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A POTENTIAL ROLE FOR CATECHOLAMINES IN THE DEVELOPMENT AND PROGRESSION OF CARCINOGEN-INDUCED MAMMARY TUMORS: HORMONAL CONTROL OF β-ADRENERGIC RECEPTORS AND CORRELATION WITH TUMOR GROWTH

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Summary—In order to gain further knowledge on the β-adrenergic receptor system in DMBA-induced rat mammary tumors, we have studied the correlation between changes in tumoral β-adrenergic receptor concentration and distribution, progesterone receptor status and tumor growth after ovariectomy and treatment with various ovarian and adrenal steroids, or induction of hyperprolactinemia. Autoradiographic localization of β-adrenergic receptors in ovariectomized (OVX) animals shows very weak labeling with [^{125}I]cyanopindolol. In these tumors, the connective tissue is predominant, while the epithelial cell content is very low. Similarly, when direct measurements of [^{125}I]cyanopindolol are performed with membrane preparations, β-adrenergic receptor concentration is sharply reduced 2–3 weeks following ovariectomy or treatment with LHRH against [D-Trp^6, des-Gly-NH$_2^{10}$]LHRH ethylamide. This effect on the β-adrenergic receptor population in the tumor is accompanied by the well known effect of castration on tumor growth and progesterone receptor levels, namely a marked regression of tumor growth and a significant decrease in progesterone receptor concentration. Treatment of OVX rats with 17β-estradiol (E_2) alone or in combination with progesterone (P) caused a highly significant increase in β-adrenergic and progesterone receptor levels, as well as tumor growth. A similar sharp increase in the value of the three parameters studied was observed following daily treatment of OVX rats with dehydroepiandrosterone (DHEA) or androst-5-ene-3β,17β-diol (5-ene-diol). The autoradiographic localization of β-adrenergic receptors in OVX rats treated with 5-ene-diol showed that the epithelial cells were numerous with a high degree of labeling. On the other hand, treatment of OVX animals with the androgen dihydrotestosterone (DHT) did not produce significant changes in β-adrenergic receptor levels or tumor growth. Finally, endogenously-induced hyperprolactinemia by implanting three anterior pituitary glands under the kidney capsule of OVX animals resulted in a significant increase in β-adrenergic and progesterone receptor levels as well as tumor growth. The positive correlation observed between changes in β-adrenergic receptor concentration, progesterone receptor levels and tumor growth indicates a high sensitivity of the β-adrenergic receptor population of DMBA-induced rat mammary tumors to the hormonal milieu, and suggests that the β-adrenergic receptor system may represent a valuable parameter of hormone responsiveness.

INTRODUCTION

The involvement of catecholamines in the growth and hypertrophy of a variety of tissues, is well established [1]. Catecholamines, particularly epinephrine, can stimulate the growth and/or proliferation of the myocardium, salivary glands and selective other tissues or cell lines, including the ovary, prostate, hepatocytes, lymphocytes and others [1]. The β-adrenergic agonist, isoproterenol, stimulates mammary epithelial cell division *in vitro* [2], as well as the development of end bud structures in the mammary gland of ovariectomized mice [3] from which arise the mammary carcinoma induced by administration of dimethylbenz(*a*)anthracene (DMBA) [4]. Specific β-adrenergic receptors of the β_2 subtype are present in epithelial cell membranes from lactating mammary gland tissue [5, 6], with an important modulation of receptor levels exerted by the steroid milieu [7].

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In DMBA-induced rat mammary tumors, β-adrenergic receptors are localized in all malignant cells and adipocytes [8]. Furthermore, spontaneous mammary tumors of aging rats contain high levels of β-adrenergic recpetors. Since a positive correlation was found between β-adrenergic receptor concentration, tumor growth and progesterone receptor levels of progressing and regressing [8] tumors, and in order to gain further knowledge of the control of the β-adrenergic receptor system in DMBA-induced rat mammary tumor growth, we have investigated the effect of surgical or chemical (LHRH-agonist) castration and treatment with various ovarian and adrenal steroids, or induction of hyperprolactinemia, on tumoral β-adrenergic receptor concentration and distribution. In addition, changes in β-adrenergic receptor levels have been correlated with tumor growth and progesterone receptor concentration in the tumors [8, 9].

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 50–55 days of age by the intragastric administration of 20 mg of DMBA (Sigma Chemicals Co., St Louis, Mo.) in 1 ml of corn oil. Animals were housed 2 per cage under a regimen of 14 h of light–10 h of darkness (lights on between 0500 h and 1900 h). Purina rat chow and tapwater were available *ad libitum*. After 3–4 months of DMBA administration, animals were palpable tumors were selected for the experiment.

Treatment of rats bearing DMBA-induced mammary tumors

After development of palpable tumors, the animals were divided into different groups. In the first series of experiments, the effect of castration and treatment with various ovarian hormones was investigated. In the second series of experiments, the effect of chemical castration or surgical castration and treatment with adrenal hormones was studied.

At the start of treatment, each group of animals (10–14/group) had an average tumor area of 3.9–4.3 cm^2 (first series) and 2.3–3.4 cm^2 (second series). The animals of the appropriate groups were bilaterally ovariectomized (OVX) under light ether anesthesia and entered in the following groups of treatment: intact + LHRH-A [D-Trp^6, des-Gly-NH_2^{10}]LHRH ethylamide, 1 μg); OVX; OVX + 17β-estradiol (E_2, 1.125 μg); OVX + progesterone (P, 2 mg); OVX + E_2 + P (1.125 μg + 2 mg) OVX + dihydrotestosterone (DHT, 250 μg). OVX + dehydroepiandrosterone (DHEA, 2 mg); OVX + androst-5-ene-3β,17β-diol (5-ene-diol, 2 mg). Endogenous hyperprolactinemia was induced in DMBA-treated animals by surgically placing three anterior pituitaries (obtained from adult female rats) under the kidney capsule, under light ether anesthesia [10]. In this experimental group, bilateral castration was simultaneously performed. Animals (10–14/group) were treated twice a day for 3–4 weeks with the subcutaneous doses of the compounds indicated above, in 0.2 ml (ovarian hormones and LHRH-A) or 0.5 ml (adrenal hormones) of 1% gelatin–0.9% NaCl. Steroids were first dissolved in a small volume of ethanol before further dilution with 1% gelatin–0.9% NaCl.

Tumor measurements were performed before the start of treatment and every 5–7 days for 21–28 days. Animals were examined for mammary tumors by palpation and the number of tumors per rat was recorded. 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 [8, 9]. At the end of the treatment period, the animals were killed by decapitation. Tumors were immediately removed, freed from connective and adipose tissue, frozen on dry ice and stored at $-80°C$ until receptor assays in the same experimental protocol.

Membrane preparation

Tissues were homogenized in 10 vol (w/v) of 0.25 M sucrose, 25 mM Tris–HCl (pH 7.5) using a Polytron PT-10 homogenizer (Brinkman Instruments, Canada) at a setting of 5 for 3 periods of 10 s with an interval of 10 s for cooling. The homogenate was then centrifuged at 600 ***g*** for 10 min. The supernatant was carefully collected and centrifuged at 105,000 ***g*** for 60 min in a Beckman L5-65 centrifuge using a 50 Ti rotor. Pellets were resuspended in assay buffer (25 mM Tris–HCl, pH 7.5, 1:20, w/v) and β-adrenergic receptors were assayed in the particulate preparation.

Cytosol preparation

Tissues were homogenized in 5 vol (w/v) of buffer A (25 mM Tris–HCl, 1.5 mM EDTA,

disodium salt, 10 mM α-monothioglycerol, 10% glycerol and 1.5 mM sodium molybdate, pH 7.4) using a Polytron PT-10 homogenizer as described above. The homogenate was then centrifuged at 105,000 *g* for 60 min and the progesterone receptor assays were performed with freshly prepared cytosol. Protein concentration was measured according to Lowry *et al.* [11] using bovine serum albumin as standard.

Progesterone receptor assay

[6,7-^{3}H]17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R5020) (87 Ci/mmol) and the corresponding unlabeled steroid were from New England Nuclear. After evaporation of the solvent under a stream of nitrogen, the labeled steroid was dissolved in buffer A. [^{3}H]R5020 binding was measured using the dextran-coated charcoal adsorption technique; 0.1 ml aliquots of cytosol preparations were incubated with 0.1 ml 16 nM [^{3}H]R5020 (200,000 cpm) and 100 nM triamcinolone acetonide in the presence or absence of a 100-fold excess of the unlabelled steroid for 18–22 h at 0–4°C as described [12]. The unbound steroid was then removed by incubation for 15 min at 0–4°C with 0.3 ml 0.5% Norit A, 0.05% Dextran T-70 (DCC) in buffer B (1.5 mM EDTA disodium salt, 10 mM monothioglycerol and 10 mM Tris–HCl, pH 7.4) and centrifugation at 3000 *g* for 15 min. Aliquots of the supernatant (0.3 ml) were then collected for radioactivity measurement. After addition of 4 ml of Formula-963 scintillation liquid (New England Nuclear), the radioactivity was measured in a Beckman counter at a counting efficiency of 32%.

Receptor [^{125}I]cyanopindolol (CYP) assay

[^{125}I]CYP was purchased from New England Nuclear at a specific activity of 2000 Ci/mmol, and receptor assay performed according to Poyet *et al.* [13]. Briefly, in the standard assay, [^{125}]CYP binding was measured by triplicate incubation for 180 min at room temperature of 100 μl of membrane preparation, 250 μl buffer (25 mM Tris–HCl, pH 7.5), 100 μl of a saturating (0.115 nM) concentration of [^{125}I]CYP in the presence or absence of 50 μl of 0.1 μM (−)propranolol. The reaction was stopped by the addition of 0.5 ml of bovine γ-globulin (0.1% (v/v) in Tris–buffer) and 1.0 ml of a solution of 24% (w/v) polyethylene glycol (PEG-6000). The assay tube was then mixed vigorously during 10 s before standing for 5 min and centrifuged for 20 min at 3000 *g*. The supernatant was discarded and the radioactivity in the pellet was counted.

Autoradiographic localization

Frozen tumor sections obtained from intact, OVX and OVX + 5-ene-diol-treated animals, were cut at 15 μm thickness and mounted onto gelatin-coated microscope slides by thawing. The sections were then dehydrated under vacuum at 4°C for 18 h and, if not immediately used, were stored at −70°C. For receptor localization and densitometry all the slides were processed in parallel under the same standard conditions (time of exposure, incubation and development). The slides were brought to room temperature and preincubated for 30 min in 0.1 M mg Tris–HCl buffer (pH 7.6). The slides were then incubated for 2 h with the same buffer containing 100,000 cpm/ml of [^{125}I]CYP in the presence or absence of unlabeled 10^{-6} M (−)propranolol according to Dubé *et al.* [14]. After rinsing, the sections were fixed in 2.5% glutaraldehyde for 10 min, dried at room temperature and placed against an Ultrafilm (LKB) or coated in liquid photographic emulsion (Kodak NTB2). The density of autoradiographic reaction was measured on 4 sections (20 measures/section) per tumor and in 3 animals/group. Arbitrary optical density units were measured by a Loats Image Analysis System (Amersham).

Materials

The LHRH agonist, [D-Trp^6,des-Gly-NH$_2^{10}$]-LHRH ethylamide was a product of Bachem, Los Angeles, Calif. The different steroids used for treatment were from Steraloids, while nuclear tract emulsion (type NTB-2) was purchased from Eastman Kodak (Rochester, N.Y.).

Calculation of affinity and number of [^{125}I]CYP binding sites

Binding data were analysed with a Hewlett–Packard calculator model 9845 using a program based on model II of Rodbard and Lewald [15, 16]. The apparent dissociation constant of [^{125}I]CYP was determined by Scatchard analysis [17]. Statistical significance was assessed according to the multiple-range test of Duncan–Kramer [18].

Table 1. Effect of ovariectomy and treatment with different hormones on β-adrenergic receptor concentration (fmol/mg protein) and average tumor area (cm^2) in rats originally treated with DMBA to induce tumor development

Treatments	Animal No.	β-Adrenergic receptor concentration (fmol/mg protein)	Average total tumor area (cm^2) 0	28 (days)
Intact progression	10	178 ± 3.4**	4.35 ± 0.98	9.69 ± 2.29**
Intact + LHRH-A regression	12	17.2 ± 2.2	4.12 ± 0.56	0.56 ± 0.24
OVX regression	14	20.5 ± 0.5	4.28 ± 0.82	0.87 ± 0.41
OVX + 5-ene-diol	11	180 ± 8**	3.62 ± 0.82	4.98 ± 0.90**
OVX + DHEA	10	170 ± 6**	3.63 ± 0.70	5.00 ± 0.90**
OVX + PRL	11	98 ± 12**	4.18 ± 0.68	4.98 ± 0.87**

**$P < 0.01$ compared to OVX animals.

Animals (10–14/group) were treated as described in Materials and Methods. Tumor measurements were performed before the start of treatment (day 0) and every 5–7 days for 28 days. Results represent the mean ± SEM of average tumor area (cm^2) on days 0 to 28 of treatment. β-Adrenergic receptors were measured in individual tumors using a saturating concentration of [^{125}I]CYP (0.115 nM) and results are the mean ± SE of 10–14 values/group.

Effect of surgical or chemical castration, treatment with adrenal hormones or hyperprolactinemia on β-adrenergic receptor levels in DMBA-induced rat mammary tumors

Tables 1 and 2 show the effect of surgical or chemical (LHRH agonist) castration and treatment with various hormones on β-adrenergic receptor number measured in individual tumors (10–14 animals/group) at a saturating concentration (0.115 nM) of [^{125}I]CYP. It can be seen that surgical or chemical castration markedly reduced β-adrenergic receptor concentration (Table 1). From control levels of 178 ± 3.4 fmol/mg protein, β-adrenergic receptors measured in regressing tumors fell to 20.5 ± 0.5 ($P < 0.01$) and 17.2 ± 2.2 ($P < 0.01$) fmol/mg protein in OVX and intact rats treated with the LHRH agonist (LHRH-A), respectively. Daily treatment for 28 days with 5-ene-diol produced a sharp increase in β-adrenergic receptor concentrations (180 ± 8.0 fmol/mg protein) ($P < 0.01$). A comparable increase followed the treatment with DHEA (170 ± 6.0 fmol/mg protein) ($P < 0.01$). The implantation of three anterior pituitary glands under the kidney capsule of OVX animals also resulted in a significant increase in β-adrenergic receptor concentration to 98 ± 12 fmol/mg protein ($P < 0.01$) (Table 1) and plasma PRL levels (not shown).

Effect of surgical castration and treatment with ovarian hormones on β-adrenergic receptor levels in DMBA-induced rat mammary tumors

In a second series of experiments (Table 2), control levels of β-adrenergic receptors in progressing tumors were measured at 169.5 ± 2.5 mg/protein, while castration resulted in a marked reduction in the number of β-adrenergic binding sites (24 ± 0.3 fmol mg/protein) in regressing tumors. Daily administration of E_2 increased β-adrenergic receptor concentration to 209 ± 25 fmol/mg protein ($P < 0.01$). A comparable increase in β-adrenergic receptor number followed treatment of OVX rats with E_2 + P (177 ± 3.0 fmol/mg protein). Treatment of OVX animals with P alone on the other hand, produced a small (35.0 ± 0.5 fmol/mg

Table 2. Effect of ovariectomy and treatment with different hormones on β-adrenergic receptor concentration (fmol/mg protein) and average total tumor area (cm^2) in rats originally treated with DMBA to induce tumor development

Treatments	Animal No.	β-Adrenergic receptor concentration (fmol/mg protein)	Average total tumor area (cm^2) 0	21 (days)
Intact progression	10	169.5 ± 2.5**	2.3 ± 0.1	6.8 ± 0.8**
OVX regression	11	24 ± 0.3	2.7 ± 0.2	1.2 ± 0.4
OVX + E_2	10	209 ± 25**	2.6 ± 0.2	11.4 ± 1.0**
OVX + P	12	35 ± 0.5*	2.9 ± 0.4	3.7 ± 0.7**
OVX + E_2 + P	10	177 ± 3**	3.4 ± 0.4	12.0 ± 1.2**
OVX + DHT	14	25.1 ± 4.7	2.8 ± 0.5	2.1 ± 0.4

*$P < 0.05$ compared to OVX animals; **$P < 0.01$ compared to OVX animals.

Animals (10–14/group) were treated as described in Materials and Methods. Tumor measurements were performed before the start of treatment (day 0) and every 5–7 days for 28 days. Results represent the mean ± SEM of average tumor area (cm^2) on days 0 and 21 of treatment. β-adrenergic receptors were measured in individual tumors using a saturating concentration of [^{125}I]CYP (0.115 nM) and results are the mean ± SE of 10–14 values/group.

protein) although significant ($P < 0.05$) increase in β-adrenergic receptor concentration, while daily administration of DHT did not modify β-adrenergic receptor concentration (25.1 ± 4.7 fmol/mg protein) in the tumors, compared to the concentration measured in OVX rats (24 ± 0.3 fmol/mg protein).

Figure 1 shows the Scatchard analysis of [^{125}I]CYP binding to membranes prepared from intact, OVX, and OVX rats treated with E_2 or 5-ene-diol. It can be seen that similar K_d values are measured in intact (68 ± 0.3 pM)-, OVX+E_2 (69 ± 0.2 pM)- and 5-ene-diol (68 ± 0.2 pM)-treated animals, while in OVX rats, a lower (40 ± 0.3 pM) K_d value was observed. On the other hand, an approx. 5-fold increase in receptor concentration followed E_2 or 5-ene-diol treatment of OVX animals (Fig. 1).

Effect of castration and hormone replacement on growth of DMBA-induced rat mammary tumors

As observed in Table 1, intact animals treated for 3 weeks with the LHRH agonist showed a marked regression of average total tumor area from 4.12 ± 0.56 cm^2 to 0.56 ± 0.24 cm^2 ($P < 0.01$), a value indistinguishable from that measured after ovariectomy (0.87 ± 0.41 cm^2). Treatment of OVX rats with DHEA or 5-ene-diol also markedly stimulated tumor growth from 0.87 ± 0.41 cm^2, after OVX to 4.98 ± 0.9 and 5.0 ± 0.9, in DHEA- and 5-ene-diol-treated animals respectively ($P < 0.01$) (Table 1).

Induction of hyperprolactinemia by implantation of three anterior pituitaries under the kidney capsule of OVX animals also resulted in a significant increase in the average total tumor area (Table 1), compared to the one measured in OVX rats (from 0.87 ± 0.41 to 4.98 ± 0.87 cm^2, $P < 0.01$).

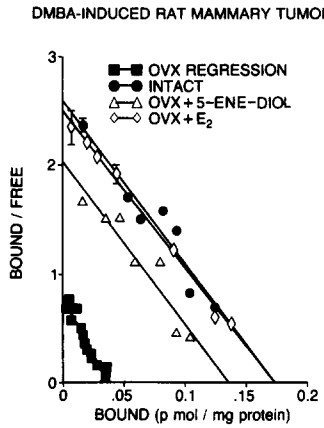

Fig. 1. Scatchard analysis of [^{125}I]CYP binding to DMBA-induced mammary tumors following castration and treatment with 5-ene-diol or E_2. Membranes were incubated for 180 min at 21–22°C with [^{125}I]CYP in the absence or presence of 0.1 μM (−)propranolol. Results shown are the mean ± SEM of triplicate determinations from a representative experiment. Data where no error is shown have a standard error of the mean smaller than the symbol used.

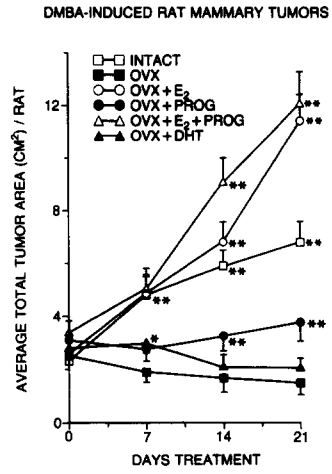

Fig. 2. Effect of ovariectomy (OVX) or 21 days treatment of ovariectomized animals with estradiol (E_2, 1.125 μg), progesterone (Prog, 2 mg), dihydrotestosterone (DHT, 250 μg), twice a day, alone or in combination, on total tumor surface area (cm^2) of DMBA-induced mammary tumors. *$P < 0.05$; **$P < 0.01$ compared to day 0.

Table 2 shows the effect of ovariectomy and treatment with ovarian hormones administered for 21 days to OVX rats on average tumor area (cm^2). In intact animals with progressing tumors, the average total tumor area increased from 2.3 ± 0.1 to 6.8 ± 0.8 cm^2 ($P < 0.01$), while, in OVX rats with regressing tumors, a marked decrease was observed (from 2.7 ± 0.2 to 1.2 ± 0.4 cm^2) ($P < 0.01$) (Table 2). In OVX animals treated with E_2, total tumor area increased from 2.6 ± 0.2 to 11.4 ± 1.0 cm^2 ($P < 0.01$). A similar sharp increase was observed following treatment with the combination E_2 + P (from 3.4 ± 0.4 to 12.0 ± 1.2 cm^2) ($P < 0.01$). Treatment of OVX animals with P alone induced a significant increase (3.7 ± 0.7 cm^2) in tumor growth compared to OVX alone (1.2 ± 0.4 cm^2) ($P < 0.01$), while daily administration of DHT to OVX rats did not interfere with the castration-induced tumor regression (from 2.8 ± 0.5 to 2.1 ± 0.4 cm^2) (not significant) (Fig. 2 and Table 2).

Effect of castration and hormone replacement on progesterone receptor concentration in DMBA-induced rat mammary tumors

Progesterone receptor levels are markedly decreased after OVX from control levels of

242 ± 63 fmol/mg protein measured in intact animals, to 16.3 ± 3.9 fmol/mg protein in OVX rats ($P < 0.01$). Treatment of OVX rats with E_2 markedly stimulated progesterone (P) receptor concentration to 344 ± 61.3 fmol/mg protein ($P < 0.01$). A similar reversal of the effect of castration on tumoral P receptor levels followed treatment with the combination of $E_2 + P$ (351 ± 72.3 fmol/mg protein), DHEA (194 ± 52.1 fmol/mg protein), 5-ene-diol (196 ± 35.8) or prolactin (187 ± 38.0 fmol/mg protein) treatments.

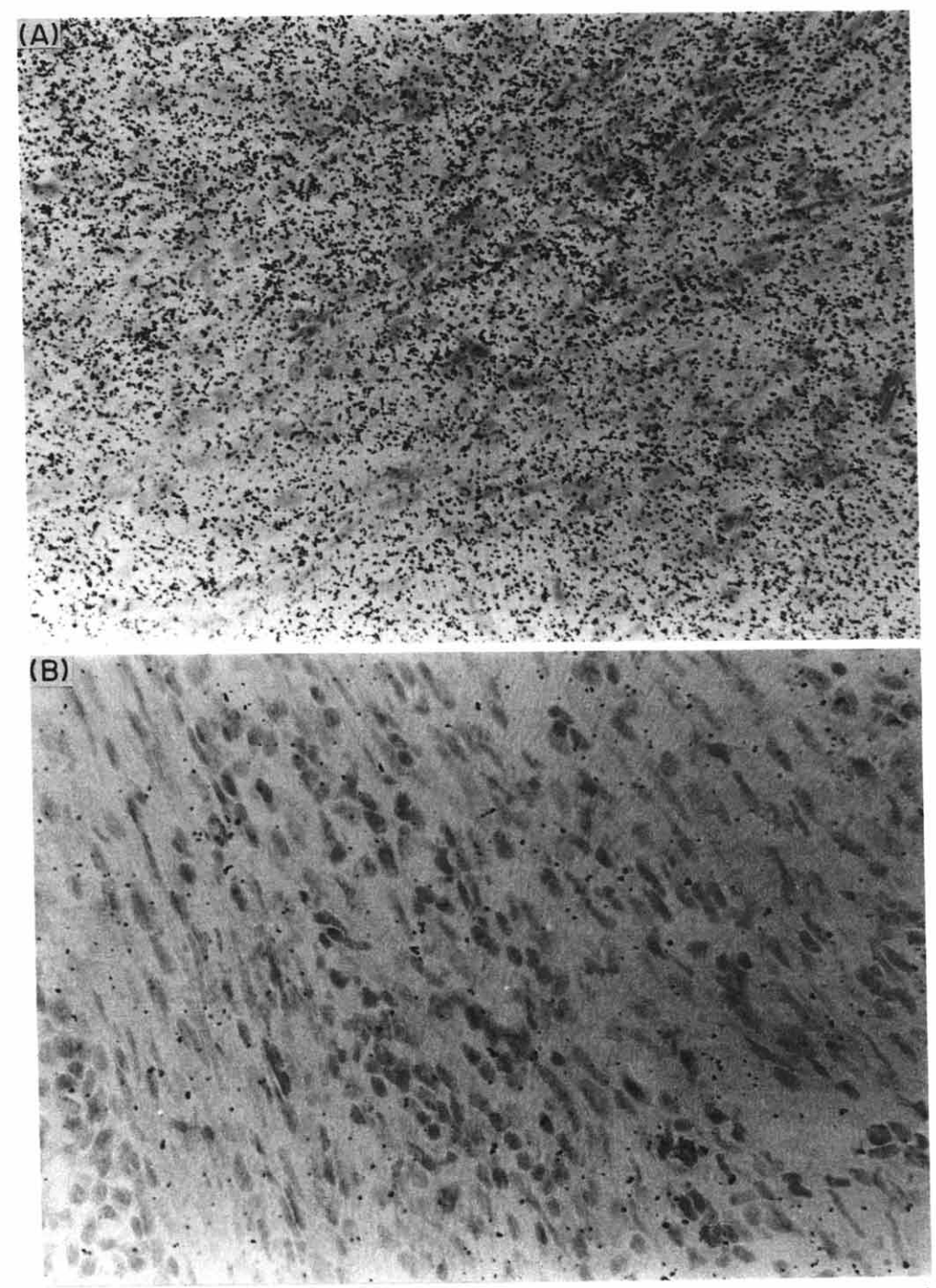

Fig. 3. Light microscopic autoradiographic localization of β-adrenergic receptors in mammary tumors of intact animals. (A) Total binding. The distribution of silver grains is heterogenous and dense over the cells. (B) Non-specific binding obtained by incubating [^{125}I]CYP with 10^{-6} M (−)propranolol. A few dispersed grains can be observed. ×800.

Autoradiography of β-adrenergic receptors following ovariectomy and hormonal treatment

As observed by light microscope autoradiography, the distribution of β-adrenergic receptors in the tumors of intact animals is quite uniform (Fig. 3A). In ovariectomized animals, the tumor contained much less epithelial cells whereas the connective tissue was predominant and the overall labeling was extremely weak (Fig. 4A). In ovariectomized animals treated with 5-ene-diol, the epithelial cells were numerous with a high degree of labeling (Fig. 5A).

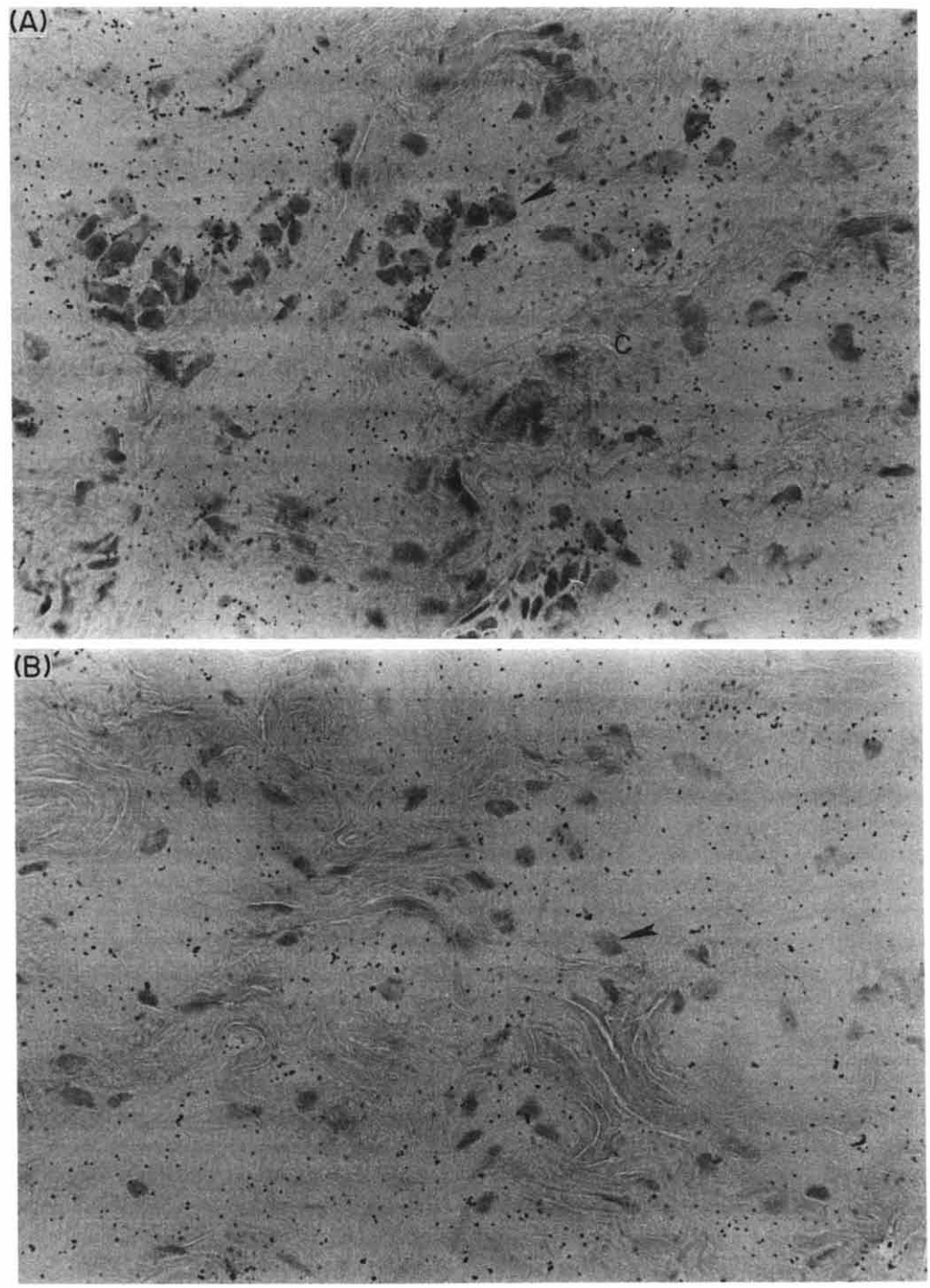

Fig. 4. Light microscope autoradiographic localization of β-adrenergic receptors in mammary tumors of an ovariectomized rat. (A) Total binding. The epithelial cells (arrows) are dispersed and weakly labeled. The connective tissue C is predominant. (B) Non-specific binding. The epithelial cells (arrows) are unlabeled. ×800.

The densitometric analysis of X-ray films in the different experimental groups after ovariectomy revealed a profound decrease in β-adrenergic receptor density/unit surface area after OVX (o.d.: 0.82 ± 0.11 in intact animals vs 0.18 ± 0.04 in OVX rats) ($P < 0.01$). Treatment of OVX rats with 5-ene-diol produced a significant increase in the density of β-adrenergic binding sites/unit surface area (o.d.: 0.76 ± 0.08) ($P < 0.01$).

To differentiate whether the observed increases in adrenergic receptors under treatment of castrated rats with steroid hormones was the result of an actual increase of recep-

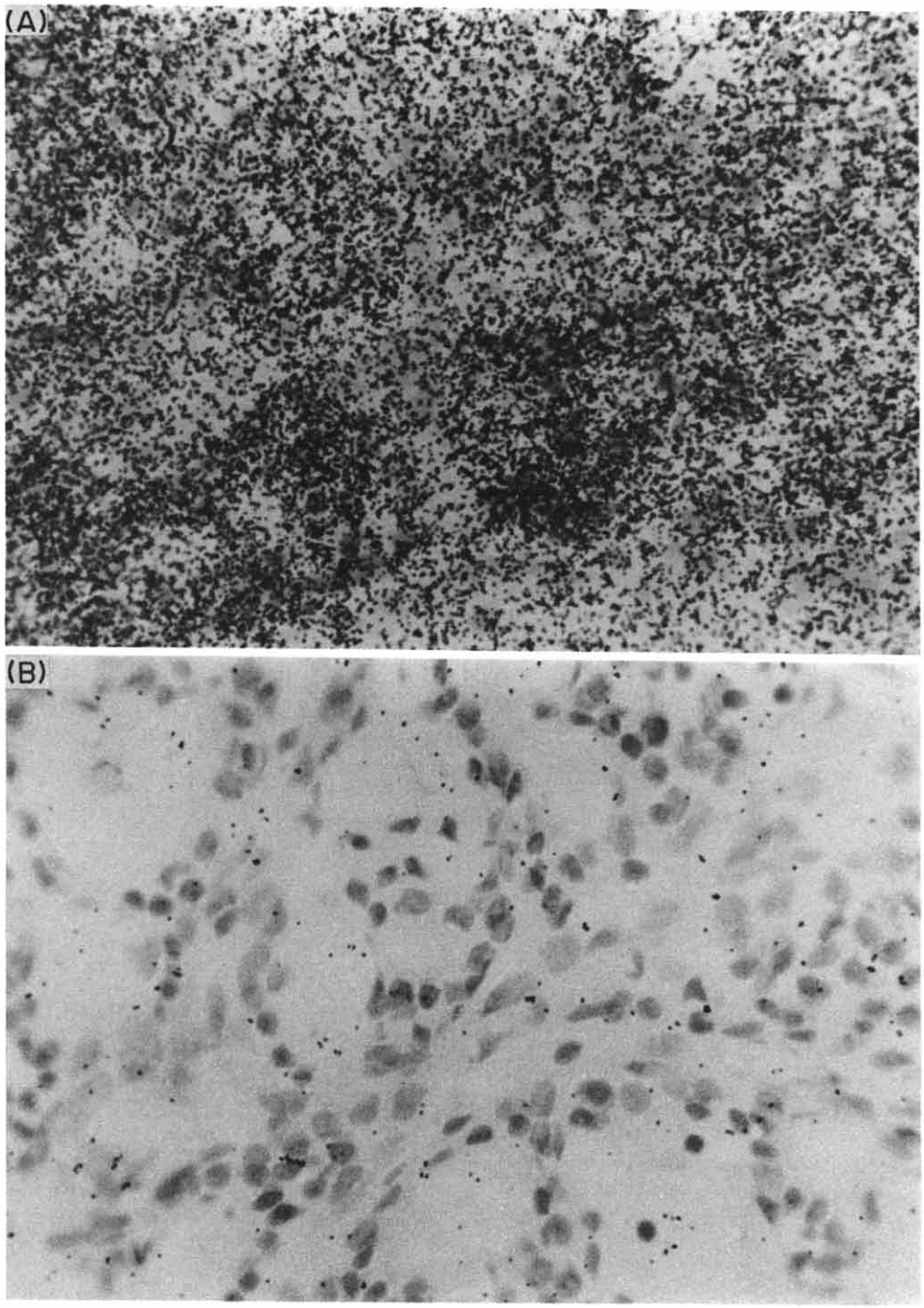

Fig. 5. Light microscope autoradiographic localization of β-adrenergic receptors in mammary tumors of ovariectomized rats treated with 5-ene-diol. (A) Total binding. The distribution of silver grains is strong and uniform. (B) Non-specific binding. $\times 800$.

Table 3. β-adrenergic receptor concentration (fmol/mg protein) and average total tumor area/rat (cm^2) in DMBA-induced mammary tumors during tumor progression in intact animals and regression after ovarectomy (OVX)

Groups	Day of observation 0	+3	+6	+9	+12	+15	+20	+30
Intact (A)	98 ± 35	140 ± 28*	168 ± 38*	160 ± 30*	156 ± 28*	168 ± 31*	157 ± 24*	170 ± 30*
(B)	1.2 ± 0.4	1.25 ± 0.5	1.7 ± 0.5	2.2 ± 0.5	2.5 ± 0.4*	3.2 ± 0.6*	4 ± 0.7*	5.1 ± 1.1*
OVX (A)	167 ± 27	98 ± 18*	84 ± 16*	41 ± 12*	20 ± 8*	16 ± 4*	17 ± 6*	14.7 ± 4.5*
(B)	5.2 ± 0.6	5.0 ± 0.5	4.7 ± 0.5	4.3 ± 0.6	3.9 ± 0.5*	3.5 ± 0.4*	2 ± 0.5*	1.9 ± 0.5*

Animals originally treated with DMBA with palpable tumors were sacrificed at different time intervals, for 30 days, or were selected according to tumor progression and bilaterally ovariectomized. The time-course effect of progression/regression (B) (expressed as average total tumor area in cm^2) and receptor levels (A) (fmol/mg protein) are indicated. Results represent the mean ± SE of 8–10 animal/experimental group.

*$P < 0.01$ compared to day 0.

tors upon individual epithelial cells, the grains were randomly counted over specific structures, in several fields and on average of 10 slide/experimental group, and epithelial cells counted using an automatic image analyzer, IBAS-2. The experiment conducted with the slides processed in parallel (same time of exposure, incubation and development in the same standard conditions) revealed a significant increase in grain count/epithelial cell in ovariectomized rats treated with E_2, $E_2 + P$, or with 5-ene-diol, in comparison with the OVX control group (not shown), clearly indicating that the observed increase in β-adrenergic receptors is a specific hormonal effect not simply related to the quantitative changes of the epithelial cell population.

Temporal changes of β-adrenergic receptor concentration during tumor development and regression after ovariectomy (OVX)

In view of the parallel changes observed in both β-adrenergic receptor concentration and average total tumoral surface area under different endocrine manipulations, we next asked: what are the temporal changes in this receptor population during tumor development or regression after OVX? Rats originally treated with DMBA, with palpable tumors (average total surface area of approximately $1.2 \pm 0.4\ cm^2$) were sacrificed at different time intervals, during tumor development and growth; while another group of DMBA-treated rats with mammary tumors ranging between 4.0 and 5.1 cm^2, were bilaterally castrated and sacrificed every 3 or 5 days. Table 3 shows β-adrenergic receptor concentration (expressed as the mean ± SE of 8–10 values/group) measured in individual tumors with a saturating concentration of [^{125}I]CYP, and average total tumor surface area during tumor progression in intact rats, and during regression after OVX. As observed, β-adrenergic receptors are at high levels in palpable tumors, similar to that measured in spontaneous mammary tumors of aged rats [7], and increase sharply well before an actual increase in tumor size, reaching a plateau during the first week of observation (Table 3). By contrast, average total tumor surface area increased slowly and progressively to reach maximal values during the first 12 days of observation, while continuing to increase until the end of the observation period.

On the other hand, when examined the time-course effect of OVX on β-adrenergic receptor decline and tumor regression, in analogy to what observed in spontaneous mammary fibroadenoma [8], β-adrenergic receptors fall dramatically by 3 days of steroid hormones removal, and after the first week, receptor levels are reduced by 50% (Table 3), while significant effects in tumor size were observed only after 12 from OVX. Two weeks after OVX, β-adrenergic receptors are maximally inhibited and tumor surface area is decreased by 50%, and continued to decrease gradually until the end of experimentation.

Such findings clearly indicate that changes in β-adrenergic receptor are not the simple result of general alterations in tumoral mass, or incipient cell death, but are a specific process preceding the actual growth or decline in tumor size.

DISCUSSION

The present data clearly show that the β-adrenergic receptor population present in DMBA-induced mammary tumors in the rat is highly sensitive to the hormonal milieu. This suggestion pertains to (1), the close correlation observed between β-adrenergic receptor levels, progesterone receptor concentration and tumor growth under different hormonal manipulations and; (2) to the autoradiographic localization of the β-adrenergic receptor within the tumor, under the same endocrine manipulations.

The specificity of the hormonal effect on β-adrenoceptors was supported by the significant increase in receptor distribution per individual epithelial cell in castrated rats treated

with steroid hormones. Moreover, high levels of β-adrenergic receptors are measured in palpable mammary tumors, reaching a maximal concentration well before the actual increase in tumor mass. Such findings, coupled to the time-course effect of castration, showing a sharp decline of β-adrenergic receptors 3 days after OVX, in the face of no change in tumor size, clearly suggest that the alterations observed in the present study in both receptor concentration and distribution, do not reflect general changes in tumoral mass, nor are the simple result of modifications in epithelial cell density, but they represent a specific process.

The hormone sensitivity of the tumoral β-adrenergic receptor is further confirmed by the high receptor concentration measured in progressing mammary tumors ([8] and Tables 1 and 2). In fact, since not all DMBA-induced mammary tumors are hormone-dependent, it is of great importance to determine changes in receptor concentration during tumor progression, stability or regression. Indeed, our recent study [8] has clearly shown that in DMBA-induced mammary tumors there is a positive correlation between β-adrenergic receptors and tumor progression and stability in intact animals, or regression after OVX [8]. The hormone dependency of this receptor system seems also well supported by our data [8] showing high levels of β-adrenergic receptors in a physiological model of hormone-dependent mammary tumor, i.e. the spontaneous mammary fibroadenoma of 18–22 month old rats, whose regression following OVX is largely preceded by a sharp inhibition of tumoral β-adrenergic binding sites.

It seems also worthwhile to mention that β-adrenergic receptor concentration, which is 10–20 times lower in the virgin mammary gland [6], than that measured in DMBA-induced or spontaneous mammary tumors, increase 3–4-fold in the pregnant mammary gland, when plasma estradiol and progesterone levels, progesterone receptors and the adenylate cylcase-cAMP system within the gland are markedly elevated [6]. Further evidence for the hormonal control of the mammary beta-adrenergic receptor pertains to the quantitative variations in β-adrenergic receptor numbers observed [7] during the different stages of the rat estrous cycle, accompanied by a predominant representation of the β_2-receptor subtype, and by the potent stimulatory effect exerted by the exogenous administration of estrogen, alone or in combination with progesterone, into ovariectomized rats [7].

On the other hand, during mid lactation, when plasma progesterone and PRL are at high levels, the β-adrenergic receptor–adenylate cyclase system of the rat mammary gland was maximally stimulated [6], supporting an important effect of the two hormones. The crucial role of PRL in maintaining beta-adrenergic receptors at high levels during lactation, was further substantiated by the marked loss of receptors observed following treatment of lactating animals with the dopaminergic (DA)-mimetic agent, 2-α-bromoergocriptine (CB-154) [7].

It seems, then, of great interest, that the tumoral β-adrenergic receptor show high sensitivity to sex steroid hormones and to the anterior pituitary hormone, PRL.

In several systems, steroid hormones have been shown to modify the adrenergic response, this effect being associated with changes in the number of adrenergic receptors [20]. In many tissues, estrogens have been reported to induce an increase in receptor expression, while progesterone antagonized many of the effects of estrogens. Heterologous stimulation of hormone receptor levels by estrogens has been reported for a number of uterine myometrial receptors. For example, serotonin, angiotensin II, oxytocin and α-adrenergic receptors in myometrium are markedly stimulated by estrogens [20–27].

β-Adrenergic receptors have been reported to either remain unchanged [28], increase [29] or decrease [30] following steroid administration. The hormonal modulation of receptors which affect uterine contractility correlates with the onset of physiological responses of the uterus, such as contraction and relaxation. In analogy with the uterus, the regulation of the physiological status of the mammary gland is achieved by modification of endocrine, autonomic and mechanical factors during adolescence, the menstrual cycle, pregnancy, parturition and lactation. The mammary gland, as other paired endocrine glands (adrenals, ovaries and testes), receives sympathetic innervation [31]. The adrenergic innervation of the gland provides the regulation of the tone of ductal smooth muscle and blood vessels within the tissue [32]. The rat ventral prostate is also innervated and prostatic β-adrenergic receptors have been shown to represent a very sensitive androgenic marker, since β-adrenergic receptor levels correlate with

intraprostatic levels of DHT and ornithine decarboxylase activity within the prostate as well as with prostatic growth [33, 34].

Clearly, ovarian hormones are crucial factors for the growth of mammary tumors induced by carcinogen administration (see Ref. [35]). Treatment of hypophysectomized animals with prolactin stimulates tumor growth as well as estradiol receptor concentration within the tumors [9, 35]. It is therefore of particular interest that ovariectomized animals, receiving 3 anterior pituitary glands under the kidney capsule, show a marked increase in both tumor growth as well as progesterone and β-adrenergic receptor concentration. Such findings demonstrate the ability of prolactin to enhance progesterone and β-adrenergic receptors via a direct effect in the tumors. In this connection, it is interesting to notice that similar numbers of β-adrenergic receptors are present in spontaneous mammary tumors of aging (18–22 months) rats [8], and the important role played by prolactin in the growth of this tumor is well recognized [see 35].

Recent data clearly indicate that the adrenal steroids dehydroepiandrosterone-sulfate (DHEA-S), DHEA, androst-5-ene-3β,17β-diol (5-ene-diol) and androstenedione (4-ene) can be converted into active estrogens (and androgens) in peripheral tissues [36–39]. Moreover, 5-ene-diol, a metabolite of DHEA-S and DHEA, has been shown to induce classical estrogenic responses in target tissues [40]. These data pertain to the stimulatory effect of 5-ene-diol on estrogen-sensitive parameters in human mammary carcinoma MCF-7 and ZR-75-1 [41, 42] cell lines. More importantly, 5-ene-diol was shown to exert mitogenic effects in ZR-75-1 human breast carcinoma cells [37]. Recently, we have, for the first time, demonstrated that the adrenal C_{19}-5-ene-steroids 5-ene-diol and DHEA possess potent stimulatory effects analogous to those of 17β-estradiol on DMBA-induced mammary tumour growth and progesterone receptor levels in the rat [43], thus supporting the suggestion of an important role or these adrenal steroids in breast cancer and other estrogen-sensitive diseases in the human.

It is, therefore, of interest that the β-adrenergic receptor concentration is sharply stimulated, in a fashion indistinguishable from that measured following treatment with estrogen, in OVX-DMBA tumor bearing rats treated with 5-ene-diol and DHEA. On the other hand, the administration of the active androgen DHT to OVX animals did not modify β-adrenergic receptor levels nor tumor growth. These and other results (see Ref. [35]) indicate that the action of androgens, like that of progesterone and other hormones on tumor development and growth, is highly sensitive to the dose used and the preexisting hormonal milieu of the animals.

Progesterone receptor levels in DMBA-induced mammary carcinomas have been proposed as a good indicator of tumor response to OVX and hormonal stimulation [8, 9, 35, 44]. In fact, restoration of progesterone receptor levels after administration of ovarian hormones and/or PRL to OVX tumor-bearing rats, is a well known phenomenon [8, 9, 12, 35, 44]. Our data are in agreement with previous findings [8, 9, 35] and indicate that changes parallel to the ones observed for the progesterone receptor are measured for the β-adrenergic receptor population in DMBA-induced rat mammary tumors. While in intact animals we have observed a good correlation between tumor progression and number of β-adrenergic binding sites (Tables 1 and 2), treatment of OVX animals with the different hormones results in different degrees of sensitivity of the tumoral β-adrenergic receptor population.

It seems apparent that the growth and dependency of rat mammary tumors on PRL and steroid hormones will vary from tumor to tumor; i.e. some tumors will require greater or lesser amounts of PRL in the absence or the presence of other hormones for optimal growth process. It is, therefore, possible that this situation is reflected by different degrees of induction of the tumoral β-adrenergic receptor.

Although the present work suggests that the tumoral β-adrenergic receptor is hormone-sensitive and that changes in receptor levels largely precede tumor development or regression, further studies are required in order to correlate β-adrenergic receptor-mediated mechanism(s) with mammary tumorigenesis, or to substantiate a possible participation of catecholamines in mammary tumor growth.

Nevertheless, we have observed [45] the presence of a β_2-subtype adrenergic receptor mechanism acting as a highly potent stimulator of adenylate cyclase activity in the androgen-sensitive mouse mammary carcinoma (Shionogi) cells in culture. Moreover, we have shown a marked androgen sensitivity of the β-adrenergic control of adenylate cyclase activity within this tumoral cell line [45]. Such findings coupled to the high

hormonal sensitivity of the β-adrenergic receptor-adenylate cyclase system of the intact mammary gland, would suggest similar post-receptor mechanism(s) acting at the tumoral level.

As to the question of what kind of relationship could exist between β-adrenergic receptors and mammary tumor growth, and the significance of such a process, it seems interesting to recall that a growing body of experimental and epidemiological evidence links psycho-social factors (such as stress) to the development and course of neoplasia [46]. Stress effects may involve ACTH, glucocorticoids, catecholamines, prolactin, opioids and immunosuppression, all factors crucially involved in tumor growth [47]. Then, changes in the circulating levels or locally secreted catecholamines, induced by physiological or pharmacological stimuli, could directly influence mammary gland function, as already suggested for adrenal, ovarian [48–51], testicular [52] and prostatic [33, 34] catecholaminergic regulation. Of major interest is the possible interplay of sex steroids (especially estrogens) and catecholamines at the mammary gland level. In fact, catecholestrogens, their receptors, together with their catabolizing enzyme, catechol-*O*-methyltransferase (COMT), are locally formed in both normal and neoplastic mammary tissues [53–55]. Furthermore, COMT levels are significantly increased in the cytosol of malignant tumor cells than in the cytosol of benign tumor or normal cells [55]. Thus changes in intra-mammary concentrations of catecholamines may provide a mechanism able to modulate the degradation of 2-hydroxyestrogens (importantly involved in malignant cell growth), within the tumors. On the other hand, the binding of the neurotransmitter to its mammary receptor might be transduced in activation of other intracellular mechanisms, including proto-oncogens mRNA expression, as has been described at the level of the brain [57].

In summary, we have correlated temporal changes in tumoral β-adrenergic receptor concentration during tumor progression and regression after ovariectomy, and described the hormonal regulation in DMBA-induced mammary tumors in the rat. While further studies are required to characterize, at a molecular level, β-adrenergic receptor gene expression and the transduction mechanism(s) at the mammary tumor level, the present findings coupled with our recent characterization of similar β-adrenergic receptors in human breast carcinoma [56], show a marked sensitivity of this receptor system to both ovarian and adrenal hormones, as well as to the anterior pituitary hormones PRL.

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