), the pE7 cDNA insert for the human EGF receptor (kindly provided by Dr. I. Pastan), a 900-base pair human TGF-α cDNA insert (kindly provided by Dr. G. I. Bell), and the human estrogen receptor cDNA insert, OR-8, and the human progesterone receptor cDNA insert (both kindly provided by Dr. P. Chambon). Each hybridization was carried out after the signal from the previous hybridization had decayed. Hybridizations, usually for 48 h, were performed at 42°C in the presence of 50% (v/v) deionized formamide, 5× Denhardt’s solution (1× Denhardt’s = 0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 5× standard saline phosphate EDTA (1× standard saline phosphate EDTA = 1.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA), 250 μg/ml denatured salmon sperm DNA, and 0.1% SDS. At the end of the hybridization period the blots were washed twice in 2× SSC, 0.1% SDS (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 15-30 min at room temperature, followed by three 20-min washes in 0.1× SSC, 0.1% SDS at 65°C. Filters were also hybridized with chicken β-actin cDNA (9) as a control for differences in the amount of RNA loaded on the gel. Filters were exposed to Kodak XAR film at -70°C with an intensifying screen.

Whole Cell Estrogen and Progesterone Receptor Assays. Cells were plated in charcoal-stripped fetal calf serum-containing medium into 24-multiwell plates. Three to 4 days later whole cell estrogen and progesterone receptor assays were performed as previously described (10). [³H]Estradiol 17β and excess unlabeled estradiol 17β, [³H]R 5020, and excess unlabeled R 5020 were used to determine estrogen and progesterone receptor binding and nonspecific binding, respectively. The progesterone receptor assay was also done in the presence of 100 nm dexamethasone to minimize binding of R 5020 to the glucocorticoid receptor in these cells.

EGF Receptor Assays. Cells were plated in medium containing 5% fetal bovine serum in 6-well plates. Three to 5 days later they were assayed for EGF-binding activity as previously described (11). Where cells were to be treated with MPA, fresh medium with the appropriate concentration of MPA was added 24 h after the binding assay was performed.

Preparation of Conditioned Medium and Radioimmunoassay for TGF-α. Cells were grown to 60-80% confluence in fetal bovine serum-containing medium as described above. The serum-containing medium was replaced with serum-free medium overnight. This latter medium was discarded and fresh serum-free medium was added and collected after 48 h of conditioning. The CM was plated on ice and the following protease inhibitors were added to give the indicated final concentrations: 1 mM phenylmethylsulfonyl fluoride, 20 kallikrein inactivating units/ml aprotinin, 20 ng/ml leupeptin, and 20 ng/ml pepstatin. The CM was centrifuged at 3000 x g for 15 min at 4°C and then filtered through a 0.45-nm filter (MSI; Fisher Scientific, Winnipeg, Manitoba, Canada). The CM was then concentrated between 100- and 200-fold using an Amicon ultrafiltration unit and Diaflo YM5 membranes (Amicon Division of W. R. Grace and Co., Danvers, MA). The concentrated conditioned media were stored frozen until assayed. Unconditioned medium was used to control and treated as described above and provided the medium blank.

TGF-α immunoreactivity in conditioned medium was assayed using a TGF-α radioimmunoassay kit (Biotope, Inc., Seattle, WA). The assay was performed in duplicate. Two independently conditioned samples of medium from T-47D-5 cells were assayed and four independent conditioned samples of medium from 5-RP cells in the presence of 10 μM MPA were assayed.

DNA Transfection and CAT Assay. T-47D-5 and 5-RP cells which had been removed from MPA for 6-8 weeks were transfected with 6 μg of MMTV-TK-CAT vector (TK-CAT-SGA kindly provided by Dr. M. Beato; Ref. 12) plus 4 μg of pCH110 (β-galactosidase expression vector: Pharmacia, Baie d’Urfe, Quebec, Canada) by the calcium phosphate glycerol shock method (13), with modifications. After 6 h incubation with the calcium phosphate-precipitated DNA, the cells were shocked with 20% (v/v) glycerol for 5 min. The cells were then treated with vehicle alone or 10 nM MPA. Forty-eight h later, the cells were harvested and a supernatant fraction was prepared as previously described (14). Chloramphenicol acetyltransferase and β-galactosidase activities of an aliquot of each supernatant containing 40 μg of protein were measured using standard protocols (14, 15).

RESULTS

Development and Stability of a Progestin-resistant Cell Line from T-47D-5 Human Breast Cancer Cells. We have previously shown that the T-47D-5 human breast cancer cell line is sensitive to the growth inhibitory effects of MPA in culture (3). Cells which are resistant to the growth inhibitory effects of MPA have been selected from the parent T-47D-5 cells by stepwise selection in increasing concentrations of MPA (from 1-10 μM). Development of the 5-RP cell line was achieved over a period of approximately 3 months. At passage 11 the cells were placed into 10 μM MPA, the concentration in which they are routinely maintained. Fig. 1 shows a comparison of the sensitivity to the growth inhibitory effects of MPA of our stock resistant line (5-RP) and the original parent T-47D-5. MPA has little, if any, effect on the proliferation of the 5-RP line but significantly inhibits the proliferation of the T-47D-5 line. The doubling time of 5-RP and T-47D-5 cells, grown in medium not contain-
removed from the growth medium were examined. Classical ligand-binding assays were initially used to measure estrogen and progesterone receptor levels. The level of ER in the 5-RP line (68171 ± 9097 sites/cell, $K_a = 0.44 ± 0.15$ nM, $n = 6$) was slightly increased compared to that of the parent T-47D-5 (56440 ± 1688 sites/cell, $K_a = 0.28 ± 0.02$ nM, $n = 6$). Little, if any, PgR is measurable in the 5-RP line maintained in MPA. This may, in part, be due to isotopic dilution as a result of the presence of high concentrations of unlabeled MPA; however, as can be seen in Fig. 2 and Table 1, the level of PgR mRNA in the 5-RP line maintained in MPA is markedly downregulated compared to the sensitive T-47D-5 parent line. When the cells were passaged in the absence of MPA, PgR was now measurable by ligand-binding assay but the PgR level was decreased on average to approximately 58% compared to that seen in the original parent, T-47D-5 line. The level of ER and PgR mRNA levels were determined by Northern blot analysis and generally reflected the results obtained from binding studies (Fig. 2, Table 1). Northern blots were quantitated by densitometry and corrected for RNA loading using actin hybridization. The signal obtained with T-47D-5 RNA of any given experiment was corrected for gel loading using the β-actin mRNA. The value of the signal obtained for T-47D-5 cells was arbitrarily given the value of 100% and the other samples were expressed relative to the T-47D-5 sample.

### Table 1: Gene expression in 5-RP cells in the presence or absence of MPA for varying periods of time

<table>
<thead>
<tr>
<th>Experiment</th>
<th>T-47D-5</th>
<th>5-RP MPA</th>
<th>5-RP NDp35</th>
<th>5-RP NDp15</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA levels (% control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PgR</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ER</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TGF-α</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EGF</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

### Fig. 2: Expression of steroid receptors and growth factors in progestin-resistant cells.

<table>
<thead>
<tr>
<th>A. PgR mRNA</th>
<th>B. ER mRNA</th>
<th>C. TGF-alpha mRNA</th>
<th>D. EGF-R mRNA</th>
<th>E. B-actin mRNA</th>
</tr>
</thead>
</table>
| Poly(A)* RNA isolated from T-47D-5 (lane 1), 5-RP maintained in MPA (lane 2), 5-RP without MPA for 35 passages (lane 3), and 5-RP without MPA for 15 passages (lane 4) was subjected to Northern blot analysis. The pattern of hybridization with the human PgR cDNA (A), with the human ER cDNA (B), with the human TGF-α cDNA (C), with the human EGF receptor (EGF-R) cDNA (D), and with the chicken β-actin (B-actin) cDNA (E). Arrows, positions of residual 28S and 18S ribosomal RNA. Estimation of molecular size of the various mRNA was made by comparison with the position of 28S and 18S ribosomal RNAs.

Steroid Hormone Receptor Status of the 5-RP Cell Line. The estrogen and progesterone receptor status of the 5-RP line in the presence of MPA and at various times after MPA had been

A. PgR mRNA

B. ER mRNA

C. TGF-alpha mRNA

D. EGF-R mRNA

E. B-actin mRNA
47D-5 cell line (Table 1). TGF-α immunoreactivity was measured in the conditioned medium of T-47D-5 and 5-RP cells maintained in the presence of MPA. The conditioned medium from T-47D-5 cells (n = 2) contained 1.12 ± 0.11 pm/10^6 cells (range, 1.01–1.22 pm), while that in 5-RP cells (n = 4) contained 1.94 ± 0.2 pm/10^6 cells (range, 1.5–2.36 pm). This represented a 1.73-fold increase in detectable TGF-α immunoreactivity in the medium of the resistant cell line. When the 5-RP cells were removed from the drug, the level of TGF-α mRNA gradually declined with increasing passages out of MPA, although even after 42 passages out of the drug a slight elevation of TGF-α mRNA was still apparent (Fig. 2C; Table 1).

The level of EGF receptor mRNA in the 5-RP line in the presence of MPA was slightly elevated compared to the T-47D-5 parent line (Fig. 2D). When data from 4 separate experiments were quantitated EGF receptor mRNA in the 5-RP line in the presence of MPA was increased 1.4 ± 0.1-fold (n = 4) compared to the T-47D-5 line. However, when the 5-RP line was removed from MPA for varying periods of time the level of EGF receptor mRNA was consistently found to be elevated 3 ± 0.5-fold (n = 6; obtained from pooling the results of single determinants obtained at passages 5, 10, 15, 21, 35, and 42 out of MPA; see Table 1).

The EGF receptor was measured by ligand-binding assay in the T-47D-5 and in the 5-RP cell line maintained in the presence and absence of MPA for varying periods of time (Table 1). In all cases the level of EGF receptor was decreased in the 5-RP line maintained in the presence of MPA when compared to T-47D-5 cells. When MPA was removed from the 5-RP for varying periods of time the level of EGF receptor was increased compared to the 5-RP in the presence of MPA (Table 2). The level of EGF receptor in the 5-RP cells without drug was found to be elevated except for one instance when compared to the T-47D-5 cell line. In contrast, EGF receptor mRNA was increased slightly in the 5-RP cells maintained in MPA and further increased when those cells were removed from the drug (Fig. 2; Table 1).

Table 2 Progesterone and EGF binding in 5-RP cells in the presence or absence of MPA for varying periods of time

<table>
<thead>
<tr>
<th>Progesterone receptor</th>
<th>Sites/cell</th>
<th>Kd (nm)</th>
<th>% control</th>
<th>EGF receptor (% control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D-5</td>
<td>356,421</td>
<td>1.9</td>
<td>100</td>
<td>100 ± 8.7</td>
</tr>
<tr>
<td>5-RP MPA</td>
<td>40,079</td>
<td>3.6</td>
<td>11.2</td>
<td>30.3 ± 7.2</td>
</tr>
<tr>
<td>5-RP NDp10</td>
<td>216,724</td>
<td>2.1</td>
<td>60.8</td>
<td>60.3 ± 6.6</td>
</tr>
<tr>
<td>5-RP NDp30</td>
<td>276,489</td>
<td>2.4</td>
<td>77.6</td>
<td>102.5 ± 5.6</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D-5</td>
<td>372,140</td>
<td>1.5</td>
<td>100</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>5-RP MPA</td>
<td>Undetected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-RP NDp15</td>
<td>150,174</td>
<td>1.3</td>
<td>40.4</td>
<td>149.2 ± 47.7</td>
</tr>
<tr>
<td>5-RP NDp35</td>
<td>207,829</td>
<td>1.7</td>
<td>55.8</td>
<td>116.7 ± 11.7</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D-5</td>
<td>323,958</td>
<td>2.2</td>
<td>100</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>5-RP MPA</td>
<td>Undetected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-RP NDp21</td>
<td>198,433</td>
<td>1.4</td>
<td>61.3</td>
<td>111.9 ± 2</td>
</tr>
<tr>
<td>5-RP NDp42</td>
<td>173,366</td>
<td>1.9</td>
<td>53.5</td>
<td>159.1 ± 32</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
# ND, no drug.

Effect of Exogenously Added TGF-α on the Growth Inhibitory Response of 5-RP Cells Removed from MPA. Since the increased endogenous expression of TGF-α was associated with progestin resistance and its decline was associated with the return to progestin sensitivity, it can be speculated that addition of exogenous TGF-α should partly reverse the increased progestin sensitivity of 5-RP cells which have been removed from MPA. The data from such an experiment are shown in Fig. 3. Addition of 10 ng/ml of recombinant human TGF-α to 5-RP cells which have been grown for 50 passages in the absence of MPA partially decreases the sensitivity of the cells to the growth inhibitory effects of MPA.

Effect of Progestins on Endogenous and Exogenous Gene Expression in the 5-RP Cell Line. The 5-RP line is resistant to the growth inhibitory effects of MPA and this resistance is relatively stable for at least 22 passages after the drug has been removed from the cells (Fig. 1). It was therefore of interest to investigate the sensitivity of endogenous genes, e.g., EGF receptor, normally responsive to MPA in the original parent line as well as an exogenous progestin-responsive gene, e.g., MMTV-TK-CAT, to progestins in the 5-RP cell line.

The effect of increasing concentrations of MPA on EGF binding in T-47D-5 cells is shown in Fig. 4. MPA increased the level of EGF receptor in these cells in a dose-dependent fashion over the concentration range of 0–10^-8 M. However, the 5-RP cell line removed from MPA for various periods of time showed a marked decrease in response to MPA in terms of increased EGF receptor levels (Fig. 4).

Similarly, the ability of MPA to activate a transiently transfected exogenous progestin-responsive gene, MMTV-TK-CAT, in the 5-RP line was markedly reduced compared to the T-47D-5 parent line (Table 3). The ability of PgR in the 5-RP line to activate transcription CAT gene ranges from 5.2% (when 2 x 10^5 cells were transfected) to 14.4% (when 1 x 10^6 cells were transfected) to 14.4% (when 1 x 10^6 cells were transfected). This range was calculated with the mean fold induction of the two experiments and the value of T-47D-5 cells. In the 5-RP line to activate transcription CAT gene ranges from 5.2% (when 2 x 10^5 cells were transfected) to 14.4% (when 1 x 10^6 cells were transfected) to 14.4% (when 1 x 10^6 cells were transfected). This range was calculated with the mean fold induction of the two experiments and the value of T-47D-5 cells.

DISCUSSION

In human breast cancer the emergence of resistant cells reduces the efficacy of many forms of drug therapy. In order to understand some of the possible mechanisms by which hormonally dependent breast cancers develop resistance to progestin therapy we have developed a breast cancer cell line which is resistant to the growth inhibitory effects of the synthetic pro-

Fig. 3. Effect of exogenously added TGF-α on the MPA-induced inhibition of proliferation of 5-RP cells withdrawn from MPA. Proliferation rate was calculated as for Fig. 1. Effect of increasing concentrations of MPA on the proliferation rate of 5-RP cells routinely grown in 10 mM MPA (*) and 5-RP cells removed from MPA for 50 passages in the absence (O) and presence (C) of 10 ng/ml human TGF-α. Points, means; bars, ± SEM; n = 3.
acetylated metabolites of \([l^{14}C]\)chloramphenicol as well as the unacetylated compound were scraped and the radioactivity was quantitated by scintillation counting.

Progestin, MPA. The progestin-resistant line, 5-RP, developed from T-47D-5 human breast cancer cells by stepwise elevation of MPA concentration over a period of 3 months, has been grown routinely in the presence of 10 \(\mu\)M MPA with a doubling time of approximately 48 h (3). Cells were treated with MPA for 24 h and then EGF binding was measured as described in Materials and Methods. *Points, means; bars, ±SEM of triplicate measurements. This entire experiment was repeated a second time with essentially similar results.

Figure 4. Effect of acute administration of increasing concentrations of MPA on \({^125}\text{I}-\text{EGF} binding in T-47D-5 cells (•), 5-RP cells removed from MPA for 11 passages (○), and S-RP cells removed from MPA for 42 passages (△). Cells were treated with MPA for 24 h and then EGF binding was measured as described in "Materials and Methods." Points, means; bars, ±SEM of triplicate measurements.

Table 3 Effect of MPA on the expression of an exogenous progestin-responsive gene (MMTV-TK-CAT) in transiently transfected cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% acetylation(a)</th>
<th>Fold induction(b) of CAT activity</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>T-47D-5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10^6</td>
<td>+</td>
<td>1.36</td>
<td>0.84</td>
</tr>
<tr>
<td>1 × 10^6</td>
<td>-</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>+</td>
<td>10.99</td>
<td>17.38</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>-</td>
<td>0.10</td>
<td>0.54</td>
</tr>
<tr>
<td>5-RP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10^6</td>
<td>+</td>
<td>0.11</td>
<td>0.38</td>
</tr>
<tr>
<td>1 × 10^6</td>
<td>-</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>+</td>
<td>0.57</td>
<td>2.38</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>-</td>
<td>0.15</td>
<td>0.64</td>
</tr>
</tbody>
</table>

\(a\) Cell number at time of transfection.

\(b\) After correction for transfection efficiency, 40 \(\mu\)g of protein were used to assay for CAT activity. The areas on the thin layer chromatograph corresponding to the acetylated metabolites of \([14]C\)chloramphenicol as well as the unacetylated compound were scraped and the radioactivity was quantitated by scintillation counting.

\[
\% \text{ acetylated} = \frac{\text{Counts in acetylated species}}{\text{Counts in acetylated species} + \text{Counts in nonacetylated chloramphenicol}} \times 100
\]

\(c\) The acetylation in the absence of MPA was given a value of 1 in each case.
TGF-α protein and activity have been shown to be present in the conditioned medium of human breast cancer cells that contain TGF-α mRNA (21). We have also shown that there is a reproducible increase in immunoreactive TGF-α in the medium of the 5-RP cells growing in the presence of MPA. Since TGF-α synthesis and secretion are increased in the 5-RP cells and TGF-α can act in an autocrine loop, it might be anticipated that it interacts with the EGF receptor on these cells and in so doing can downregulate it. When the level of EGF binding in the 5-RP line maintained in MPA was compared to that of the T-47D-5 cell line, a marked reduction was found. These data are consistent with a higher level of endogenous expression of TGF-α in the 5-RP cell line. The endogenous ligand is available to interact with its receptor, resulting in enhanced internalization, making the receptor unavailable to the radioactive ligand but enhancing receptor expression, as determined by EGF receptor mRNA abundance. The discrepancy which we have seen between TGF-α mRNA levels (increased 3-fold over T-47D-5 cells) in the 5-RP cells and the level of TGF-α immunoreactivity in the conditioned medium (increased 1.73-fold over T-47D-5 cells) may be explained by TGF-α rapidly interacting with the EGF receptor. This suggestion is supported by the observed downregulated level of EGF binding under the conditions used for conditioning the medium.

The presence of high concentrations of MPA appears to be required to maintain the marked elevation in TGF-α expression. Removal of the drug for varying periods of time results in TGF-α mRNA levels gradually declining back toward those present in the sensitive T-47D-5 parent line. It should be noted, however, that even at passage 42, i.e., 30–36 weeks, without MPA, there is still a slight elevation in TGF-α mRNA levels. The decrease in TGF-α expression seen when MPA is withdrawn is consistent with the concomitant increase in EGF binding seen in the 5-RP cells after MPA withdrawal. Interestingly, the increase in EGF binding in these cells is accompanied by an even further increase in EGF receptor mRNA. The mechanism of this increase remains unclear since acute administration of progestins and EGF both enhance EGF receptor expression (11, 16, 22).

The ability of exogenously added TGF-α to partially reverse the increased progestin sensitivity of the MPA-withdrawn 5-RP cells is consistent with increased TGF-α expression being involved with the resistant state. If elevated TGF-α were the only factor responsible for the resistance of the 5-RP line to the growth inhibitory effects of MPA, then the return to sensitivity might be expected to be more rapid and parallel more closely the declining TGF-α mRNA levels. Moreover, effects such as the decreased ability of MPA to activate endogenous gene expression, i.e., EGF receptor, and exogenous gene expression, i.e., transiently transfected MMTV-TK-CAT, as we have demonstrated, would not be expected. However, we have found that the 5-RP cell line in the presence of MPA, as might be expected (23), has markedly downregulated expression of the progesterone receptor. This occurred at both the binding and mRNA levels. Although the level of PgR expression increased after MPA removal, the level of PgR is found to be permanently decreased to approximately 58% of that seen in the original T-47D-5 parent line. This decreased level of receptor is associated with decreased ability to respond to progestins as determined by the decreased ability of MPA to activate expression of both endogenous (EGF receptor) and exogenous (MMTV-TK-CAT plasmid) target genes. While the reduction in the MPA-induced increase in the EGF receptor appeared to correlate with the reduction in PgR, the marked impairment of the response of the transiently expressed MMTV-TK-CAT is unlikely to be explained on this basis. The PgR level in the 5-RP line was reduced to approximately 58% of that of the wild type, while the MPA-induced CAT activity in the transient expression study was reduced to between 5 and 14% of the wild type.

It has been shown previously that exposure of MCF-7 and T-47D human breast cancer cells to the synthetic progestin, R 5020, caused a marked downregulation of the progesterone receptor mRNA and protein (23). When 10 nM R 5020 was used, this downregulation was maintained for at least 48 h. The very high levels of MPA in which the 5-RP line was selected and routinely grown similarly caused PgR downregulation. However, even after the drug had been removed for many passages when no residual MPA would be expected to remain, the PgR levels both at the binding and mRNA level were still reduced. Moreover, this reduction was accompanied by a decreased ability of the cells to respond to MPA. However, the amount of PgR remaining was still sizable, suggesting that either these receptors were a population that was intrinsically less active than the population in the original parent line (i.e., receptor heterogeneity) or that other factors, separate from the receptor but involved in modulation of PgR activity, were altered in some way. Consistent with the latter hypothesis is the observation that, as the 5-RP cells gradually increase in their sensitivity to MPA with longer periods of time out of the drug, there appeared to be no concomitant increase in PgR levels. This would be consistent with an effect on a factor(s) distal to the ligand/receptor complex.

In normal estrogen and progestin target tissues such as the uterus (24) and in T-47D human breast cancer cells (25), progestins have been found to downregulate the ER. This occurred at both the mRNA and the protein levels. The down-regulation of ER by progestin is thought to be one way in which progestins antagonize the effects of estrogens. However, in the 5-RP cell line, even in the presence of MPA, ER gene expression, if anything, is slightly increased (Fig. 2; Table 2).

In summary, progestin resistance in the 5-RP cell line appears to be multifactorial, involving both increased growth factor expression and decreased progesterone receptor levels. It is likely that these two aspects do not account entirely for the progestin-resistant phenotype and as yet other unidentified mechanisms may also be involved.

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Mechanisms Involved in the Evolution of Progestin Resistance in Human Breast Cancer Cells

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