Additive Inhibitory Effects of an Androgen and the Antiestrogen EM-170 on Estradiol-stimulated Growth of Human ZR-75-1 Breast Tumors in Athymic Mice

Sophie Dauvois, Chang-Shan Geng, Charles Lévesque, Yves Mérand, and Fernand Labrie

Medical Research Council Group in Molecular Endocrinology, CHUL Research Center and Laval University, Quebec G1V 4G2, Canada

ABSTRACT

The effects of the androgen dihydrotestosterone (DHT) and of the androgenic steroid medroxyprogesterone acetate were studied on the growth of human ZR-75-1 breast carcinoma in athymic mice. The possibility of additive inhibitory effects of DHT and of the new steroidal antiestrogen N-a-butyl, N-methyl-16'-chloro-3',17'-dihydroxy-estra-1',3',5'(10')-tri-en-7'-a-ylundecanamide (EM-170) was also investigated on tumor growth. Removal of the high dose 17β-estradiol (E2) implants used to optimally stimulate initial ZR-75-1 tumor development in ovariectomized mice led to a progressive decrease in tumor area to 50.2 ± 8% (SEM) of original tumor size 40 days after E2 deprivation. Additional treatment with the androgen DHT led to a more rapid fall in tumor volume, which already reached 57% of pretreatment values at 11 days. Whereas physiological implants of E2 led to a progressive increase in tumor size to about 180% above original size after 40 days, physiological plasma levels (205 ± 37.2 pg/ml or ~0.67 nM) of DHT completely reversed the stimulatory effect of E2. Similar inhibitory effects on E2-stimulated tumor growth were achieved with the synthetic androgenic steroid medroxyprogesterone acetate. When the steroidal antiestrogen EM-170 at the dose of 30 µg/day was used simultaneously with DHT, tumor area was further reduced from 99.0 ± 5.9% (DHT alone) to 58.8 ± 18% when both DHT and EM-170 were administered together for 40 days compared with 169 ± 22.2% in control E2-stimulated animals. The present data show that the androgen DHT as well as medroxyprogesterone acetate are potent inhibitors of E2-stimulated human ZR-75-1 breast cancer cell growth in vitro. Moreover, the inhibitory effect of DHT can be further increased by addition of the antiestrogen EM-170, thus suggesting the interest of combining these 2 classes of compounds acting, at least partially, through different mechanisms, in order to improve breast cancer therapy in women.

INTRODUCTION

While estrogens have long been recognized as playing an important role as stimulators of human breast cancer growth (see Refs. 1–3 for review), recent experimental data have demonstrated that androgens exert a direct inhibitory effect on the proliferation of human breast cancer ZR-75-1 cells (4–9). Such data support the well-known observation that androgens used for the treatment of advanced breast cancer in both pre- and postmenopausal women (10–13) have a success rate comparable with that achieved with other endocrine therapies (12, 14, 15). Whereas the beneficial effects of androgens in intact women may be partially mediated by blockade of pituitary gonadotrophin secretion and secondarily, inhibition of ovarian estrogen secretion, the above-mentioned data (4–9) provide strong evidence for a direct inhibitory action of androgens on breast cancer cells. In fact, the role of androgens as direct regulatory factors of breast cancer cell growth is well supported by the presence of androgen receptors in a large proportion of human breast cancers (16–20).

In addition to the direct growth inhibitory effect of physiological concentrations of androgens demonstrated in human breast cancer ZR-75-1 cells in vitro (4–9), we have recently observed a potent inhibitory effect of androgens, namely DHT, a nonaromatizable androgen with high specificity for the androgen receptor, on the growth of DMBA-induced mammary tumors in ovariectomized rats supplemented with estrogens (21). Moreover, the inhibitory effect of DHT was reversed by simultaneous treatment with the antiandrogen flutamide, thus further supporting an action of DHT via specific binding to the androgen receptor (21). DMBA-induced mammary tumors are known to possess androgen receptor (22). A similar direct inhibitory effect on the growth of DMBA-induced mammary tumors has been achieved with medroxyprogesterone acetate (23), a compound possessing a relatively high level of androgenic activity (5, 24–27).

Following demonstration of the additivity of the inhibitory effect of DHT and antiestrogens on ZR-75-1 cell proliferation in vitro (4–9), we have extended our study in vivo in athymic mice using the same human breast cancer cells to more closely mimic the clinical situation in women. The present study thus examines the effect of low doses of the androgen DHT and of the androgenic steroid MPA on the growth of ZR-75-1 tumors in athymic mice as well as the potential beneficial effect of additional treatment with a steroidal antiestrogen possessing pure antiestrogenic activity.

MATERIALS AND METHODS

ZR-75-1 Cells. ZR-75-1 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and routinely cultured in RPMI 1640 supplemented with 2 mm l-glutamine, 1 mm sodium pyruvate, 100 IU penicillin/ml, 100 µg streptomycin/ml, and 10% fetal bovine serum, under a humidified atmosphere of 95% air/5% CO2 at 37°C as described (4, 28). Cells were passaged weekly by treatment with 0.05% trypsin/0.02% EDTA (w/v). The cell cultures used for the experiments herein described were between passages 87 and 96.

Animals. Female homozygous CD-1 (nu/nu) athymic mice (28- to 42-day-old) were obtained from Charles River Canada, Inc. (St. Constant, Quebec, Canada). Mice were housed in vinyl cages with air filter tops in laminar air flow hoods and maintained under pathogen-limited conditions. Cages, bedding, and food were autoclaved before use. Water was autoclaved, acidified to pH 2.8, and provided ad libitum. Mice were bilaterally OVX under anesthesia achieved by i.p. injection of 0.25

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3 Holder of a Fonds de la Recherche en Santé du Québec Studentship.

4 To whom requests for reprints should be addressed, at Medical Research Council Group in Molecular Endocrinology, CHUL Research Center, 2705 Laurier Boulevard, Quebec G1V 4G2, Canada.

The abbreviations used are: DHT, 5α-dihydrotestosterone; MPA, medroxyprogesterone acetate; EM-170, N-a-butyl,N-methyl-16'-chloro-3',17'-dihydroxy-estra-1',3',5'(10')-tri-en-7'-a-ylundecanamide; DMBA, dimethylbenz[a]anthracene; OVX, ovariectomized; E2, 17β-estradiol.
RESULTS

In preliminary experiments, we studied the influence of the number of injected cells and their growth phase on the latent period and incidence of palpable tumors in the athymic mice. The best results were obtained after injection of 2 to 4 x 10^6 ZR-75-1 cells collected during their logarithmic phase of growth. Under such conditions, tumors with a diameter of 0.5 cm or larger were found in 11.2 ± 4.2% (SEM), 50.6 ± 7.27%, and 69.6 ± 8.22% of animals after 2, 4, and 6 weeks, respectively, in O VX mice supplemented with E₂. Tumor measurements were performed 3 times a week using Vernier calipers. Two perpendicular tumor diameters were measured and tumor area (in cm²) was calculated using the formula \( \frac{1}{2} \times w \times l \). Tumor area calculated on the first day of treatment was taken as 100%.

Statistical significance was measured according to the multiple-range test of Duncan-Kramer (30). Data are expressed as means ± SEM.

Fig. 1. A, Effect of 8-day treatment with increasing doses of E₂ on uterine weight in OVX BALB/c female mice. Comparison is made with intact animals (C). E₂ was administered by silastic implants containing E₂ at decreasing dilutions with cholesterol (1/5000, 1/3000, 1/500, and 1/250, E₂/cholesterol, w/w). B, Effect of 8-day treatment with silastic implants of DHT (1/5, DHT/cholesterol, w/w) on seminal vesicle weight and DHT plasma levels in castrated (CX) BALB/c male mice in comparison with intact mice (C). Results are expressed as means ± SEM of 10 mice/group.

Fig. 2. Effect of OVX or additional treatment of OVX athymic mice with silastic implants of DHT (1/5, DHT/cholesterol, w/w) on average total ZR-75-1 tumor area. Results are expressed as percent of pretreatment values (means ± SEM of 8 tumors/group).
vals, tumor size remained at approximately 50% of original tumor area.

We next examined the effect of DHT on tumor growth stimulated by the "physiological" E2 implant. As illustrated in Fig. 3, E2 caused a constant increase in total tumor area from 100% (which corresponds to an average of 0.23 ± 0.08 cm²) to 145 ± 13.7%, 180 ± 19.0%, 179.2 ± 16.0%, 230 ± 32.0%, and 226 ± 31.0% after 20, 40, 60, 80, and 100 days of treatment, respectively. Treatment with DHT, on the other hand, completely reversed the stimulatory effect of E2 of tumor growth and decreased total tumor area to 102 ± 10.0%, 75.2 ± 10.3%, 60.1 ± 11.4%, 53.0 ± 13.0%, and 48.0 ± 9.5% of the original size after 20, 40, 60, 80, and 100 days, respectively (P < 0.01 at all time intervals).

We next studied the effect of constant delivery of MPA from osmotic pumps at the dose of 300 µg/day on E2-stimulated tumor growth. As illustrated in Fig. 4, E2, as observed in the previous experiment, induced a constant increase in tumor growth to 174 ± 22.0% of original size at 40 days (100% = 0.31 ± 0.03 cm²), whereas MPA reversed the effect of E2 at all time intervals. In fact, after 40 days of treatment, tumor size decreased from 174 ± 22.0% (E2 alone) to 61.2 ± 9.1% (E2 + MPA) of original size (100% = 0.27 ± 0.03 cm²).

After demonstration of the potent inhibitory effect of the androgen DHT and of the androgenic compound MPA (5, 24-27), we next examined the effect of combination treatment with an androgen and an antiestrogen. For this experiment, we used a new steroidal antiestrogen developed in our laboratories that shows pure antiestrogenic activity and is thus devoid of any estrogenic effect in all the in vitro and in vivo assays used (29). As illustrated in Fig. 5, E2 (100% = 0.20 ± 0.025 cm²) caused an increase in total tumor area to 169 ± 22.2% of the original size after 26 days with a plateau at later time intervals. On the other hand, it can be seen that DHT inhibited the stimulatory effect of E2 to 99.0 ± 9.5% of the original size (100% = 0.23 ± 0.05 cm²) after 40 days of treatment (P < 0.01), whereas addition of the antiestrogen EM-170 to DHT further inhibited tumor size to 58.2 ± 18.0% of original size (P < 0.01 versus E2 and P < 0.05 versus E2 + DHT).

**DISCUSSION**

The present data demonstrate that the androgen DHT is a potent inhibitor of the stimulatory effect of E2 on ZR-75-1 human breast carcinoma growth in athymic mice. While our original observations of the inhibitory effect of androgens on human breast cancer ZR-75-1 growth were obtained in cells in culture (4-9), the present study performed under in vivo conditions more closely mimics the clinical situation in women. Moreover, MPA, a synthetic steroid well recognized for its androgenic activity in many systems (24-27), including breast cancer, was shown to be effective in reversing the effect of E2 on breast tumor growth in vivo.
cancer cell growth (5), exerts inhibitory effects similar to those of DHT on E2-stimulated ZR-75-1 tumor growth in athymic mice.

Tumor regression of MCF-7 cells has been observed upon estrogen withdrawal and tamoxifen treatment (31–33) in athymic mice, whereas other reports failed to show the efficacy of estrogen withdrawal on tumor regression but rather observed tumor stabilization (34–36). The present data (Fig. 2) clearly show a regression of ZR-75-1 tumors upon removal of E2 implants in OVX animals.

In a previous study, it has been observed that the estrogen-dependent breast carcinoma Br-10 had a slow growth rate in female athymic mice but that the same tumor inoculated in untreated male mice or female animals treated with testosterone propionate did not grow (37). Such data obtained with the Br-10 mammary carcinoma suggest that androgens, at the opposite of estrogens, do not support the growth of this human mammary tumor type in nude mice. In the present study, the inhibitory effect of DHT alone in OVX animals is characterized by a more rapid regression of tumor volume followed by a period of growth stabilization during the following month. No appearance of new tumors was detectable during the whole observation period in animals OVX treated with vehicle or DHT.

Studies in the athymic mouse have shown that MCF-7 cells may develop resistance to tamoxifen after long-term treatment and that, furthermore, the growth of such tumors is stimulated by tamoxifen (38). However, the growth of tamoxifen-stimulated tumors was inhibited by new pure antiestrogens (39), thus suggesting that such tamoxifen-resistant tumors remain estrogen-sensitive. It would certainly be of interest to investigate the possible inhibitory effect of androgens on tamoxifen-resistant tumors. In fact, some clinical studies have suggested the benefit of combined therapy with an antiestrogen and an androgen (40, 41). Thus, treatment with fluoxymesterone was found to be more efficient than tamoxifen alone (42, 43), with the addition of the androgenic compound or anabolic steroid leading to a 33% remission rate in tamoxifen-insensitive tumors (43).

Our previous in vitro data have demonstrated that concentrations of DHT similar to the plasma levels found in men are potent inhibitors of the mitogenic effect of E2 in human ZR-75-1 cells and also inhibit growth in the absence of estrogens. The present findings of additional inhibitory effects upon addition of the pure antiestrogen EM-170 to DHT are also well supported by in vitro data obtained with the same cell line (4). Whereas, in Fig. 5, DHT caused stabilization of tumor size in the presence of E2, a higher degree of inhibition of tumor volume was observed with the same treatment in Fig. 3, thus illustrating the well-recognized variable degree of response to inhibitors of tumor growth (31–36, 38, 39).

Whereas part of the inhibitory effects of DHT could be due to a down-regulation of estrogen receptors (6), an inhibitory effect of androgens on ZR-75-1 cell growth has also been observed in the presence or maximally inhibitory concentrations of antiestrogens (4), thus clearly suggesting that androgens exert a direct inhibitory effect on breast cancer cell growth that is independent of the estrogen receptor. Such data provide an explanation for the additional beneficial effects of fluoxymesterone over tamoxifen alone (42, 43). In fact, we have found that androgens inhibit cell proliferation stimulated by IGF-1 and EGF in a dose-dependent manner, this effect being also reversed by hydroxyflutamide. In addition, androgens induce the expression of specific proteins such as GCDFP-15 (7, 8) and GCDFP-24 (9).

The observation of a stimulatory effect of androgens on breast cancer growth (44–47) may be due to the use, in most of these studies, of the aromatizable androgen testosterone, which is converted into estrogens in breast cancer cells (48). Furthermore, the high supraphysiological doses of androgens used are likely to permit their binding to the estrogen receptor (4, 49), thus inducing estrogen-specific effects such as stimulation of cell proliferation, induction of M, 52,000 protein expression (50), and inhibition of GCDFP-15 expression (7). It should be mentioned that the biphasic effect of DHT on breast cancer cell growth has been clearly demonstrated in ZR-75-1 cells in vitro (4). In fact, at low concentrations (0.01 to 10 nm), DHT interacts exclusively with the androgen receptor, thus leading to inhibition of cell growth while, at concentrations above 10 nm, the androgen also interacts with the estrogen receptor, thus progressively counteracting its androgen receptor-mediated inhibitory effect on cell growth (4).

To avoid the possibility of aromatization to estrogens and the potential interaction of high-dose androgen with the estrogen receptor, we have used the nonaromatizable androgen DHT at levels that closely resemble physiological concentrations in humans. Under such conditions, as mentioned above, we have observed specific androgen receptor-mediated activity of DHT on breast cancer cells that are all reversed by the pure antiandrogen hydroxyflutamide (4–9).

The inhibitory effect of DHT on ZR-75-1 tumor growth observed in this study does not result from an effect of DHT on the pituitary-gonadal axis causing a secondary decrease in E2 secretion by the ovaries, since OVX animals supplemented by continuous release of physiological levels of E2 were used. Such data provide further support for a direct inhibitory action of androgens at the tumor cell level under in vivo conditions, thus adding to the inhibitory effect of androgens on pituitary gonadotropin release in intact subjects.

As mentioned above, the mechanisms by which androgens exert a direct inhibitory effect on breast cancer growth may be explained, at least in part, by the recent observation that androgens inhibit estrogen receptor expression (6) and cell cycle kinetic parameters (51). Moreover, it has recently been found that androgens stimulate the conversion of E2 into estrone (E1) by enhancing 17β-hydroxy-steroid dehydrogenase activity (52), thus decreasing the intracellular concentration of E2.

The additive inhibitory effects of androgens in combination with antiestrogen therapy may offer an improvement of endocrine therapy of breast cancer in both pre- and postmenopausal women. In fact, since androgens and antiestrogens appear to act, at least in part, through different mechanisms, it is reasonable to suggest that an additional number of cancer cells should become responsive to such endocrine therapy and that a greater degree of tumor regression should be achieved.

REFERENCES


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EFFECTS OF ANDROGEN AND ANTIESTROGEN ON BREAST TUMOR GROWTH


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