Androgen Sensitivity of the New Human Breast Cancer Cell Line MFM-223

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ABSTRACT

The mammary carcinoma cell line MFM-223 is characterized by high androgen and low estrogen and progesterone receptor levels. With the dextran charcoal method, androgen binding was determined at 160 fmol/mg protein corresponding to approximately 100,000 binding sites per cell in whole cell binding assays. The estrogen and progesterone receptor contents were between 8 and 18 fmol/mg protein. The proliferation of MFM-223 cells was significantly inhibited by doses >0.01 nM dihydrotestosterone. The androgenic inhibition of cell proliferation was antagonized by antiandrogens cyproterone acetate and hydroxyflutamide. In spite of the low estrogen receptor content, MFM-223 cell proliferation was slightly enhanced by 10 nM 17β-estradiol. Treatment with 17β-estradiol or dihydrotestosterone failed to provoke an increase of the progesterone receptor level. MFM-223 cells have characteristic patterns of isoenzyme polymorphism and of karyotype alterations revealing marker chromosomes and homogeneously staining regions. In the spectrum of human mammary carcinoma cell lines, MFM-223 cells offer a unique model to investigate molecular mechanisms of androgen receptor action.

INTRODUCTION

Breast cancer growth is presumed to be under the influence of a variety of steroid hormones. The stimulatory effect of estrogens on the progression of breast cancer is well known, and was confirmed in vitro by the enhancement of proliferation of human mammary cancer cells by 17β-estradiol (1). Androgens can have differential effects on the progression of breast cancer. After aromatization to estrogenic steroids, androgens may enhance tumor progression indirectly (2). On the other hand, androgens may be therapeutically effective by direct interaction with the appropriate receptor at the cellular level. Androgen receptors were observed in approximately 50% of breast cancer specimens (3–5). By an improved detection method, 85% of breast cancer samples contained androgen receptor levels above 10 fmol/mg protein (6). However, there is only limited knowledge on the functional role of androgens in development and progression of mammary tumors. Based on the postulated antagonistic effect on estrogenic enhancement of breast cancer growth, pharmacological doses of testosterone propionate had been used in systemic palliative treatment regimens (7).

In vitro, receptors for androgen, estrogen, and progesterone were found in the permanent human mammary cancer cell lines MCF-7 (8), T-47D (9), ZR-75-1 (10), and EFM-19 (11). Cell proliferation in these lines is enhanced by estrogen, but the reaction to androgens varies. Enhancement of cell proliferation by 1 μM DHT is reported for MCF-7 (12) and EFM-19 (11), whereas in ZR-75-1 cell cultures a biphasic inhibitory effect on the proliferation was observed (13). Proliferation of T-47D cells was not affected in the range of 0.01 to 1 μM (14). Cells of the newly established mammary carcinoma cell line MFM-223 have comparatively high androgen receptor levels in correspondence with the original tumor tissue. In vitro proliferation of MFM-223 cells is inhibited by concentrations above 0.01 nM DHT.

MATERIALS AND METHODS

Origin. MFM-223 cells were obtained from the pleural effusion of a postmenopausal breast cancer patient. The patient had not received any prior treatment. The tumor was classified as a widespread (T4 N2 M1) ductal mammary carcinoma with the histological grade of III (15). In the primary tumor tissue analyzed by using the dextran-coated charcoal method (16), androgen, estrogen, and progesterone receptors were found (94, 63, and 6 fmol/mg protein, respectively).

Cell Culture and Media. After dilution with an equal volume of GM, the pleural exudate was transferred to plastic culture flasks (Nunc, Roskilde, Denmark). Once a week, the culture medium was replaced by fresh medium after centrifugation of the cell suspension (50 × g for 5 min). During the first 4 months of in vitro culture, gradual attachment of the cells was attained. Thereafter, the cells were consistently adherent and were routinely passaged at confluence by trypsinization.

GM was based on Eagle’s minimal essential medium and was enriched with 4 mM L-glutamine, 1 mM sodium pyruvate, 67 mg/liter gentamicin sulfate (Biochrom, Berlin, Federal Republic of Germany), 5 mg/liter fetuin, 2.5 mg/liter transferrin, 250 ng/ml L-histidyl-L-lysine (Serva, Heidelberg, Federal Republic of Germany), 40 IU/liter insulin (Hoechst, Frankfurt, Federal Republic of Germany) and 10% fetal bovine serum (Boehringer, Mannheim, Federal Republic of Germany). DCM was prepared from Eagle’s minimal essential medium plus L-glutamine and gentamicin but free of phenol red and supplemented with 10% fetal bovine serum pretreated with dextran-coated charcoal for removal of steroids (17).

Tumorigenicity. Two female athymic nude mice were given injections s.c. of 106 cells at passage 40. After 6 weeks, the tumors, grown at the injection sites, were removed from both animals. The histological evaluation revealed moderate to poorly differentiated adenocarcinomas resembling the primary human tumor.

Immunocytochemistry. Cells were seeded on standard microscope slides in Petri dishes and cultivated to confluence. The cell layers were fixed with acetone at 4°C and air dried. The following antibodies were applied: anti-pancytokeratin KL1, anti-human milk fat globulins HMFG1 and HMFG2 (Dianova, Hamburg); anti-vimentin Vim-3BH; anti-cytokeratins 8 and 18 (Boehringer); and anti-epithelial membrane antigen (Dakopatts, Hamburg). For the detection of estrogen receptors, the ER-ICA kit (Abbott, Wiesbaden, Federal Republic of Germany) was used after preincubation of the cells in DCM for 5 days.

Enzyme Polymorphism. The cells were harvested by trypsin/EDTA treatment, washed 3 times with PBS, frozen in liquid nitrogen, and homogenized by using a dismembrator (Braun, Melsungen, Federal Republic of Germany). After thawing, 1–5 μl of the supernatant resulting from centrifugation were analyzed by polyacrylamide gel electrophoresis and subsequent staining (18, 19).

Karyotype Analysis. After preparation of metaphases, trypsin Giemsa banding of the chromosomes was performed, and the morphological aberration pattern was evaluated microscopically (20).

Steroid Hormone Receptor Assays. Cells seeded in 4-well cluster dishes were grown for 6 days to reach near confluence. The cultures...
ANDROGEN-SENSITIVE BREAST CANCER CELLS were rinsed with PBS, and 0.5 ml DCM was added per well. 

**Results**

**Phenotype of MFM-223 Cells.** At the beginning of the *in vitro* culture, the cells grew in suspension as single cells and cellular aggregates (Fig. 1A). During the first 4 months, the cells became gradually adherent to the bottom of the plastic culture flasks, and the monolayers adopted a characteristic reticulated growth pattern (Fig. 1B). Individual cells carried extensions of several cell diameters. The cell cultures were trypsinized only 12 times during the first year. At passage 12, the cell doubling time was 184 h. Subsequently, the cell growth accelerated and at passage 35 the doubling time was less than 70 h.

Cells grown as monolayer on microscope slides and stained with antibodies to intermediate filaments showed only marginal staining with anti-vimentin. They were immuncytochemically positive for pancytokeratin and epithelial membrane antigen, indicating their epithelial character. The presence of the human milk fat globulins HMFG 1 and 2 (22) and of the cytokeratins 8 and 18 (23) was demonstrated with the appropriate antibodies (not shown). The isoenzymes of MFM-223 cells were identical with the isoenzyme pattern of the erythrocytes of the same patient with one exception (Table 1). The cultured MFM-223 cells lacked allele 2 of the glyoxalase system. The isoenzyme pattern of MFM-223 cells was clearly distinguishable from that of MCF-7 and EFM-19 cells.

**Karyotype.** In the analysis performed at passage 35, the chromosome distribution pattern revealed near diploidy for most of the MFM-223 cells (Fig. 2); 10% of the cells were near tetraploid. After trypsin Giemsa banding, HSRs were observed in at least 3 marker chromosomes (Fig. 3). The chromosomes carrying HSRs were identified as X and 5 (Fig. 4). Additionally, one chromosome 7 with HSR was rearranged with an unidentified fragment. Furthermore, 2 markers involving fragments of chromosome 7 were observed with high incidence. These structural aberrations, apparently characteristic of MFM-223 cells were found to be monosomic in near-diploid cells and mostly disomic in near-tetraploid cells.

**Steroid Receptors.** Hormone binding was analyzed repeatedly at various cultivation passages by whole cell binding assays. At passage 25 to 30 (Fig. 5), Scatchard analyses demonstrated 23,000 sites/cell for the estrogen receptor and the unexpectedly high number of 126,500 sites/cell for the androgen receptor. Binding of both ligands occurred with high affinity; the $K_d$...
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Fig. 3. A representative metaphase of MFM-223 cells at passage 35 of the culture. Homogeneously staining regions (HSRs) are indicated by arrows, other marker chromosomes by asterisks.

Table 1

<table>
<thead>
<tr>
<th>Mar</th>
<th>Morphology</th>
<th>Designation</th>
<th>Incidence per 10 metaph.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>HSR(X) (q21)</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>HSR(5) (q11)</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>(?;7; HSR) (?;q36; HSR)</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>(?;7;?) (q21; ?)</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>del(7q)</td>
<td>9</td>
</tr>
</tbody>
</table>

Fig. 4. Designation and incidence of marker chromosomes observed in MFM-223 cells.

values were 0.23 and 0.13 nM, respectively. At later passages (Table 2), estrogen receptors were no longer detectable, whereas consistently high numbers of androgen receptors were determined at approximately 100,000/cell. The lower limit of detection in the estrogen whole cell binding assay was approximately 5,000 binding sites/cell. This was confirmed by lack of staining of estrogen receptor in the immunocytochemical assay after passage 40, whereas at passages 25 to 30 approximately 5% of the cells stained positively. Estrogen binding determined in parallel by the dextran-coated charcoal method revealed 5 to 10 fmol/mg protein, a value considered near the detection limit. In whole cell binding assays, progesterone receptors were not found at all passages of culture. Binding of progesterone was not detectable even after preincubation of the cells with 10 nM E2 or 1 μM DHT for 7 days. The preincubation experiments were performed to reveal induction of progesterone binding, since human mammary carcinoma cells were reported to increase the number of progesterone receptors in response to estrogen treatment (24). For the synthetic glucocorticoid dexamethasone, 7,500 binding sites/cell were found (Kd = 1.7 nM) at passage 69 (data not shown).

A comparison of steroid hormone receptor levels based on determinations using the dextran-coated charcoal method (Table 3) shows that MFM-223 cells have the highest level of androgen receptors so far observed in human mammary carcinoma cell lines. Estrogen and progesterone receptors were determined at very low levels in MFM-233 cells. Although the data shown in Table 3 were obtained in several laboratories under different culture conditions and at different culture passages, the ratios of the receptor levels indicate characteristic properties of the cell lines investigated. The comparison emphasizes the unique property of high androgen binding capacity in combination with extremely low estrogen and progesterone receptor levels in MFM-223 cells.

Table 2

<table>
<thead>
<tr>
<th>Cultivation passages</th>
<th>Cellular properties</th>
<th>25–30</th>
<th>35–40</th>
<th>50–60</th>
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<tbody>
<tr>
<td>Androgen receptor (sites/cell)</td>
<td>126,500</td>
<td>90,000</td>
<td>99,000</td>
<td></td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>0.13</td>
<td>0.17</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor (sites/cell)</td>
<td>23,000</td>
<td>&lt;5,000&quot;</td>
<td>&lt;5,000&quot;</td>
<td></td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubling time (h)</td>
<td>70</td>
<td>65</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Growth inhibition by 1 μM DHT (% of control)</td>
<td>41</td>
<td>52 ± 14&quot;</td>
<td>64 ± 7&quot;</td>
<td></td>
</tr>
<tr>
<td>Growth stimulation by 10 nm E2 (% of control)&quot;</td>
<td>115</td>
<td>105 ± 3&quot;</td>
<td>139 ± 22&quot;</td>
<td></td>
</tr>
</tbody>
</table>

* Below the level of detection.
* Determined from quadruplicate cultures in 7-day proliferation assays in GM.
" Means ± SD of 4 independent experiments.
* Assays performed in DCM.
**Table 3.** Steroid hormone receptor content of MFM-223 cells determined by the dextran-coated charcoal method in comparison with EFM-19 cells and published data on human mammary carcinoma cell lines.

The values are expressed as fmol/mg protein.

<table>
<thead>
<tr>
<th>Steroid receptor</th>
<th>Cell line</th>
<th>Androgen</th>
<th>Estrogen</th>
<th>Progesterone</th>
<th>References</th>
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</thead>
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<tr>
<td></td>
<td>MFM-223</td>
<td>±750</td>
<td>±340</td>
<td>8±5</td>
<td>Present study*</td>
</tr>
<tr>
<td></td>
<td>EFM-19</td>
<td>50±3</td>
<td>±343</td>
<td>±18±11</td>
<td>Present study*</td>
</tr>
<tr>
<td>MCF-7</td>
<td>40±100</td>
<td>&gt;300</td>
<td>29±11</td>
<td>4±3±11</td>
<td>(8)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>34</td>
<td>29</td>
<td>43±14±9</td>
<td>14±8</td>
<td>(9)</td>
</tr>
<tr>
<td>T47-D</td>
<td>3</td>
<td>17</td>
<td>254</td>
<td>4±12</td>
<td>(10)</td>
</tr>
</tbody>
</table>

*Means ± SD of 3 separately collected and analyzed batches of cells at passages 45–50 (MFM-223) or 80–85 (EFM-19).

**DISCUSSION**

Among the variety of human mammary carcinoma cell lines established, MFM-223 cells are distinguished by their extreme sensitivity to androgens.
sensitivity to androgen in combination with an elevated androgen receptor content. During the development to a permanent line, MFM-223 cells had acquired a reticulate growth pattern which is characteristic compared with MCF-7 and EFM-19 cells, forming colonies under the same culture conditions (21). The pattern of chromosomal aberrations in MFM-223 cells containing typical HSRs and unique markers was different from that of MCF-7 (25), EFM-19 (26), or other breast cancer cell populations (27). The isoenzyme pattern of MFM-223 cells was special when compared with that of other human mammary carcinoma cells analyzed in parallel. In respect to the erythrocyte receptor content. During the development to a permanent sensitivity to androgen in combination with an elevated androgen receptor appears unlikely. After incubation with 10 nM E2 using comparable culture conditions, we determined a Ka value of 9.1 nM for cyproterone acetate and hydroxyflutamide, providing further support for the involvement of the androgen receptor. The more pronounced effect of cyproterone acetate may be due to its higher affinity to the androgen receptor under in vitro conditions. This was demonstrated in earlier experiments with MCF-7 cells, in which a Ka value of 9.1 nM for cyproterone acetate versus a Ka value of 27 nM for hydroxyflutamide was determined (21).

In vitro growth inhibition by androgens was shown previously for ZR-75-1 human mammary cancer cells. In the biphasic inhibition pattern, the maximum effect was at 1 to 10 nM DHT and much lower at 1 µM, apparently due to interaction with the estrogen receptor at the high concentration (13). In MFM-223 cell cultures, a biphasic dose effect of androgenic inhibition of the proliferation was not observed. With respect to the very low estrogen receptor level, significant interaction of DHT with the estrogen receptor appears unlikely. After incubation with 10 nM E2 using comparable culture conditions, we determined a 40% increase in the number of MFM-223 cells, compared with an increase by 200% in MCF-7 cells (28). The growth-stimulatory effects in these cell lines corresponded to estrogen levels of 8 fmol/mg protein in MFM-223 and 100 fmol/mg protein in MCF-7 cells.

In comparison with MFM-223 cells, ZR-75-1 cell proliferation was markedly stimulated by estrogens (13). The inhibition of ZR-75-1 and MCF-223 cell proliferation by androgens is in contrast with the stimulatory effect of DHT in MCF-7 and EFM-19 cell cultures. DHT (1 µM) enhanced MCF-7 and EFM-19 cell proliferation and synthesis of the progesterone receptor, thus imitating estrogenic properties (12, 21, 29). Low doses, e.g., 10 nM DHT, interfered with the induction of the progesterone receptor by 1 or 10 nM E2 in MCF-7 cells, but had no effect in the absence of E2 (29–31). Concentrations of 0.1 to 10 nM DHT which were effective in ZR-75-1 and MFM-223 cells do not have stimulatory or inhibitory effects on the proliferation of androgen receptor-positive MCF-7 and EFM-19 cells. In MCF-7 cells DHT is rapidly converted to androstane-3β,17β-diol (12), possibly exerting estrogenic effects in this cell line (32). Variations in androgen metabolism may thus contribute to the diverging effects of DHT on the in vitro growth of human mammary carcinoma cell lines.

Predominance of the androgen receptor in MFM-223 cells is a novel property and adds another experimental aspect to the spectrum of human breast cancer cell lines. In this cellular model system for androgenic manipulation, interference with other steroid receptors is greatly reduced. MFM-223 cells facilitate the exploration of molecular mechanisms of the androgen receptor acting at the genomic level and influencing cell proliferation.

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