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Androgens and Breast Cancer

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ABSTRACT

We have recently demonstrated that physiological levels of androgens exert direct and potent inhibitory effects on the growth of human breast cancer ZR-75-1 cells *in vivo* in nude mice as well as *in vitro* under both basal and estrogen-stimulated conditions. The inhibitory effect of androgens has also been confirmed on the growth of dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in the rat. Such observations are in close agreement with the clinical data showing that androgens and the androgenic compound medroxyprogesterone acetate (MPA) have beneficial effects in breast cancer in women comparable to other endocrine therapies, including tamoxifen. Although the inhibitory action of androgens on cell proliferation in estrogen-induced ZR-75-1 cells results, in part, from their suppressive effect on expression of the estrogen receptor, the androgens also exert a direct inhibitory effect independent of estrogens. Androgens cause a global slowing effect on the duration of the cell cycle. These observations support clinical data showing that androgenic compounds induce an objective remission after failure of antiestrogen therapy as well as those indicating that the antiproliferative action of androgens is additive to that of antiestrogens. We have also recently demonstrated in ZR-75-1 human breast cancer cells the antagonism between androgens and estrogens on the expression of GCDFP-15 and GCDFP-24 which are two major proteins secreted in human gross cystic disease fluid. The effects of androgens and estrogens as well as those of progestins and glucocorticoids on GCDFP-15 and GCDFP-24 mRNA levels and secretion are opposite to those induced by the same steroids on cell growth in ZR-75-1 cells. In fact, the changes in secretion of these two glycoproteins could be useful markers of the action of androgenic and antiestrogenic compounds during the course of breast cancer therapy in women.

Key Words: androgens, estrogens, sex steroid formation, gross cystic disease fluid proteins, intracrinology, cell cycle kinetics, antiestrogens.

I. INTRODUCTION

While estrogens have long been known to play an important role as stimulants of human breast cancer growth,[1-3] our recent data have demonstrated that androgens exert an opposite and 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 to that achieved with other endocrine therapies.[12,14] Moreover, a higher response rate and a longer time to disease progression are observed when androgens are combined with an antiestrogen compared to an antiestrogen alone.[15,16] It should also be mentioned that androgens induce an objective remission after failure of antiestrogen therapy and hypophysectomy. These clinical and experimental observations indicate that the benefits obtained with androgen therapy cannot solely be due to a suppression of pituitary gonadotropin secretion but result, at least in part, from a direct effect on tumor growth. The role of androgens as direct regulatory factors of breast cancer growth is well supported by the presence of androgen receptors in a large proportion of human breast cancers.[17-20]

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 5α-dihydrotestosterone (DHT), a non-aromatizable androgen with high specificity for the androgen receptor, on 17β-estradiol (E_2)-stimulated human ZR-75-1 breast cancer cell growth in athymic mice. Moreover, we have also demonstrated that DHT exerts an inhibitory effect on the growth of DMBA-induced mammary tumors in ovariectomized rats supplemented with estrogens.[21] 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 in these mammary tumors known to possess the androgen receptor.[21,22] We have also recently demonstrated the marked antagonism between androgens and estrogens on the expression of the two glycoproteins GCDFP-15 and GCDFP-24, which are major proteins present in human breast gross cyst fluid.[7,9]

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II. CRUCIAL IMPORTANCE OF INTRACRINE FORMATION OF ANDROGENS AND ESTROGENS

While the above-mentioned data describe the direct inhibitory effects of androgens on breast cancer growth, it is likely that endogenous androgens play an important role in the control of normal and tumoral breast tissue growth and function. In fact, men and women are unique with other primates in having adrenals that secrete large amounts of the precursor steroids dehydroepiandrosterone (DHEA) and especially DHEA-sulfate (DHEA-S) which are converted into androstenedione (Δ^4-dione) and then into potent androgens and estrogens in peripheral tissues. This situation of a high secretion rate of adrenal precursor sex steroids in men and women is thus completely different from all current animal models used in the laboratory, namely, rats, mice, guinea pigs, and all others (except monkeys), where the secretion of sex steroids takes place exclusively in the gonads.[23,24] These recent observations have opened a new field of endocrinology, that of "intracrinology".[25] Through intracrine activity, locally produced androgens and/or estrogens exert their action inside the same cells where synthesis takes place. Intracrinology is thus a terminology complementary to the well-known autocrine, paracrine, and endocrine activities wherein a hormone acts at the surface of the producing cells (autocrine activity), a hormone acts on neighboring cells (paracrine activity), or a hormone released in the circulation acts on distant target tissues (classical endocrine activity).

A major problem which is at least partially responsible for the delayed progress in intracrinology is the fact that the animal models usually used in the laboratory do not secrete significant amounts of adrenal precursor sex steroids, thus focusing all attention on the testes and ovaries as the exclusive sources of androgens and estrogens for target tissue growth and function. Since local formation of androgens and estrogens plays such a major role in both normal and tumoral hormone-sensitive tissues in the human, a major proportion of our research program has recently been devoted to this exciting and therapeutically promising area.

For example, as a measure of the importance of adrenal precursor sex steroids in adult men, the serum levels of the main metabolites of androgens, i.e., 5α-androstane-3α, 17β-diol (3α-diol), androsterone (ADT), and their glucuronidated derivatives, 3α-diol-G and ADT-G, are only reduced by 50 to 70% following surgical or medical castration,[26,27] thus demonstrating that the conversion of adrenal precursor sex steroids accounts for 30 to 50% of total androgens in adult men. In women, the role of the adrenal steroids DHEA-S, DHEA, and Δ^4-dione as precursors of estrogens is likely to be even more important.[28] In fact, our best estimate of the intracrine formation of estrogens in peripheral tissues in women is likely to be in the order of 75% before menopause and almost 100% after menopause. It thus becomes of major importance to obtain precise knowledge of the factors regulating sex steroid formation and degradation in breast, skin, adipose tissue as well as in other peripheral tissues.

The precursors DHEA-S and DHEA secreted by the adrenals require the action of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3Δ-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase, and/or aromatase in order to form the androgen DHT or the estrogens E_2 and androst-5-ene-3β-17β-diol (Δ^5-diol). Since the structure of the enzymes 3β-HSD and 17β-HSD was not known, we cloned cDNAs encoding human, macaque, bovine, and rat 3β-HSD as well as human 17β-HSD and have deduced the amino acid sequences of the corresponding proteins.[29–35] We have also elucidated the structure of two in tandem human 17β-HSD genes[36] and a human 3β-HSD gene.[37] Both 3β-HSD and 17β-HSD are expressed in breast and in a large series of peripheral tissues, thus permitting the local biosynthesis of active androgens and estrogens from the adrenal precursor sex steroids DHEA and DHEA-S.

III. SOURCES OF ANDROGENS IN WOMEN

While there is essentially no arterovenous gradient of testosterone (T) across the adrenal gland, similar measurements in the ovary indicate that 20% of the 150 to 200-μg daily production rate of T can be accounted by direct ovarian production.[38] On the other hand, the ovary and adrenal do not secrete DHT directly. It can thus be concluded that, in women, circulating T and DHT are essentially derived from peripheral transformation of DHEA and Δ^4-dione by 3β-HSD, 17β-HSD, and 5α-reductase. In fact, the daily secretion rate of Δ^4-dione in normal cycling women is 1.5 mg, equal amounts being of ovarian and adrenal origins in premenopausal women.[39,40] Δ^4-dione can be transformed into either T and DHT[41] or into the estrogen E_2, depending upon the ratio of 17β-HSD, 5α-reductase, and aromatase activities present in peripheral tissues. In postmenopausal women, about 70% of circulating Δ^4-dione is of adrenal origin, the rest being of ovarian origin.[42] Not only is DHT formed locally in peripheral tissues from T and Δ^4-dione, but DHT is also largely metabolized intracellularly prior to entering the circulation, mainly as ADT-G and 3α-diol-G.

IV. SEX STEROID METABOLISM IN ZR-75-1 HUMAN BREAST CANCER CELLS

In order to better understand the mechanisms regulating androgen and estrogen biosynthesis and metabolism in breast cancer cells, we have identified and quantified the main metabolic pathways of androgens and estrogens in the well-known ZR-75-1 cell line, including the formation of steroid fatty acid esters (FAEs), glucuronides, and sulfates.[43–45] Among the en-

zymes involved in sex steroid biosynthesis and metabolism, 17β-HSD plays a crucial role since it catalyzes the interconversion of E_2 and E_1, T and Δ^4-dione, Δ^5-diol and DHEA, 5α-androstane-3, 17-dione (A-dione) and DHT, ADT and 3α-diol, as well as epiandrosterone (Epi-ADT) and 5α-androstane-3β, 17β-diol (3β-diol) (Figure 1). Following incubation with E_2, Δ^5-diol or T, E_1, DHEA, and Δ^4-dione were the main products, respectively, thus indicating high levels of 17β-HSD.[43] When Δ^4-dione was used, on the other hand, a high level of transformation into A-dione, Epi-ADT, and ADT was found, thus indicating the presence of high levels of 5α-reductase as well as 3α- and 3β-hydroxysteroid dehydrogenase.[43]

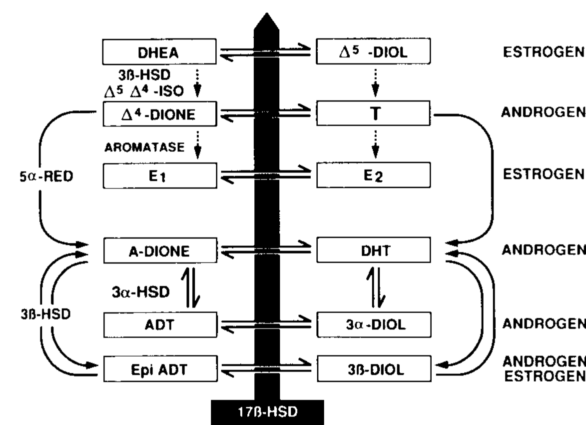

FIGURE 1. Schematic representation of the enzymatic activities demonstrated in human breast cancer cell lines ZR-75-1 following incubation with the estrogenic and androgenic steroids and precursors, namely, E_2, E_1, Δ^5-diol, DHEA, T, Δ^4-dione, DHT, and ADT (bold characters). Note the presence of the four enzymes (solid lines) and the absence of two enzymes (broken lines) in this model.

As evidenced by the above-mentioned data, 5α-reductase activity is present at a particularly high level in ZR-75-1 cells. The best precursor for the production of the above-mentioned metabolites was DHT with ~ 68% of total radioactivity transformed into A-dione, ADT, epi-ADT, DHT, 3α-diol, and 3β-diol. These data demonstrate that the ZR-75-1 human mammary cancer cells can synthesize androgens and estrogens very efficiently from inactive adrenal steroid precursors, thus well illustrating the importance of intracrine function in breast cancer cells. The enzymatic systems involved not only synthesize the active steroids, but also metabolize them into inactive derivatives, thus controlling the level of intracellular active steroids.

V. MECHANISMS OF ANTIPROLIFERATIVE ACTION OF ANDROGENS IN BREAST CANCER CELLS

In addition to the well-recognized inhibitory effects of antiestrogens on breast cancer cell proliferation, as previously mentioned, DHT is a potent inhibitor of growth of the estrogen-sensitive breast cancer cell line ZR-75-1 through a specific interaction with the androgen receptor.[4–9] Since the benefits of combined treatment with an androgen and an antiestrogen have already been reported in women with breast cancer,[15,16] thus supporting the *in vitro* data mentioned earlier,[4–9] a more precise understanding of the mechanisms of action of androgens and antiestrogens in breast cancer cells becomes important.

After a 12-day incubation of ZR-75-1 cells in the presence of 0.1 n*M* E_2 in phenol red-free medium, cell number was increased 2.8-fold above control ($p < 0.01$) (Figure 2A). The addition of 1 n*M* DHT, on the other hand, caused a 78% blockade ($p < 0.01$) of E_2-induced ZR-75-1 cell growth, while the new pure steroidal antiestrogen EM-139,[46] on the other hand, not only completely reversed the effect of E_2 but further inhibited cell number by 30% below control values ($p < 0.01$) (Figure 2B). It can also be seen in Figure 2B that, in the absence of E_2, EM-139 and DHT alone caused 21% ($p < 0.01$) and 43% ($p < 0.01$) inhibitions of basal cell growth, respectively.

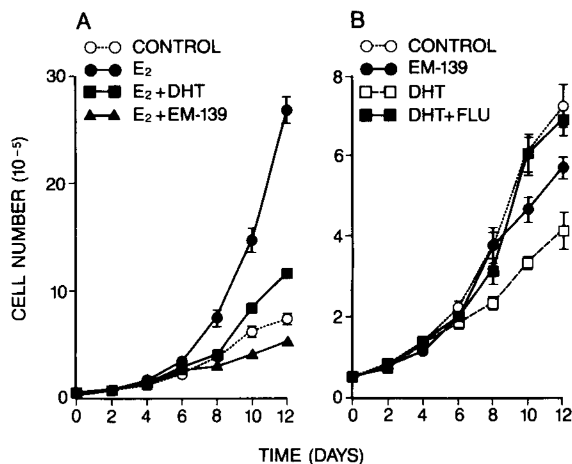

FIGURE 2. (A) Time course of the effect of 0.1 n*M* E_2, 1 n*M* DHT + E_2, 0.3 μ*M* EM-139 + E_2, or control medium on the proliferation of ZR-75-1 cells during a 12-day incubation period. (B) Time course of the effect of 1 n*M* DHT, 0.3 μ*M* EM-139, DHT + EM-139, DHT + 0.3 μ*M* OH-FLU, or control medium on the proliferation of ZR-75-1 cells. Three days after plating at an initial density of 5 × 10^5 cells/10 cm^2 per well, cells were incubated with the indicated concentrations of the compounds with medium changes every 48 h for the indicated time periods. At the end of the indicated incubation periods, cell number was determined with a Coulter counter. Data are expressed as means ± SEM of quadriplicate wells.[47]

In order to more easily visualize the effect of treatment with E_2, DHT, and EM-139 on the cell cycle kinetic parameters, the data describing the G_0-G_1 fraction in Figure 3 are presented as percentage change relative to control cells taken as 100%. E_2 treatment caused a 12.4% decrease ($p < 0.01$) in G_0-G_1 cells 24 and 30 h after addition of the estrogen (Figure 3A). The

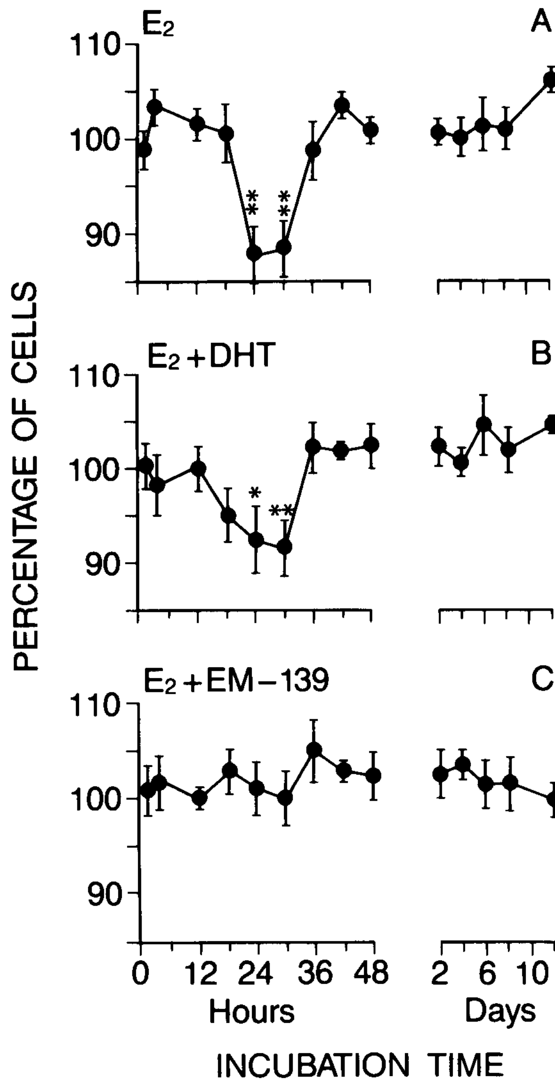

FIGURE 3. Effect of treatment with E_2 in the presence or absence of DHT or EM-139 alone or in combination on the G_0-G_1 fraction of the ZR-75-1 cell cycle. The indicated steroids were used at the following concentrations: E_2, 0.1 n*M*; DHT, 1 n*M*; and EM-139, 0.3 μ*M*. At the indicated time intervals, cells were harvested and fixed before staining with the DNA dye propidium iodide and analyzed with an EPICS 753 flow cytometer. The distribution of the cells in the G_0-G_1, S, and G_2 + M fractions was calculated using the PARA 1 software. Data representing the proportion of cells in the G_0-G_1 phase of the cycle in the indicated treatment groups are expressed as the percentage of control G_0-G_1 cells harvested at the same time. Cells were incubated for the indicated time periods with steroids and treated as described in Figure 2.[47]

antiestrogen EM-139, on the other hand, completely prevented the reduction by E_2 of the proportion of ZR-75-1 cells in the G_0-G_1 phase of the cycle (Figure 3C), while DHT, at the dose used (1 n*M*), only partially reversed the stimulatory effect of E_2 (DHT + E_2: 92.5 ± 2.5% vs. E_2:87.6 ± 1.6%, N.S.).

In order to assess the percentage of cells which advanced through the S phase during the hormonal and antihormonal treatments mentioned above, we have used the continuous BrdUrd exposure technique.[47] It can be seen in Figure 4 that after 48-h exposure to BrdUrd in the absence of estrogen, 10 n*M* DHT alone caused a 13% (p <0.01) decrease in the percentage of cells having incorporated the nucleotide analog. The antiestrogen alone, on the other hand, caused an inhibition slightly greater than that observed with DHT. E_2, on the other hand, increased the percentage of cells having incorporated BrdUrd by 23%, the effect of the estrogen being completely reversed by DHT or EM-139.

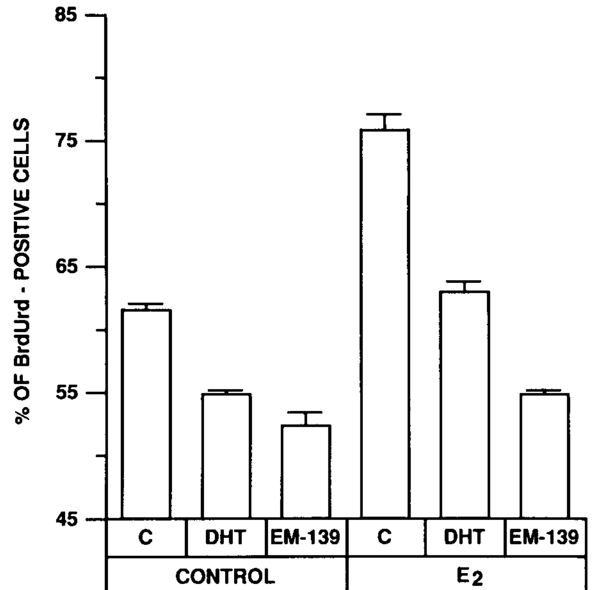

FIGURE 4. Effect of DHT or the new antiestrogen EM-139 in the presence or absence of E_2 on the proportion of cycling ZR-75-1 cells. Cells were pretreated for 2 days with 10 n*M* DHT or 1 μ*M* EM-139 in the presence or absence of 0.1 n*M* E_2 before change for medium containing the same indicated steroids plus 10 μg/ml 5'bromodeoxyuridine (BrdUrd). Following a 2-day incubation, cells were then harvested, fixed, and stained with the DNA dye Hoechst 33342. The percentage of BrdUrd-positive cells was calculated as described.[47]

The present data demonstrate that when concomitantly added with E_2, DHT or EM-139 inhibited the stimulatory effect of E_2 on cell proliferation, while only EM-139 significantly reversed the decrease induced by E_2 on the G_0-G_1 cell fraction. However, DHT and EM-139 alone did not affect the distribution of ZR-75-1 cells between the different phases of the cell cycle. In fact, continuous labeling with BrdUrd showed that EM-139 and DHT uniformly increased the duration of the cell cycle, thus explaining, at least in part, the inhibitory effect of these compounds on cell proliferation. This effect of androgens on the cell cycle is accompanied by their marked suppression of estrogen receptor expression and thus provides

an explanation for the benefits of androgens in breast cancer therapy. Moreover, the present data indicate that the inhibitory effects of androgen therapy could be additive to the standard treatment limited to blockade of estrogen action by antiestrogens.

VI. INHIBITORY EFFECTS OF ANDROGENS ON ZR-75-1 TUMOR GROWTH IN ATHYMIC MICE

Following our demonstration of the inhibitory effect of DHT and antiestrogens on ZR-75-1 cell proliferation *in vitro*,[4–9] we extended our study *in vivo* to ovariectomized athymic mice using the same human breast cancer cells in order to more closely mimic the clinical situation in women. We thus examined the effect of DHT on tumor growth stimulated by "physiological" doses of E_2 administered by silastic implants.

As illustrated in Figure 5, E_2 caused a constant increase in total tumor area from 100% (which corresponds to an average of 0.23 ± 0.08 cm^2) to $145 \pm 14\%$, $180 \pm 19\%$, $179 \pm 16\%$, $230 \pm 32\%$, and $226 \pm 31\%$ after 20, 40, 60, 80, and 100 days of treatment, respectively. Treatment with DHT, on the other hand, completely reversed the stimulatory effect of E_2 on tumor growth and decreased total tumor area to $102 \pm 10\%$, $75 \pm 10\%$, $60 \pm 11\%$, $53 \pm 13\%$, and $48 \pm 10\%$ of the original size after 20, 40, 60, 80, and 100 days, respectively. The androgen DHT is thus a potent inhibitor of the stimulatory effect of E_2 on ZR-75-1 human breast carcinoma growth in athymic mice. Similar inhibitory effects on E_2-stimulated tumor growth were achieved with the synthetic androgenic compound medroxyprogesterone acetate (Provera).[48] 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 well-known inhibitory effect of androgens exerted on the pituitary gonadal axis in intact subjects.

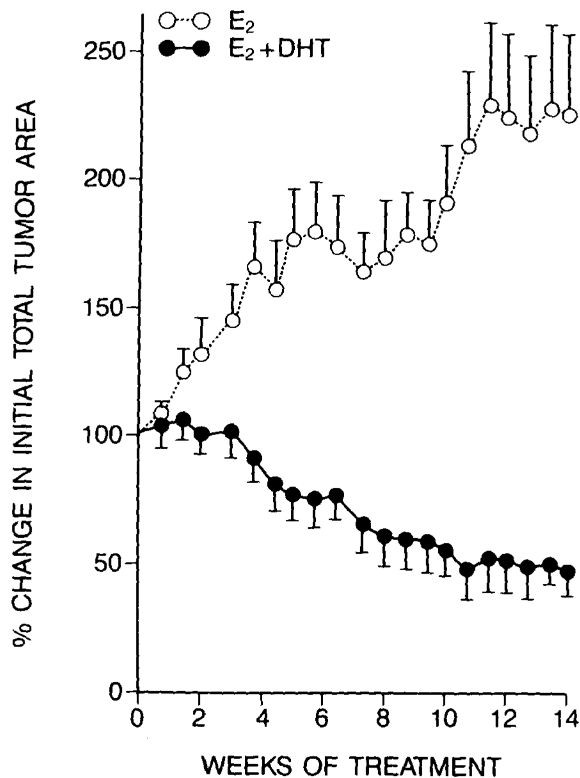

FIGURE 5. Effect of 100-day treatment of ovariectomized (OVX) athymic mice with silastic implants of 17β-estradiol (E_2) (1/3000, E_2/cholesterol, w/w) alone or in combination with silastic implants of dihydrotestosterone (DHT) (1/5, DHT/cholesterol, w/w) on average total ZR-75-1 tumor area in nude mice. Results are expressed as percentage of pretreatment values (means ± SEM of 11 tumors in the E_2 group and 9 tumors in the E_2 + DHT group).[48]

VII. REGULATION OF THE EXPRESSION OF GCDFP-15 AND GCDFP-24 IN ZR-75-1 CELLS BY STEROIDS

The management of breast cancer could be facilitated by the availability of prognostic factors indicating which patients may be at increased risk for relapse and death. The human breast gross cystic disease fluid protein-24 (GCDFP-24), also named progesterone-binding breast cyst protein, accounts for up to 50% of the total protein in breast cyst fluid.[49–51] Furthermore, GCDFP-24 is also detected by immunohistochemistry at relatively high concentrations in several steroid-sensitive cancers, including those of the endometrium, ovary, and prostate.[52] Moreover, the concentration of GCDFP-24 in breast tumor cytosol is markedly higher in well-differentiated carcinomas than in poorly differentiated tumors.[53] In addition, GCDFP-24 shows a high degree of binding specificity for the C-17 and C-21 regions of progesterone and could well have an influence on the accumulation of the steroid precursors pregnenolone and progesterone, especially in breast cysts and adenocarcinomas, as well as in other steroid-producing normal and tumoral tissues.[50,54] It was demonstrated very recently that the GCDFP-24 corresponds to the apolipoprotein D.[55]

The progesterone-binding breast cyst protein GCDFP-24 is one of the rare examples of an androgen-stimulated protein in human breast cancer cells,[9] the others being the prolactin-inducible protein, which corresponds to GCDFP-15[7,8,56,57] and the zinc α_2-glycoprotein,[58] also named GCDFP-44. It is noteworthy that all three of these androgen-stimulated proteins in human breast cancer cells represent the major proteins found in human breast gross cystic disease fluid.[50] Moreover, these three breast cystic disease fluid proteins have been found in 50% of breast carcinoma samples, while 80% of breast carcinomas expressed at least one of the three proteins.[52] A

positive correlation of immunohistochemical staining was found only between GCDFP-15 and GCDFP-44, thus showing that there exists a divergence of expression of at least some of these proteins with malignant transformation.

We have recently demonstrated that androgens, the progestin R5020, and glucocorticoids stimulate GCDFP-15 and GCPFP-24 mRNA levels and protein secretion while estrogens markedly inhibit these two parameters in ZR-75-1 cells.[7-9,35,59] The effects of androgens, the progestin R5020, glucocorticoids, and estrogens on GCDFP-15 expression are thus opposite to their respective action on ZR-75-1 cell growth. The interest of GCDFP-15 as a tumor marker also pertains to the recent observation that the presence of GCDFP-15 mRNA in human breast cancer samples is positively correlated with the incidence of estrogen, progesterone, and androgen receptors.[8,57,60]

It is thus of interest to summarize the data obtained following detailed investigation of the effect of steroids on both GCDFP-15 and GCDFP-24 secretion in the human breast cancer cell line ZR-75-1. We have investigated in detail the interactions between R5020, DHT, the synthetic glucocorticoid dexamethasone (DEX), and E_2 on cell growth as well as on GCDFP-24 and GCDFP-15 release. As illustrated in Figure 6B, a 10-day incubation with DHT or DEX, alone or in combination, increased GCDFP-24 release by 2.8-, 3.7-, and 6.0-fold, respectively. It can also be seen in the same figure that the amount of GCDFP-24 released by E_2-treated cells was 56% lower than that secreted by control cells. In E_2-treated cells, simultaneous exposure to DHT, DEX, or DHT plus DEX markedly increased GCDFP-24 release by 5.2-, 4.2-, and 10.2-fold, respectively. A similar additive stimulatory effect of androgens and glucocorticoids was also observed on GCDFP-15 release, although the amplitude of the effect of the same steroids is twofold greater on GCDFP-15 compared to GCDFP-24 secretion (Figure 6C). Furthermore, it can also be seen in Figure 6 that exposure to R5020 increased by 1.7- and 6.0-fold the release of GCDFP-24 and GCDFP-15, respectively, in E_2-treated cells. The effect of R5020 (in the presence of E_2) was also additive to that of DHT or DEX, especially on GCDFP-15 release (Figure 6C).

The present data demonstrate that the effect of all steroids studied on both GCDFP-24 and GCDFP-15 secretion is inversely correlated with their respective action on ZR-75-1 cell proliferation, thus suggesting that the synthesis of these glycoproteins could be involved in cell growth arrest, tumor regression and/or cell differentiation.[7,9,59,61] The multiple hormonal regulation of these two glycoproteins also suggests that GCDFP-24 and/or GCDFP-15 could well be a good biochemical marker(s) for monitoring the response to androgenic and antiestrogenic compounds during breast cancer therapy.

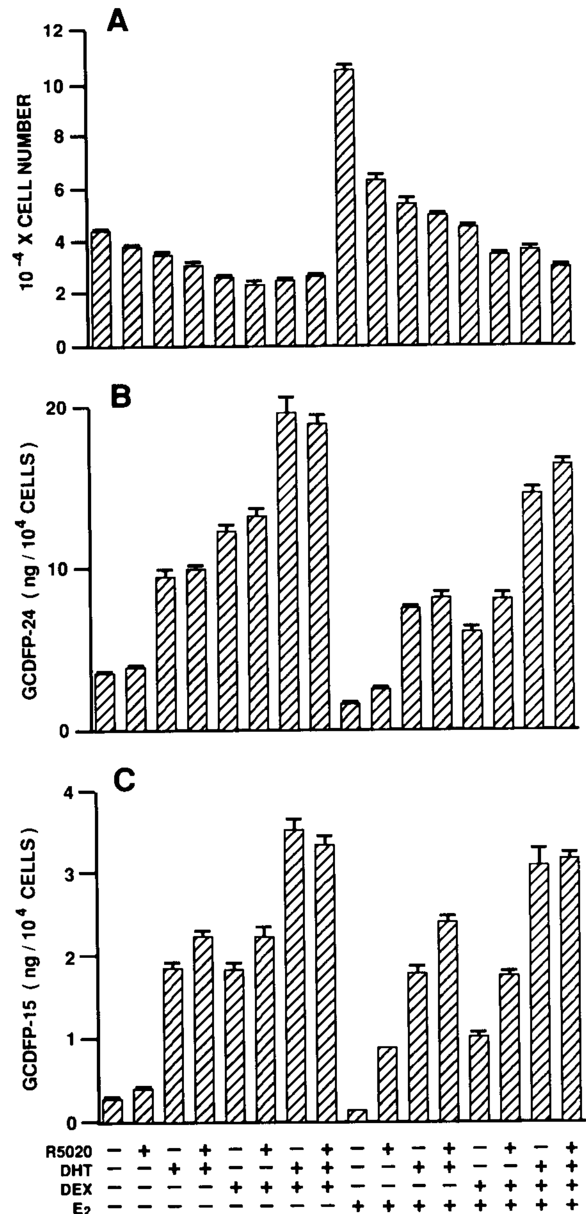

FIGURE 6. Effect of the progestin R5020 (10 n*M*), DHT (10 n*M*), and the synthetic glucocorticoid dexamethasone (DEX; 300 n*M*) alone or in combination in the presence or absence of E_2 (0.1 n*M*) on cell proliferation (A), GCDFP-24 secretion (B), and GCDFP-15 release (C) in ZR-75-1 human breast cancer cells. Cells were incubated for 10 days with the indicated steroids with changes of media at 2-day intervals. At the end of the incubation period, cell number was determined with a Coulter counter, while GCDFP-24 and GCDFP-15 released during the last 48 h was determined by radioimmunoassays using reagents kindly provided by Dr. Darrow E. Haagensen. Data are expressed as means ± SEM of quadriplicate dishes.

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