Stimulatory effects of androgen and antiandrogen on the in vitro proliferation of human mammary carcinoma cells*,

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Summary. The proliferation of three mammary carcinoma cell lines was explored for the effectiveness of dihydrotestosterone (DHT) and the antiandrogenic substances cyproterone acetate (CPA) or hydroxyflutamide. The cell growth, determined in multiple experimental cultures of the estrogen-sensitive lines MCF-7 and EFM-19, was stimulated by \(10^{-9} M\) to \(10^{-6} M\) DHT, whereas estrogen-resistant MCF-21 cells were unresponsive to the hormonal factors applied. Growth-promoting effects of \(10^{-8} M\) to \(10^{-6} M\) CPA were detected in cultures of those cell lines which were sensitive to estrogen and androgen. Competition experiments with DHT and the antiandrogens suggested involvement of the androgen receptor in the stimulation of cell growth by CPA. Participation of the estrogen receptor was excluded by lack of competition between CPA and the enhancement of proliferation by estradiol-17β. At the receptor level the antiandrogens were able to compete with androgen binding. The results of the study demonstrate androgenic properties of CPA in regard to the growth of human mammary carcinoma cells.

Keywords: Mammary carcinoma — Cell culture — Androgenic stimulation — Growth promotion by antiandrogens

Introduction

The origin and growth of breast cancer are presumed to be under the influence of a variety of steroid hormones including androgens, which are mediated by the appropriate cellular receptors (Wagner and Jungblut 1976; Trams and Maass 1977; Vorherr and Messer 1978; Allegra et al. 1979; Thomas 1986). This is underlined by the demonstration of stimulatory effects of androgens (Lippman et al. 1975, 1976b; Simon et al. 1984a) and estrogens (Lippman et al. 1976a; Darbe et al. 1983; Simon et al. 1984a) on the proliferation of cell lines derived from advanced mammary carcinomas. Inhibitory effects on mammary carcinoma cell growth were reported for antiandrogens (Lippman et al. 1976b) and antiestrogens (Lippman et al. 1976a; Simon et al. 1984a). High doses of androgens such as \(10^{-6} M\) dihydrotestosterone (DHT), which are well above the physiological serum levels of \(3 \times 10^{-11} M\) to \(10^{-9} M\) in females (Dawood and Saxena 1976), were shown to enhance the progesterone receptor level in cells of the MCF-7 line (Zava and McGuire 1978), mimicking the induction effect of estrogen. In contrast, the comparatively low dose of \(10^{-8} M\) DHT was ineffective on the progesterone receptor level of untreated cells, but inhibited the induction of progesterone receptor by \(10^{-9}\) or \(10^{-8} M\) estradiol (Zava and McGuire 1978; McIndoe and Etre 1981; Shapiro and Lipman 1985). Thus, with respect to the progesterone receptor level, DHT exerted an antiestrogenic effect at \(10^{-9} M\), and acted as an estrogen in higher doses. However, dose-dependent effects of androgens and their antagonists on the in vitro growth of mammary carcinoma cells have not been studied extensively.

In primary breast cancer, androgen receptors were found in 54% of premenopausal and in 48% of postmenopausal patients (Würz et al. 1982; Miller et al. 1982), attracting clinical interest in the role of androgens as growthregulatory factors (Blossey et al. 1982; Bryan et al. 1984). Among the various modalities of endocrine therapy, adrenalectomy had been described to lower the serum levels of androgens and the products of their peripheral aromatization, the estrogens (Mac-
Donald et al. 1967). On the other hand, testosterone and other androgens have been recommended as additive endocrine treatment of breast cancer (CCNSC 1964). Although these endocrine therapy regimens were replaced by the introduction of more effective endocrine factors with fewer side-effects, such as antiestrogenic tamoxifen (Cole et al. 1971), they corroborate the possible clinical significance of androgens as regulatory factors in breast cancer.

By application of antiandrogenic cyproterone acetate (CPA) temporary remission had been achieved in a few cases of breast cancer (Schulz et al. 1982; Hackenberg et al. 1986). In the present study, monolayer cultures of three human mammary carcinoma cell lines were used to investigate in vitro effects of androgens and their interaction with antiandrogens in regard to cell proliferation and hormone binding. Since CPA is known to have additional progestagenic activity (Neumann and Steinbeck 1974; Neri and Kassem 1984), the effect of this antiandrogenic compound was compared with progesterone.

**Materials and methods**

**Origin of tumor cells.** MCF-7 cells (Soule et al. 1973) were kindly provided by Dr. G. Daxenbichler, Universitäts-Frauenklinik Innsbruck, Austria. EFM-19 cells (Simon et al. 1984a, b) were recultivated from frozen stock in passage 23. By using methods described previously (Hölzel et al. 1985), MFM-21 cells were established as a permanent line from a pleural effusion of a patient with recurrent mammary carcinoma. The patient had been admitted to the Zentrum für Frauenkunde, Marburg in March 1984, and had not been treated systemically at the time of cell explantation. The histological type of the primary tumor was that of a carcinoma simplex simplex of low differentiation. After 24 passages of in vitro cultivation, MFM-21 cells were injected s.c. in female nude mice. The histology of the nude mouse tumor was identical to that of the original tumor tissue. In addition, the karyotype of cells recultured from the nude mouse tumor revealed the same chromosome distribution pattern as MFM-21 cells before the animal passage, thus confirming the identity of the cell populations investigated. Karyotyping was described previously (Hölzel et al. 1985; Kunzmann and Hölzel 1987).

**Cell Cultivation.** The monolayer cell cultures were grown in plastic culture flasks (Nunc, Roskilde, Denmark), which were kept in humidified incubators with a 5% CO₂ atmosphere. The standard growth medium consisted of Earle’s salts, Eagle’s minimal essential medium amino acids and vitamins (Biochrom, Berlin). The pH value was adjusted to 7.2 by the addition of 16% NaHCO₃. The growth medium was enriched with 4 × 10⁻³ M t-glutamine, 10⁻³ M sodium pyruvate (Biochrom, Berlin), 5 mg/l fetuin, 2.5 mg/l transferrin, 2.5 × 10⁻³ M glycyl-L-histidyl-L-lysine, 10⁻³ M triiodothyronine, 10⁻³ M estradiol-17β (all from Serva, Heidelberg, FRG), 40 IU/l insulin (Hoechst, Frankfurt), 50 ng/ml ovine prolactin (Sigma, Munich, FRG) and 10% fetal calf serum (Boehringer, Mannheim, FRG). The culture medium was renewed every 3–4 days. At confluence, the cells were detached from the bottom of the flasks with 0.05% trypsin/5 × 10⁻³ M EDTA (Serva) in phosphate-buffered saline (PBS: 0.14 M NaCl; 3 × 10⁻³ M KCl; 8 × 10⁻³ M Na₂HPO₄; 1.5 × 10⁻³ M KH₂PO₄).

**Experimental growth conditions.** The reduced medium used in proliferation experiments consisted of the same components as growth medium with the exception of the hormones and growth factors. Fetal calf serum applied to the experimental cultures was preincubated twice with 0.5% charcoal (Norit A, Serva) and 0.05% deuterium₂ (Pharmacia, Uppsala, Sweden) for 30 min at 55 °C to remove endogenous steroids (Darbre et al. 1983).

**Proliferation assay.** The cells were detached from the stock culture flasks by trypsinization, suspended in standard growth medium and seeded at a concentration of 10⁴ per well in 4× cluster dishes (Nunc, Roskilde, Denmark). After the attachment period of 1 day, the cell layer was washed with PBS and supplied with 1 ml reduced medium. The hormonal factors under investigation were dissolved in absolute ethanol and further diluted with the appropriate volume of PBS. In 10-fold dilution steps, 50 µl was added to each culture to obtain the final hormone concentrations desired. The final ethanol concentrations were less than 0.1% and had no influence on the proliferation rates. Medium and hormones were renewed after 3 days. At the end of the 7-day experimental incubation period, cells were detached from the bottom of the culture dishes by trypsinization and counted in a hemocytometer.

**Hormones and antihormones.** The DHT was purchased from Sigma, estradiol-17β (E-2) and progesterone were obtained from Merck, Darmstadt, and CPA was supplied by Schering (Berlin). Hydroxyflutamide was a gift from Essex (Munich, FRG).

**Binding studies with [³H]R1881 and competition by antiandrogens.** As described above, 5 × 10⁴ cells were seeded per culture dish. After the attachment period of 1 day, the cell layer was rinsed with PBS and supplied with 1 ml reduced medium. The cells were grown for 4 days before [³H]R1881 (methyltrienolone-17β-hydroxy-17α-nortestosterone-4,9,11-triene-3-one, sp. act. 80 Ci/mmol, NEN, Frankfurt, Dreieichshain, FRG) was added in the range of 2.5 × 10⁻¹¹ M; 5 × 10⁻⁹ M with or without 100-fold concentration of unlabeled R1881, and in the presence of a 1500-fold excess of tamoxifen (Sigma) to block binding to the glucocorticoid receptors. After an incubation period of 4 h at 37 °C the cultures were disassembled twice with PBS, and 0.5 ml 1 M NaOH was added to solubilize the bound radioactivity. The samples were neutralized with 10% HCl, and the radioactivity was determined in a β-scintillation counter using 10 ml Scinti-G (Roth, Karlsruhe, FRG). Parallel incubations were performed with 10⁻⁷ M or 10⁻⁵ M [³H]R1881 and 10⁻⁸ M or 10⁻⁶ M unlabeled CPA or hydroxyflutamide. The binding data were analyzed by linear regression. In the Scatchard and the affinity of the unlabeled antiandrogens were calculated indirectly from the displacement of [³H]R1881 from the receptor by an approach for competitive binding (Dixon and Webb 1966; Hofmann and Sernetz 1983). The Kᵦ of the unlabeled substances is equivalent to Kᵦ × x divided by (Kᵦ – Kᵦx), in which x was the concentration of the unlabeled substance, Kᵦ was the [³H]R1881 at Kᵦ was the reduced apparent Kᵦ of [³H]R1881 partially displaced, the unlabeled substance.

**Results**

**Growth properties and antiandrogenic stimulation.** The cell lines investigated were different in their morphological growth patterns (Fig. 1). The attachment MCF-7 cells was partly diffuse and partly in colonies (Fig. 1a), whereas EFM-19 cells were growing in distinct colonies (Fig. 1b). The growth pattern of MFM-21 cells was diffuse (Fig. 1c). As demonstrated...
karyotype analyses (Table 1), the range of numerical chromosome aberrations was individually different in the three cell lines; detailed analyses of structural chromosome aberrations confirmed the individuality of the karyotypes (data not shown). Under standard growth conditions the doubling time of MCF-7 and MFM-21 cells was in the range of 40–50 h; the proliferation of EFM-19 cells was considerably slower. When tested in reduced medium with dextran-charcoal-treated fetal calf serum, the proliferation of MCF-7 and EFM-19 cells was enhanced by E-2, whereas MFM-21 cells were estrogen-resistant.

The growth of cells of the estrogen-responsive lines MCF-7 and EFM-19 was enhanced by the androgen DHT (Fig. 2a, b). The growth stimulation by DHT was dose-dependent; measurable effects were observed at the concentration of $10^{-9} M$, and the highest stimulation by a factor of 2 was found at $10^{-6} M$ DHT in both cell lines. The growth of cells of the line MFM-21 was not affected by DHT in the concentration range examined (Fig. 2c).

**Stimulatory effects of antiandrogenic CPA**

The incubation with CPA provoked enhancement of cell growth in the same lines (Fig. 3a, b) which had shown responsiveness to E-2 and to DHT. The proliferation of MCF-7 cells was stimulated twofold by $10^{-7} M$ CPA; higher or lower doses of CPA were less effective. EFM-19 cell growth was enhanced twofold
Fig. 2. Dose-dependent stimulation of the proliferation of MCF-7 (a) and EFM-19 (b) cells by dihydrotestosterone (DHT). Cells of the line MFM-21 (c) were unresponsive to DHT in the concentration range tested. Each point represents the mean cell count of quadruplicate cultures ± SD. The growth of the appropriate control cultures is indicated by the open bars.

Fig. 3a-c. Stimulatory effect of antiandrogenic CPA on the growth of MCF-7 cells (a) and EFM-19 cells (b). MFM-21 cells (c) were unresponsive to CPA.

by CPA in the concentration range of $10^{-8} \text{M}$ to $10^{-5} \text{M}$. Cells of the line MFM-21, which were resistant to E-2 and to DHT showed no reaction to CPA in the dose range examined (Fig. 3c). When incubations of MCF-7 cells were performed in the presence of increasing doses of progesterone, no significant stimulatory effects were noticeable (data not shown). Thus the stimulatory effect of CPA apparently was not due to its progestagenic potential.

In order to test the effectiveness of an antiandrogenic compound with a chemical configuration other than that of CPA, experimental cultures of the three cell lines were exposed to hydroxyflutamide under

Fig. 4. Biphasic efficacy of hydroxyflutamide (HF) on the growth of MCF-7 cells as shown by minor stimulation in concentrations of $10^{-7} \text{M}$ and slight reduction in higher dosages.
comparable conditions. The growth of MCF-7 cells was slightly stimulated by low concentrations of hydroxyflutamide (Fig. 4); doses higher than $10^{-7}$ M reduced the proliferation. The growth of EFM-19 and MFM-21 cells was not significantly altered by hydroxyflutamide (data not shown).

**Interaction of androgen and antiandrogens**

Interference of antiandrogenic CPA or hydroxyflutamide with the androgenic stimulation by DHT was tested in MCF-7 cell cultures (Fig. 5). CPA induced a dose-dependent inhibition of the enhancement of cell proliferation in the presence of $10^{-6}$ M DHT (Fig. 5a). $10^{-8}$ M CPA was ineffective, whereas increasing doses of CPA up to $10^{-5}$ M reduced the enhancement of MCF-7 cell growth down to the level of the untreated control cultures kept in the absence of the hormonal factors. The application of hydroxyflutamide reduced the androgenic stimulation of MCF-7 cell growth by approximately 50% in the dose range of $10^{-8}$ M to $10^{-5}$ M (Fig. 5b). Correspondingly, in EFM-19 cell cultures the stimulatory effect of $10^{-6}$ M DHT was abolished by CPA in the concentration range of $10^{-8}$ M to $10^{-5}$ M (data not shown).

Conversely, the antiandrogenic stimulation of cell growth was subject to interference by the androgen DHT. When the enhancement of the proliferation of MCF-7 cells by $10^{-6}$ M CPA was challenged with increasing doses of DHT (Fig. 6), the effectiveness of CPA was reduced to the level of the untreated control cell growth. Thus an androgenic stimulation by DHT was not observed in the presence of $10^{-6}$ M CPA. In this experiment the stimulatory effect of the sole application of CPA was only half that of $10^{-6}$ M DHT alone which enhanced the cell number more than twofold compared with the control cultures without any hormones. This is in agreement with results shown in the previous experiments with MCF-7 cells in which $10^{-6}$ M DHT was more effective than the equimolar dose of CPA (cf. Figs. 2a, 3a).

**Fig. 5.** Reduction of the growth-enhancing effect of $10^{-6}$ M DHT on MCF-7 cells by the simultaneous application of increasing doses of testosterone acetate (CPA) (a) or hydroxyflutamide (HF) (b). The growth of the untreated control cultures is shown by the open bars; the shaded bars represent the growth in cultures stimulated by the sole application of $10^{-6}$ M DHT.

**Fig. 6.** Reduction of the stimulatory effect of CPA on the growth of MCF-7 cells by increasing doses of DHT. The open bar represents the growth of the untreated control cultures. The enhancement of cell growth by the sole application of $10^{-6}$ M DHT is indicated by the filled bar; the effect of $10^{-6}$ M CPA alone is shown by the dotted line.

**Fig. 7.** Estrogen-induced growth of MCF-7 cells in the presence of increasing doses of antiandrogenic CPA (a) or hydroxyflutamide (HF) (b). The growth of the untreated control cultures is represented by the open bars; the shaded bars show the stimulatory effect of $10^{-8}$ M E-2 alone.
Lack of interference of antiandrogens with estrogentic stimulation

The growth-promoting effect of E-2 on MCF-7 cells was not affected by the simultaneous application of increasing doses of antiandrogens (Fig. 7). CPA was unable to reduce the stimulatory effect of $10^{-8} \, M$ E-2 (Fig. 7a). A slight enhancement of MCF-7 cell growth beyond the effect produced by the sole administration of $10^{-8} \, M$ E-2, although statistically not significant, was observed in the presence of $10^{-7} \, M$ and $10^{-6} \, M$ CPA; this is in the same dose range in which the single application of CPA led to an increase of MCF-7 cell number (cf. Fig. 3a). Similarly, the simultaneous application of increasing concentrations of hydroxylflu- tamide in combination with $10^{-8} \, M$ E-2 was not effective in reducing the stimulation of MCF-7 cell growth by the estrogen (Fig. 7b), indicating the lack of interference.

Binding of antiandrogen to intact MCF-7 cells

For the determination of the affinity of the androgen receptor for the antiandrogens CPA and hydroxylflu- tamide, competitive binding assays were performed with the synthetic compound $[^3]HR1881$. In these experiments MCF-7 cell cultures were preincubated for 7 days in reduced, steroid-free medium. As demonstrated by Scatchard analysis (Fig. 8), androgenic $[^3]HR1881$ was bound by a high-affinity receptor in MCF-7 cells ($K_d = 1.7 \times 10^{-10} \, M$). We found 34,600 androgen-binding sites per cell. The incubation of the presence of $10^{-8} \, M$ or $10^{-7} \, M$ CPA resulted in competitive displacement of $[^3]HR1881$ from the androgen receptor. The apparent affinities of $[^3]HR1881$ were deduced from the Scatchard plots, corresponding to $K_d = 2.0 \times 10^{-10}$ and $K_d = 3.5 \times 10^{-10} \, M$, respectively. The affinity of CPA, calculated from competition curves, was $K_d = 9.1 \times 10^{-9} \, M$. The affinity of hydroxylflu- tamide (data not shown) was estimated in a similar fashion from the reduced apparent binding of $[^3]HR1881$ in the presence of $10^{-8} \, M$ and $10^{-7} \, M$ hydroxylflu- tamide (apparent $K_d = 2.3 \times 10^{-10}$ and $8.0 \times 10^{-10} \, M$, respectively). The binding of hydroxylflu- tamide to the androgen receptor was calculated to occur with $K_d = 2.7 \times 10^{-8} \, M$.

Discussion

The carcinoma cell character of the three lines compared in this study is indicated by the abnormal chromosome distribution patterns. In addition, the tumorigenicity of EFM-19 and MFM-21 cells was confirmed by heterotransplantation in nude mice. For MCF-7 cells, estrogen-sensitive growth in athymic mice had been demonstrated previously (Sauter and McCrath 1980; Shafie and Grantham 1981; Osborn et al. 1985). The in vitro aspect of the cell culture investigated showed EFM-19 cells consistently growing in colonies with the doubling time twice as long as that of the more diffusely arranged MCF-7 or MFM-21 cells. Stimulatory effects of E-2 on the in vitro growth of MCF-7 cells (Lippman et al. 1976a) and EFM-19 cells (Simon et al. 1984a) have been described earlier whereas MFM-21 cells did not react to E-2 in the culture medium, as shown in earlier experiments (unpublished). It is interesting to note that only the growth of the estrogen-responsive cell lines MCF-7 and EFM-19 was enhanced by $10^{-9} \, M$ to $10^{-6} \, M$ DHT applied to this investigation.

The stimulation of MCF-7 and EFM-19 cell growth by DHT initiated the simultaneous application of antiandrogenic CPA, since it appeared possible that the androgenic stimulation of proliferation in the estrogen-responsive cell lines might be mediated through the estrogen receptor. A similar mechanism was proposed for the androgen-induced elevation of the progesterone receptor level in MCF-7 mammary carcinoma cells (Zava and McGuire 1978). Since androgens can be converted to estrogens by aromatization (MacDonald et al. 1967; Longcope et al. 1964), the stimulatory effect of relatively high doses of DHT on the cell growth might be caused by estrogen via the estrogen receptor (Sisteri et al. 1974; Santen 1980). In the case of participation of the estrogen receptor in the androgenic stimulation of cell growth, CPA should not act competitively in the proliferation assay. H-
The stimulatory effect of CPA on MCF-7 cell growth is unexpected, since this antiandrogenic substance is not known to have any estrogenic or androgenic potency (Neumann and Steinbeck 1974). A progestagenic effect of CPA was ruled out in this investigation by experiments that showed progesterone to be rather inhibitory than stimulatory on the growth of MCF-7 and EFM-19 cells (data not shown).

The growth-promoting effect of CPA and hydroxylutamide in this study is in conflict with earlier reports on the inhibitory action of antiandrogens on the growth of MCF-7 cell cultures (Lippman et al. 1976b). Alterations of the steroid receptor levels and of the responsiveness to endocrine factors are not unusual in human mammary carcinoma cell cultures and may arise even during relatively short cultivation periods (Simon et al. 1984b; Hölzle et al. 1986). Clonal variation among MCF-7 sublines with distinct karyotypic alterations (Whang-Peng et al. 1983) was responsible for heterogeneity in tumor growth after transplantation in nude mice (Seibert et al. 1983; Butler et al. 1986). Some of the MCF-7 sublines had developed estrogen-independent growth (Nakata et al. 1981 b) and resistance to the antiestrogen tamoxifen (Nakata et al. 1981 a; Reddel et al. 1985), although antiestrogen binding (Miller et al. 1984) and inducibility of estrogen-responsive proteins may be maintained (Vignon et al. 1984). Clonal variations also explained contradictory reports on the stimulation of MCF-7 cell growth by LHRH agonists (Miller et al. 1985a; Wilding et al. 1987). It appears that the enhancement of cell growth by antiandrogens demonstrated in this investigation can be regarded as another example of the development of clonal variants in mammary carcinoma cell populations based on positive selection mechanisms during the extended in vitro cultivation.

The results of this study, providing evidence for growth-stimulatory effects of antiandrogens on human carcinoma cells, suggest caution in regard to the usefulness of CPA in the endocrine treatment of mammary tumor patients, unless the desired carcinostatic effect can be demonstrated in the individual case. Further investigations with mammary carcinoma sublines containing androgen-binding activity may be helpful to explore the promotion of cell growth by CPA and its androgenic property.


R. Hackenberg et al.: Antiandrogens and breast cancer 259

Received May 13, 1988/Accepted July 14, 1988