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Report

Androstenedione and androst-5-ene-3β,17β-diol stimulate DMBA-induced rat mammary tumors – role of aromatase

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Summary

The effect of the adrenal steroids androst-5-ene-3β,17β-diol (Δ^5-diol) and androstenedione (Δ^4-dione) was studied on the growth of mammary carcinoma induced in the rat by dimethylbenz[a]anthracene (DMBA). The plasma levels of the two steroids were maintained at values within the range of those found in the circulation of post-menopausal women by constant release from osmotic pumps in ovariectomized animals. Δ^5-diol and Δ^4-dione, at the daily release rate of 500 μg, led to plasma levels of 1.26 ± 0.19 and 1.72 ± 0.75 ng/ml, respectively. At these physiologically relevant plasma concentrations, both Δ^5-diol and Δ^4-dione caused a marked stimulation of tumor growth while having minimal or no effect on uterine weight or on plasma prolactin and LH levels.

Concomitant treatment with the aromatase inhibitor aminoglutethimide completely blocked the stimulatory effect of Δ^4-dione released from silastic implants on tumor growth, while simultaneous administration of the antiandrogen flutamide had no significant effect. On the other hand, when aminoglutethimide was administered with Δ^5-diol, the stimulatory effect of the adrenal steroid on tumor growth was not affected. Such data indicate that, under the present experimental conditions, transformation of Δ^4-dione into androgens plays a minor role, the predominant effect of the adrenal steroid being stimulation of tumor growth through conversion into estrogens, while Δ^5-diol exerts a direct estrogenic effect independent from aromatase activity. The minimal or absent effect of the same treatment on uterine weight and on plasma prolactin and LH levels indicates the tissue specificity of the effects observed, the mammary tissue being most sensitive to the action of adrenal steroids.

Introduction

The best known characteristic of human breast cancer is its responsiveness to estrogens in a large proportion of cases [1, 2]. Mammary carcinoma induced in the rat by dimethylbenz[a]anthracene (DMBA) is the most widely used *in vivo* model of breast cancer [3–5]. The development and growth of these tumors are particularly sensitive to the stimulatory action of estrogens and prolactin [3–7]. In addition to the well documented role of ovarian estrogens on the growth of DMBA-induced tumors, we have recently found a potent growth stimulatory effect of the adrenal steroids dehydro-

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epiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (Δ^5-diol) [8].

Since aromatase activity has been demonstrated in DMBA-induced mammary tumors [9], and since the adrenal steroids androstenedione (Δ^4-dione) and Δ^5-diol are present at relatively high concentrations in the plasma of post-menopausal women [10, 11], we have studied the effect of constant plasma levels of the two steroids (within the range of plasma concentrations found in post-menopausal women) on the growth of DMBA-induced mammary tumors in ovariectomized rats, and have investigated the effect of simultaneous treatment with the aromatase inhibitor aminoglutethimide on tumor growth induced by the two adrenal steroids.

Materials and methods

Animals

Mammary tumors were induced in female Sprague-Dawley (Crl : CD(SD)Br) rats (obtained from Charles River Canada Inc., St-Constant, Quebec) at 50 to 55 days of age by a single intragastric administration of 20 mg of DMBA (Sigma Chemical Co., St. Louis Mo) in 1 ml of corn oil. Animals were housed two per cage under a regimen of 14 h of light and 10 h of darkness (lights on at 05:00 h). Purina rat chow and water were given ad libitum.

Treatments

Three months after DMBA administration, animals bearing tumors having a diameter of 1 cm or more were selected and tumor number and size were recorded. The animals were then divided into homogeneous groups.

For the first experiment, the six groups were: intact, ovariectomized (OVX), OVX + Δ^4-dione 200 μg/day, OVX + Δ^4-dione 500 μg/day, OVX + Δ^5-diol 200 μg/day, and OVX + Δ^5-diol 500 μg/day. Steroids dissolved in DMSO were administered by Alzet osmotic pumps at the rate of 4.6 μl/h at a steroid concentration of 1.8 μg/μl to release 200 μg/day, and at a concentration of 4.5 μg/μl to release 500 μg/day. Control animals received pumps containing DMSO alone. All animals, except those of the intact group, were bilaterally ovariectomized under ether anesthesia. At the time of surgery, Alzet pumps were placed subcutaneously in the abdominal area.

In the second experiment, the five groups were: OVX, OVX + Δ^4-dione implant, OVX + Δ^4-dione implant + aminoglutethimide (AG), OVX + Δ^4-dione implant + flutamide (FLU), OVX + Δ^4-dione implant + AG + FLU. Δ^4-dione was inserted in Silastic implants (Medical Grade Tubing, Dow Corning) at the time of surgery. Silastic implants had the following size: 1 cm (length), 0.155 cm (inner diameter), and 0.313 cm (outer diameter). Rats of the appropriate groups were treated twice daily for 18 days with 5 mg AG or 10 mg FLU. Both compounds were injected subcutaneously (s.c.) in 0.5 ml of 1% gelatin – 0.9% NaCl, while OVX only animals received the vehicle alone.

In the third experiment, ovariectomized animals received either Δ^5-diol by osmotic pumps (500 μg daily) or AG alone or the combination of both compounds.

Tumor measurements were performed the day of ovariectomy and then at 2- to 3-day intervals for 12 or 18 days according to the experiments. The two largest perpendicular diameters of each tumor were measured with calipers and the product of these diameters was used to estimate tumor size as described [4, 7]. Tumor size measured on the day of OVX was taken as 100%. Results are thus expressed as percent of growth in relation with the size on the day of OVX. Twenty to 30 tumors were present in each group. The animals were killed by decapitation. Blood samples were collected and the plasma was frozen at −20° C until assayed. Tumors and uteri were immediately removed, freed from connective and adipose tissue, weighed, frozen in liquid nitrogen, and stored at −80° C until assayed.

Radioimmunoassays

Plasma PRL and LH were measured by double-

antibody radioimmunoassays using rat LH-I-6 and rat PRL-I-5 for iodination, rat LH-RP-2 and rat PRL-RP-3 as standards, and the rabbit antisera anti-LH-S-8 and anti-PRL-S-8, generously supplied by the National Pituitary Program, Baltimore, USA.

Steroid assays

Steroid concentrations were measured by radioimmunoassays following methanol and diethyl ether extraction for tissue samples and diethyl ether extraction for plasma samples and chromatography on LH-20 columns as described in detail elsewhere [13]. All samples were chromatographed and radioimmunoassayed (RIA) simultaneously.

Calculations

Radioimmunoassay data were analyzed using a program based on Model II of Rodbard and Lewald [14]. Statistical significance was calculated according to the multiple range test of Duncan-Kramer [15].

Results

Figure 1 illustrates the changes in total tumor area under the influence of the various treatments. While there is a constant increase in total tumor area in the intact animals from 100 to 165% ($p<0.01$) during the 12 days of observation, there is a marked reduction to 20% of the original tumor size in the ovariectomized (OVX) animals ($p<0.01$). More importantly, it can be seen in the same figure that constant daily release of 500 μg of Δ^5-diol or Δ^4-dione by Alzet osmotic pumps maintains total tumor area to 115% and 85% of the original values, respectively ($p<0.01$ vs OVX in both groups). The 200 μg daily dose of the two adrenal steroids exerts intermediate stimulatory effects on tumor growth.

When the effect of the same treatment is studied on uterine weight in OVX animals, it can be seen in

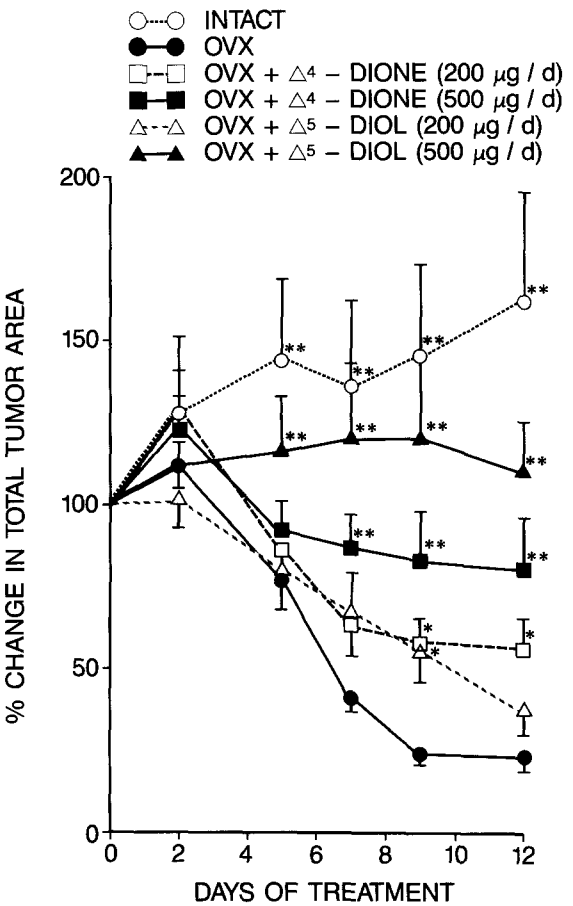

Fig. 1. Effect of ovariectomy only (OVX) or of 12-day treatment of OVX animals with the C_{19} adrenal steroids Δ^4-dione or Δ^5-diol continuously delivered by Alzet pumps at daily doses of 200 or 500 μg on the percentage of change in total area of DMBA-induced mammary tumors in the rat (*, $p<0.05$; **, $p<0.01$; exp. versus OVX). Results are expressed as means ± SEM.

Fig. 2 that at the doses used, Δ^4-dione has no effect on this parameter while the highest dose of Δ^5-diol (500 μg/day) causes a small but significant 25% ($p<0.01$) stimulation of uterine weight. On the other hand, at the doses of the two steroids, no effect is observed on plasma LH or prolactin (PRL) levels (Fig. 3).

It is of interest to see, in Fig. 4, the plasma and tissue levels of Δ^5-diol and Δ^4-dione achieved in the same experiment. The constant daily release of 500 μg of Δ^5-diol led to plasma and tissue levels of the steroid of 1.26 ± 0.19 ng/ml plasma and 2.05 ± 0.35 ng/g tissue, respectively. For Δ^4-dione, constant release of 500 μg per day led to plasma levels

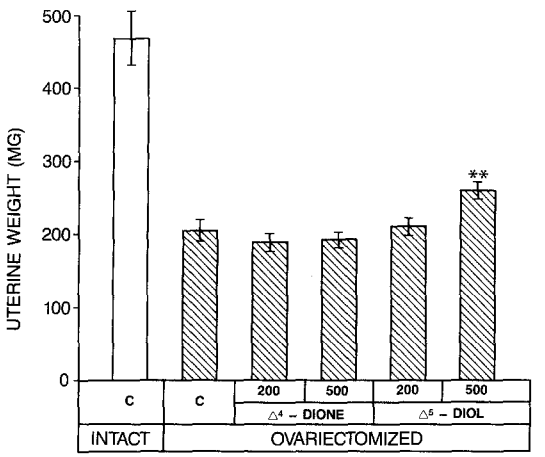

Fig. 2. Effect of ovariectomy only (OVX) or of 12-day treatment of OVX animals with the adrenal steroids Δ^4-dione or Δ^5-diol continuously delivered by Alzet pumps at daily doses of 200 or 500 μg on uterine weight in rats bearing DMBA-induced mammary tumors. (**, $p<0.01$, exp. versus OVX). Results are expressed as means ± SEM.

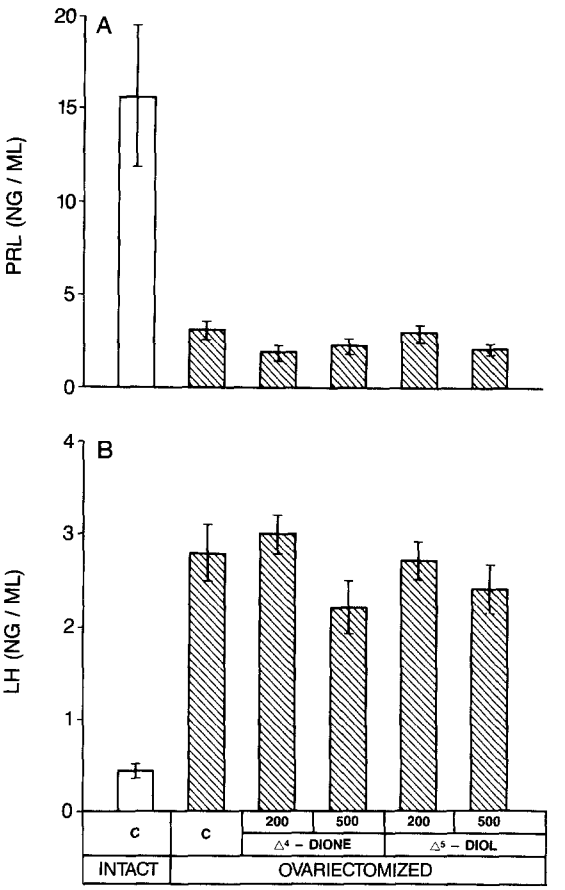

Fig. 3. Effect of ovariectomy only (OVX) or of 12-day treatment of OVX animals with the adrenal steroids Δ^4-dione or Δ^5-diol continuously delivered by Alzet pumps at daily doses of 200 or 500 μg on plasma PRL (A) or LH (B) levels in rats bearing DMBA-induced mammary tumors. Results are expressed as means ± SEM.

of 1.72 ± 0.75 ng/ml while concentrations of 4.70 ± 1.25 ng/g were measured in the tumor tissue.

Since Δ^4-dione can be converted into both androgens and estrogens in both normal rat mammary tissue and DMBA-induced carcinoma [9], we next studied the effect of the aromatase inhibitor aminoglutethimide (AG) and of the antiandrogen flutamide (FLU) on the same parameters in OVX animals supplemented with Δ^4-dione constantly released from silastic implants. As illustrated in Fig. 5, total tumor area decreased to 38 ± 5% of the pretreatment value 18 days after ovariectomy (OVX). In OVX animals bearing silastic implants giving plasma Δ^4-dione levels of 1.26 ± 0.15 ng/ml, total tumor area was maintained at 92 ± 11% of the control pretreatment value ($p<0.01$ vs OVX). The marked stimulatory effect of Δ^4-dione was unaffected by simultaneous treatment with FLU (84 ± 12 vs 92 ± 11%, N.S.). Treatment with AG, on the other hand, decreased total tumor area measured at the end of the observation period from 92 ± 11% to 27 ± 7.0% ($p<0.01$). Addition of flutamide to animals treated with AG increased total tumor area from 27 ± 7.0% to 44 ± 8.0%, a difference which is not statistically significant. As observed in the previous experiment, neither uterine weight nor plasma LH and PRL concentrations were affected by any of the treatments administered to OVX animals (data not shown).

It was then of interest to study the possible role of aromatase on the stimulatory action of Δ^5-diol on DMBA-induced tumor growth. As shown in Fig. 6, AG had no effect on tumor growth when administered alone or in association with Δ^5-diol in OVX animals.

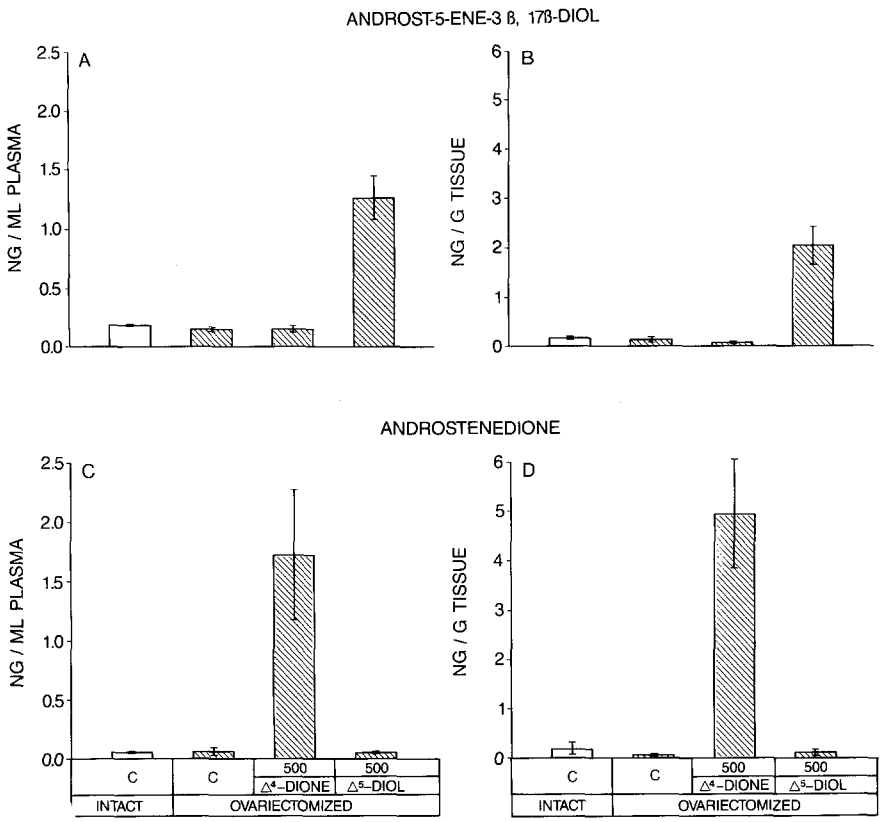

Fig. 4. Effect of ovariectomy only (OVX) or of 12-day treatment with the adrenal steroids Δ^4-dione or Δ^5-diol continuously delivered by Alzet pumps at the daily dose of 500 μg on plasma Δ^5-diol (A) or Δ^4-dione (C) levels as well as on tissue Δ^5-diol (B) or Δ^4-dione (D) concentration. Results are expressed as means ± SEM.

Discussion

The present data demonstrate that maintenance of plasma levels of Δ^5-diol or Δ^4-dione at concentrations comparable to those found in postmenopausal women can independently induce a potent stimulation of DMBA-induced mammary cancer growth. Moreover, the absence of effect of the antiandrogen flutamide on the stimulatory action of Δ^4-dione, together with the complete reversal of this action by simultaneous treatment with the aromatase inhibitor aminoglutethimide, indicates that conversion into estrogens is the predominant effect of Δ^4-dione in this system. The action of Δ^5-diol, on the other hand, is clearly independent of aromatase activity.

Our previous studies have shown that twice daily subcutaneous injection of 2 mg Δ^5-diol or DHEA prevented the loss in tumor number and area induced by OVX [8]. Although those data were the first demonstration of the effect of a C_{19}-Δ^5 steroid on the growth of an estrogen-sensitive cancer *in vivo*, the high levels of circulating Δ^5-diol or DHEA presumably occuring shortly after each injection of the steroids complicated the assessment of their physiological importance. In fact, under normal conditions in women suffering from breast cancer, approximately constant levels of steroids are maintained in the circulation during each 24-h period and no such elevations of steroid levels similar to those found after subcutaneous steroid injection occur. The present data obtained following constant release of Δ^5-diol with Alzet osmotic pumps clearly demonstrate that plasma levels of Δ^5-diol similar to those found in adult women (0.3 to 1.0 ng/ml) [10, 11] are a potent stimulus for tu-

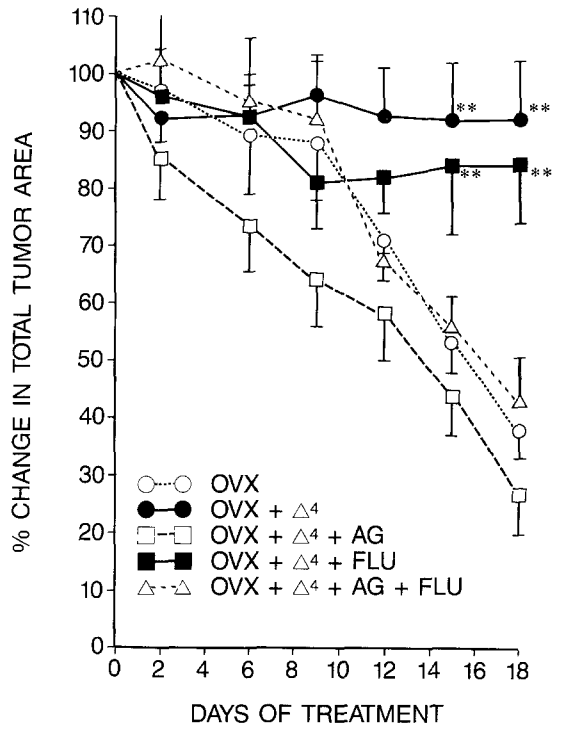

Fig. 5. Effect of ovariectomy only (OVX) or 18-day treatment with Δ^4-dione (silastic implants) alone or with aminoglutethimide (AG, 5 mg twice daily) or flutamide (Flu, 10 mg twice daily) on the percentage of change in total area of DMBA-induced mammary tumors in rats. (**, $p<0.01$, exp. versus OVX). Results are expressed as means ± SEM.

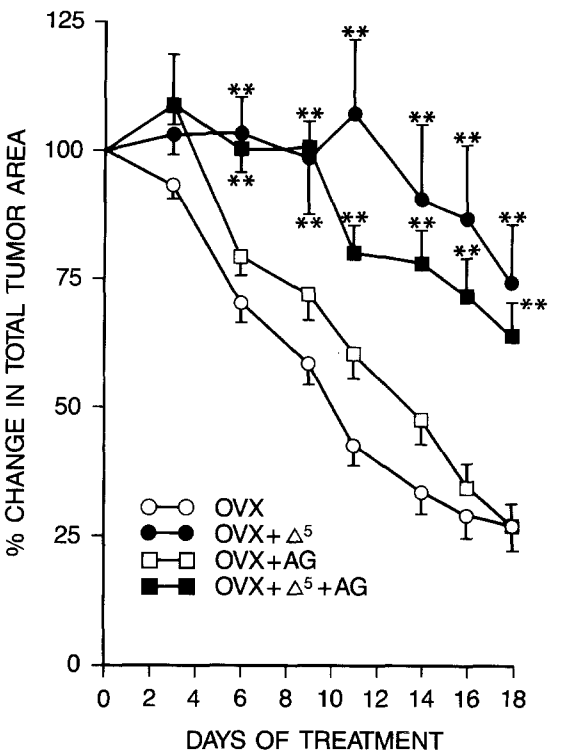

Fig. 6. Effect of ovariectomy only (OVX), or 18-day treatment with Δ^5-diol (Alzet osmotic pumps) at the daily dose of 500 μg, or Δ^5-diol with simultaneous aminoglutethimide (AG, 5 mg twice daily) on the percentage of change in total area of DMBA-induced mammary tumors in the rat. (**, $p<0.01$, exp. versus OVX). Results are expressed as means ± SEM.

mor growth in the DMBA model, thus strongly supporting our previous suggestions [8]. Moreover, the plasma Δ^4-dione levels of 1.72 ± 0.75 and 1.26 ± 0.5 ng/ml after osmotic pump and implant administration, respectively, are similar to the plasma Δ^4-dione of 1.0 to 1.7 ng/ml measured in adult women [12].

The role of the 'classical' estrogens (estradiol, estrone, and estriol) in promoting the growth of estrogen-sensitive breast cancer is well recognized [16–18]. In recent years, however, several observations suggest that C_{19}-Δ^5 steroids of adrenal origin, especially Δ^5-diol, could induce estrogenic effects in target tissues, (see [19, 20] for review). For instance, plasma levels of Δ^5-diol typical of those found in the blood of Western women cause a uterotropic response in the sexually immature rat uterus [21]. Moreover, in the MCF-7 human breast cancer cell line, physiological concentrations of Δ^5-diol and DHEA-S stimulate the secretion of a 52,000 kDa glycoprotein which is under specific estrogenic control in these cells [22]. Moreover, high (100 nM) concentrations of Δ^5-diol have been shown to stimulate thymidine kinase activity and to increase cellular levels of progesterone receptors in MCF-7 cells [23].

More importantly, we have recently found that incubation of the human breast carcinoma cell line ZR-75-1 with Δ^5-diol at concentrations similar to those found in the serum of adult women induces a marked stimulation of the growth of these cells [24]. This was the first demonstration of the stimulatory effect of an adrenal steroid on estrogen-sensitive cancer growth. In this cell line, our metabolic studies indicate that DHEA is converted into Δ^5-diol with no or minimal further metabolism and no detectable formation of 17β-estradiol, thus sug-

gesting that Δ^5-diol exerts its stimulatory action on cancer cell growth by a direct interaction with the estrogen receptor [24]. Such studies remain to be done in the DMBA-induced mammary tumor in order to assess the possible transformation of Δ^5-diol into 17β-estradiol. However, published evidence, in addition to our own studies with the ZR-75-1 cell line, shows little conversion of DHEA into 17β-estradiol in MCF-7 breast carcinoma cells [21, 22].

Although the main estrogenic activity in postmenopausal women has been attributed to estrone [25] and estrone sulfate [26], and Δ^4-dione is considered the main precursor of these estrogens, no information was previously available on the action of this steroid in estrogen-sensitive breast cancer models. The present data show that Δ^4-dione, at concentrations within the range of those found in adult women, is also a potent stimulus of DMBA-induced tumor growth.

Since androgens have been reported to inhibit the growth of DMBA-induced mammary tumors in intact animals [27], as well as that of mammary fibroadenoma [28], and some C_{19} 17β-hydroxysteroid dehydrogenase activity has been observed in these tumors [9, 29], it is of interest to see that the predominant effect of Δ^4-dione is stimulatory, thus indicating the high and predominant level of aromatase activity in these tumors [9]. In fact, addition of the pure antiandrogen flutamide did not modify the stimulatory effect of Δ^4-dione administered alone, thus indicating a minimal androgenic effect under the present experimental conditions. On the other hand, when the formation of estrogens from Δ^4-dione was blocked by AG, inhibition of the androgen receptor by flutamide in animals treated with Δ^4-dione and AG caused a 80% increase in total tumor area which, however, did not reach the level of significance. Since flutamide is a pure nonsteroidal antiandrogen having no other known activity than blocking the androgen receptor [30–33], the present results indicate that estrogen formation following maintenance of plasma Δ^4-dione at human physiological levels is the main factor controlling DMBA-induced mammary carcinoma growth.

When AG was administered to intact rats during the induction and development phases of DMBA-induced mammary carcinogenesis, there was a decrease in the number of tumor-bearing animals as well as in the number of tumors per rat [34], thus suggesting an action of AG at the ovarian level. The present data obtained in OVX animals indicate for the first time that AG can also inhibit aromatase activity in peripheral tissues.

DMBA-induced mammary tumor tissue has been shown to metabolize DHEA into dihydrotestosterone (DHT), androstane-3α,17β-diol (3α-diol), and some E_2, while testosterone (T) was converted into DHT and a small amount of E_2 [9]. Similar conversions are found in human breast cancer tissue [9]. Normal rat mammary gland and DMBA-induced mammary tumors have been shown in enzymatic assays to contain 17β-hydroxysteroid dehydrogenase, 3-ketosteroid reductase, and Δ^4-5α-hydrogenase activities [29, 35]. The enzymatic systems which process steroids in human breast cancer tissue are also present in DMBA-induced mammary tumors, thus supporting the use of DMBA-induced tumors as models of human breast cancer [4–9].

The implications of the present findings for breast cancer are important. As mentioned above, the average plasma concentration of Δ^5-diol in women (1 to 3 nM) [10, 11] is sufficient to cause a sustained estrogenic stimulus in responsive breast tumor cells, the serum level of Δ^5-diol being maintained by continuous peripheral conversion from DHEAS and DHEA [19, 36, 37]. Moreover, the cytosolic concentrations of Δ^5-diol and DHEA in breast tissue are 2- to 5-fold higher than in plasma [38–40], and breast cancer tissue is known to convert DHEA into Δ^5-diol [24, 41, 42].

This first demonstration that physiological plasma concentrations of C_{19}-Δ^5 steroids can stimulate breast cancer cell growth in an animal model indicates that estrogens of adrenal origin, in the form of Δ^5-diol, Δ^4-dione, or their precursors, should be taken into consideration for the efficient control of estrogen-sensitive breast cancer. Physiological concentrations of Δ^5-diol or Δ^4-dione, considered separately, can exert a potent stimulation of tumor growth. Consideration should also be given to the fact that Δ^5-diol does not need to be converted into E_2 to exert its estrogenic action [24], while aroma-

tase activity appears to play the predominant role in the stimulatory action of Δ^4-dione.

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