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Progesterone receptor involvement in independent tumor growth in MPA-induced murine mammary adenocarcinomas

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Abstract

We have developed a model of hormonal carcinogenesis in BALB/c female mice, in which MPA induced ductal mammary adenocarcinomas, expressing high levels of estrogen and progesterone receptors (ER and PR). A series of tumor lines, retaining both PR and ER expression, were obtained from selected tumors, which are maintained by syngeneic passages. In this model progesterone behaves as the growth-stimulating hormone (progesterone-dependent or PD tumors), whereas estrogens induce tumor regression. Through selective treatments we were able to derive a series of progesterone-independent (PI) variants. These lines do not require progesterone treatment to grow in ovariectomized female BALB/c mice, but retain, however, the expression of ER and PR.

The aim of this paper is to investigate a possible regulatory role of the progesterone receptor (PR) on PI tumor growth. ER and PR were detected by immunocytochemistry in all lines studied. They were also characterized using biochemical assays and Scatchard plots. No differences in K_d of PR or ER were detected in PI variants. AR or GR were not detected in tumor samples using biochemical assays. Estradiol (5 mg silastic pellet) induced complete tumor regression in all tumors tested. We also evaluated the effects of different antiprogestins on tumor growth. Onapristone (10 mg/kg/day) and mifepristone (4.5 mg/kg/day) were able to induce complete tumor regression. The antiandrogen flutamide (5 mg silastic pellet) had no effect on tumor growth in agreement with the lack of androgen receptors. We used an in vitro approach to corroborate that the antiprogestin-induced inhibition was not attributable to an intrinsic effect. Cultures of a selected PI line were treated with PR antisense oligodeoxynucleotides (ASPR) to inhibit in vitro cell proliferation. A significant decrease of ^{3}H-thymidine uptake was observed in cells of a PI line growing in the presence of 2.5% charcoalized fetal calf serum and 0.8–20 μg/ml ASPR. It can be concluded that the PR pathway is an essential path in the growth stimulation of PI tumors.

Keywords: Progesterone receptors; Mammary adenocarcinomas; Mice; Antiprogestins; Antisense

1. Introduction

Steroid hormones influence breast cancer development and progression. A great proportion of these tumors arise as hormone-dependent (HD) lesions and they might evolve to a hormone-independent (HI) status, but even at this stage they may respond to hormone therapy, providing they retain functional steroid receptors [1]. The transition to a HI state entails mechanisms of a complexity far beyond a simple receptor-ligand interaction or lack of it; in humans clinical evidence shows that tumors that develop tamoxifen resistance retain the expression of functional estrogen receptors (ER) [2]. Darbre and Daly [3] reported that the transition to a estrogen-insensitive state, in the ZR-75-1 human cell line, does not involve any reduction in the number or functionality of ER. The determinants of acquisition of hormone independence

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remain to be fully elucidated, although in the last few years novel mechanisms of steroid hormone action have been described that may account for some of the phenomena observed. In vitro studies showed that the direct activation of second messengers systems, in the absence of steroid hormone, could trigger steroid receptor-mediated cellular responses [4]. Thus, other biological activators may be using the steroid receptor pathway to stimulate tumor growth in HI tumors. Epidermal growth factor, transforming growth factor-α and insulin-like growth factor-I are each able to induce an increase in the mRNAs of two estrogen-induced genes in the absence of 17-β-estradiol (E_2), and this effect is reverted by antiestrogens [5]. Transfection studies in HD cell lines with different growth factors and experiments with transgenic mice [6,7] lend further support to this hypothesis.

Several models of mammary tumorigenesis have been developed in mice. In most models, although hormones are necessary for tumor induction, the tumors developed are hormone-independent [8]. Some strains of mice carrying an infective mouse mammary tumor virus develop pregnancy-dependent tumors which are ER(+) and PR(+). In these models HI variants lack ER and PR, suggesting that HD tumors are composed of a mixture of ER and PR positive and negative cells from which the receptor negative population is preferentially selected during the transition to hormone independence [9]. In a different model, the MXT mouse mammary tumor, hormone independent variants maintain the expression of PR at levels similar to those observed in the parental line [10]. This observation suggested that mechanisms other than the selection of a cell population negative for steroid receptor, bearing a growth advantage, might be playing a role in the acquisition of HI.

In this work we have explored some of the variables implicated in the selection of independent-lines originated in medroxyprogesterone acetate (MPA)-induced tumors in BALB/c mice [11,12].

In vivo progestin-dependent (PD) lines originated in these ductal metastatic carcinomas, which express high levels of ER and PR [13], are maintained through syngeneic serial passages in MPA-treated mice. By transplantation into untreated mice, we generated progestin-independent (PI) tumor lines that retain the expression of ER and PR [14]. In this model of hormonal carcinogenesis progesterone is the growth-stimulating hormone while estrogens induce tumor regression [15]. The persistent expression of PR in PI tumor lines, even after several cycles of serial transplantation in untreated mice (for more than 10 years) led us to hypothesize that this steroid receptor may still be playing a role in the stimulation of tumor growth. In this paper we report our findings as regard to the binding properties of these receptors and, using an antisense approach and antiprogestins, we define their functional role in PI cell growth.

2. Materials and methods

2.1. Animals and tumors

All experiments were carried out using 2-month-old virgin female BALB/c mice raised at the National Academy of Medicine, Buenos Aires. They were housed 3–6 per cage in air conditioned rooms at $20 \pm 2°C$, kept under an automatic 12 h light/12 h darkness schedule and given pellets and tap water ad libitum. Animal care was in accordance with institutional guidelines. Ductal mammary adenocarcinomas were induced by MPA as previously described [11] and maintained by syngeneic transplants in female mice treated with 40 mg of MPA depot s.c. contralateral to tumor inoculum. C4-HD and C7-HD were obtained in 1985 [11] and 59-HD in 1989 [14]. From the formers we generated their PI variants, C4-HI and C7-HI, which were able to grow in the absence of MPA. The BET tumor was originated after treating the C7-HD line with E_2 [15]. This variant was originally PI and estrogen-resistant, but it became estrogen-sensitive after one year of successive transplants in untreated animals (unpublished data).

2.2. Tumor growth on ovariectomized mice

These experiments were performed using the 22nd passage of the PD ductal mammary tumor line 59-HD. Twelve female BALB/c mice per group were inoculated with 10^5 cells s.c. in the inguinal flank of MPA-treated, intact, sham or ovariectomized (ovx) mice. Tumor growth was measured with a caliper (width and length) twice a week in the first two months and once a week thereafter. Tumor latency was considered as the time elapsed between tumor inoculum and the date tumors became palpable. Tumors growing in ovx animals were evaluated for hormone receptors and hormone dependency.

2.3. Hormone-dependence

Tumors that grew in ovx mice were transplanted s.c. by trocar in the inguinal flank of intact or ovx mice ($n = 3$). MPA-treated mice were used as controls. Tumors were considered PD if, after 5 months of observation they did not grow in ovx. Tumors were considered PI if they were able to grow in ovx animals.

2.4. ER and PR studies

2.4.1. Reagents

[2,4,6,7-^{3}H(N)]-E_2 (specific activity: 99.5 Ci/mmol), [17α-methyl-^{3}H]-promegestone (^{3}H-R5020) (specific activity: 86.0 Ci/mmol), 6α-[1,2-^{3}H(N)]-methyl-17α-hydroxyprogesterone acetate (specific activity: 56.8 Ci/mmol), nonradiactive R5020 and nonradioactive methyltrienolone (R1881) were purchased from New England Nuclear, USA. Diethylstilbestrol (DES), cortisol (F), dihydrotestosterone (DHT); TRIS base, dithiotreitol and EDTA were obtained from Sigma Chem. Co., St Louis, MO. Mifepristone (RU 38.486) was a gift of Roussel Uclaf, Romainville. All reagents were of analytical grade.

2.4.2. Preparation of tumor samples

Frozen tumor samples were weighed and homogenized in a ratio 1:4 W/V in buffer A (20 mM TRIS–HCl [pH = 7.4], 1.5 mM EDTA, 0.25 mM dithiotreitol, 20 mM Na_2MoO_4 and 10% V/V glycerol). They were centrifuged for 20 min at 12,000 rpm in cold and the supernatant was used to carry out the receptor assays.

2.4.3. Receptor assays

ER and PR were evaluated by single saturation dose as previously described (Molinolo et al., 1987), in both the parental tumor line (59-HD) and in the tumors arising in ovx animals, using [^{3}H]-E_2 or [^{3}H]-R5020 respectively. All steps were performed at 0–4°C. Scatchard analysis [16] were performed using doses ranging from 0.2 to 30 nM. Briefly, duplicate cytosol aliquots of 100 μl were incubated with tritriated hormones in the presence or absence of 100-fold molar excess of nonlabeled hormones, and with 200-fold molar excess of DHT (for ER) or F (for PR) to suppress nonspecific binding to plasma proteins. The samples were incubated 18 h and then 100 μl of a suspension of 1% charcoal–0.1% dextran in buffer A were added to each sample. The free fraction was separated by centrifugation at 3500 rpm for 10 min and counted in a β Beckman Counter. The results were expressed as fmol/mg cytosol protein. Protein concentrations were determined according to the method of Lowry et al. [17]. In in vitro studies PR were measured using the whole cell technique [18].

2.4.4. Immunocytochemistry of ER and PR

Samples of selected tumors were fixed for 24 h in chilled 15% formaldehyde in PBS, dehydrated through graded ethanols to xylene, and embedded in paraffin. Several 5 mm tissue sections were cut from the paraffin blocks for immunostaining. ER and PR were localized essentially as described by Silberstein et al. [19] using MC-20 polyclonal anti-ER (Santa Cruz Biotechnology Inc, CA) at a 1:50 dilution and C-20 polyclonal anti-PR (Santa Cruz Biotechnology Inc, CA) at a 1:100 dilution. The sections were incubated with the primary antibodies for 48 h at 4°C. Antibody binding was detected using a biotynilated goat anti-rabbit secondary antibody and the avidin–biotin–peroxidase system (Vector Laboratories, Inc., Burlingame, CA) according to Hsu et al. [20]. Peroxidase activity was developed with 3–3′ diaminobenzidine.

2.5. MPA binding sites in PD and PI tumors

MPA binding sites were evaluated using ^{3}H-MPA at doses ranging from 0.2 to 30 nM, in the presence or absence of 100-fold molar excess of nonlabeled MPA, and with 200-fold molar excess F to suppress nonspecific binding to plasma proteins. The maximum binding sites for the specific binding value and the K_d were estimated by Scatchard plot analyses. To avoid interference with circulating MPA, hormone treatment was stopped one week prior to tumor dissection.

2.6. Competitive binding analysis

Aliquots of PD tumor extracts were incubated for 24 h at 0°C with ^{3}H-R5020 to a final concentration of 2.5 nM together with increasing concentrations of unlabeled R5020, Pg, MPA, DHT, R1881, DES or mifepristone ranging from 0.01 to 20,000 nM. Specific binding was evaluated as described above. Similar experiments were performed in the presence of a 200 fold molar excess of F.

2.7. Effect of mifepristone, onapristone, flutamide and 17-β-estradiol on PI tumor growth

Four different PI tumor lines (C4-HI; 59-2-HI, which originated in the experiment mentioned above; C7-HI and BET) selected for these experiments were inoculated s.c. in the inguinal flank of intact female mice ($n = 3$–6); hormonal treatments were started when the tumors reached approximately 25–50 mm^2. Onapristone [Schering, Germany, (ZK 28299)] was inoculated daily in doses of 1 or 10 mg/kg s.c. [21] during 30 days or in silastic pellets of 1.7 mg. Mifepristone was administered in daily doses of 4.5 mg/kg during 9 days in C7-HI tumor line and during 30 days in the 59-2-HI line. Flutamide and E_2 (Hormone Pellet Press, KS) were administered in pellets of 5 mg during 30 days. All pellets were implanted s.c. in the back of the animals. Tumor growth was evaluated twice a week, as previously described.

2.8. Use of antisense oligodeoxynucleotides to evaluate the role of PR in PI tumor growth

The ASPR and SPR used in this study, previously described by Mani et al., in in vivo studies in rat [22], were modified to reflect the sequence of the mouse PR [23]. It includes the initial codon of the PR A isoform. ASPR (5′-ACTCATGAGCGGGGACAACA-3′) and SRP (5′-TGTTGTCCCC GCTCATGAGT-3′) were synthesized by Cybersin PA and by Fagos S.A, Buenos Aires, and purified by high pressure chromatography. A NCBI BLAST search in the Gene Bank database revealed no ASPR homology higher than 70% with any known mouse gene. Primary cultures of 59-2-HI were performed as previously described [18] with some modifications. Briefly, the epithelial enriched tumor fraction was purified and cells were seeded in 24 multiwell plates in the presence of Dulbecco's modified MEM F12-HAM without phenol red and 5% charcoalized fetal calf serum (chFCS). After 48 h, this medium was replaced by fresh medium with 2.5% chFCS, and the cells were incubated with 0.16–20 μg/ml of ASPR or SPR for 96 h. After an incubation period of 72 h, the experimental solution was replaced with fresh medium and the cells incubated with 1 μCi/ml of ^{3}H-thymidine (NEN, Dupont Boston Ma, 70–90 Ci/mmol). After 24 h, the cells were trypsinized in a final volume of 400 μl, which was distributed in 2 wells of a 96 microplate and then harvested. Nonneoplastic fibroblasts obtained from tumor stroma [18], MCF-7 and 3T3 Swiss cells were used as controls to test the specificity of ASPR and SPR inhibition. A scrambled ASPR (ScASPR) was also used once to further confirm the specificity of the ASPR effect. (ScASPR: 5′-ACACCGAGACTCTGGACG TT-3′). The ability of ASPR to inhibit PR was tested by binding techniques using C4-HD primary cultures. The cells were incubated with or without 20 μg/ml of ASRP in the same experimental conditions used in the experiments of cell proliferation and PR were evaluated using the whole cell method [18]. The cells were counted in Neubauer chambers and receptor values were expressed as fmol/10^{5} cells.

2.9. Statistical analysis

Tumor growth in ovx and control mice was compared using the tumor incidence as calculated with the product limit estimate of the survival distribution of Kaplan–Meier [24]. Distribution equality was assessed with the log rank test [25]. In in vivo and in vitro experiments the differences between tumor size or cell proliferation were evaluated using ANOVA and Tukey multiple post t tests.

3. Results

3.1. Tumors arising in ovx animals

The 59-HD tumor line was used to obtain variants able to grow in ovx mice. Tumors were transplanted into MPA-treated and untreated virgin mice and in sham operated or ovx mice. All tumors grew in MPA-treated mice with a median tumor latency of 20.5 days (Table 1). Untreated animals had a median tumor latency of 228 days; a shorter, although marginally significant latency ($p < 0.053$), was observed in sham-operated untreated mice (control group). Ovx inhibited tumor growth ($p < 0.001$). Six tumors that had started to grow in ovx animals, were transplanted into MPA-treated, untreated, and ovx. Upon growth, all tumors remained MPA-responsive and five out of six were able to grow in untreated ovx. This growth was considered as PI because tumors were able to grow without the exogenous administration of MPA. The remaining tumor disclosed a growth pattern similar to that of the parental line. These results suggest that tumors arising in ovx animals, which may disclose an apparent autonomous growth, are still MPA-responsive.

3.2. ER and PR studies

Scatchard analysis of ER and PR were performed in samples of the parental line and in the PD and PI variants to investigate a possible modification of PR receptor constants. No significant differences were observed in K_d and total number of receptors between the parental PD and the PD or PI variants arising in ovx animals (Table 2). There were wide variations in K_d values among different tumors; this contributed to generate a mean value almost an order of magnitude higher than the standard K_d reported for uterus. Values higher than the standards have also been reported in other tumor models [10]. ER and PR were also identified using immunocytochemistry in both PD and PI tumor lines. Strong specific nuclear staining

Table 1
Tumor latency and tumor take of a PD tumor line transplanted in ovx mice

Groups	Tumor Latency (days) median (range)	Tumor take
Control[a]	228 (73–483)	92% (11/12)
Control + MPA[a]	20.5 (17–32)	100% (6/6)
Sham[b]	140.5 (32–285)	83% (10/12)
Ovx[b]	306 (258–390)	67% (8/12)

[a] $p < 0.001$ (log rank test).
[b] $p < 0.001$ (log rank test).

Table 2
ER and PR binding parameters of a PD parental tumor line and of several PI variants

Lines	Progesterone receptor (mean±S.D.)[a]		Estrogen receptor (mean±S.D.)[a]	
	K_d (nM)	Q (fmol/mg protein)	K_d (nM)	Q (fmol/mg protein)
Parental	24.3±12.3	548.0±306.9	9.6±1.4	165±11.1
Variants				
PD ($n=1$)	23.7±4.1	375.8±42.9	–	–
PI ($n=5$)	45.7±6.7	820.8±477.0	8.4±3.9	143±43.1

[a] Values represent the mean of at least three determinations for each tumor.

was observed in almost 70% of the epithelial cells (Fig. 1).

To rule out MPA binding to receptors other than PR, we used different approaches. Scatchard analysis was performed using ^{3}H-MPA instead of ^{3}H-R5020 and specific binding was displaced with MPA or R5020 (Fig. 2). Similar results were obtained in both cases.

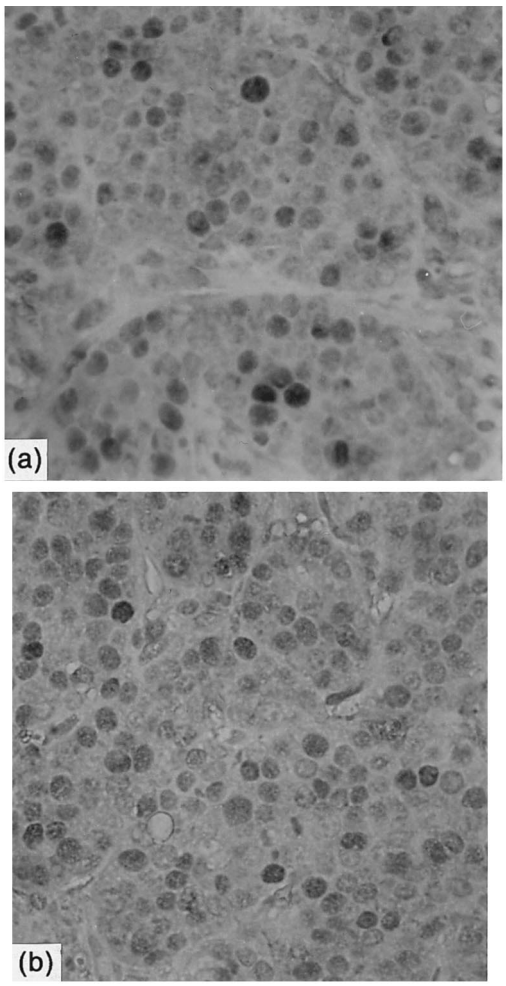

Fig. 1. Immunostaining of ER (a) and PR (b) in a PI tumor line. Strong nuclear staining is observed in nearly 70% of the cells.

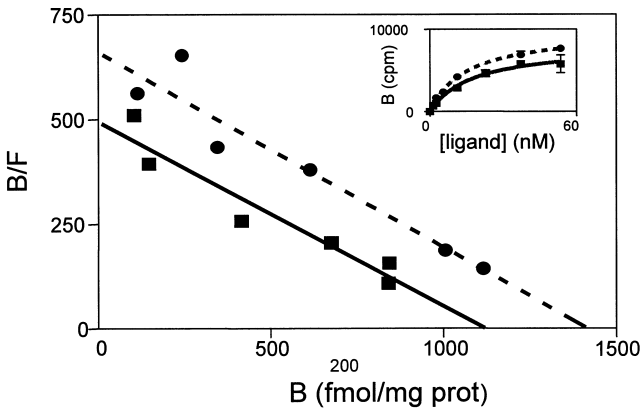

Fig. 2. Scatchard analysis of ^{3}H-MPA competed with unlabeled MPA (■) ($K_d=16.9$ nM; $Q=1120$ fmol/mg prot) or unlabeled R5020 (●) ($K_d=16$ nM; $Q=1410$ fmol/mg prot) of a PD adenocarcinoma. K_d and Q were calculated by nonlinear regression analysis. Inset: saturation curves.

We performed competitive binding assays in PD tumor samples from animals in which MPA was removed one week before the assay. Aliquots of extracts were incubated for 24 h at 0–4 °C with different concentrations of unlabelled R5020, Pg, MPA, DHT, R1881, DES or mifepristone followed by the addition of ^{3}H-R5020 to a final concentration of 2.5 nM. Specific binding was obtained as described above. Pg was as effective as MPA to displace ^{3}H-R5020 specific binding (Fig. 3). DHT was less effective and DES did not induce displacement. The same pattern was obtained using a PI tumor variant, and uterus. Similar results were obtained when the samples were incubated in the presence of 200 fold molar excess of F.

To further extend these findings, we assayed four different PD tumor lines (D5-HD, 5A-HD, 48-HD and 59-HD), four PI variants (C4-HI, 59-2-HI, D5-HI and 5A-HI) and uteri from E_2 primed animals in displacement studies. The samples were incubated with 30 nM of ^{3}H-R5020 or ^{3}H-MPA and 200-molar excess of different steroid hormones. This approach is similar to

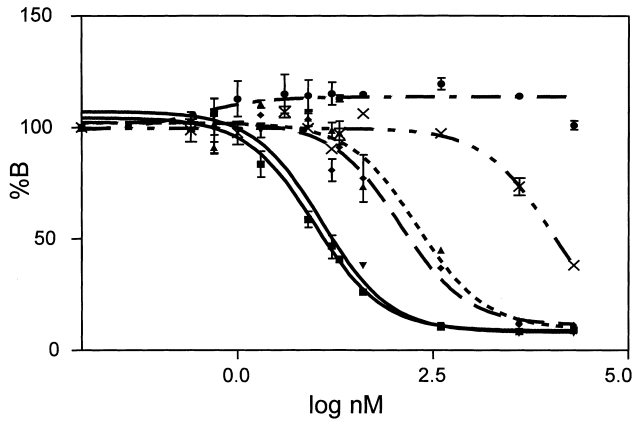

Fig. 3. Displacement of ^{3}H-R5020 (2.5 nM) binding by increasing concentrations (0.01 to 20 μM) of unlabeled R5020, mifepristone, MPA, Pg, DHT and DES in a representative PD tumor line.EC_{50}: R5020 (■) = 9.7 nM, mifepristone (▼) = 9.6 nM, Pg (◆) = 96 nM, MPA (▲) = 245 nM, DHT (×) = 10290 nM and DES (●) = nondisplaced. Similar results were obtained in the presence of 200 fold molar excess of F.

perform the previous displacement assays using a single concentration (30 nM). A representative example of the pattern of displacement of one of these tumors and uterus is shown in Fig. 4. R5020, MPA, and Pg had the same ability to displace the specific binding of ^{3}H-R5020, DHT has an intermediate value and DES induced no displacement. This displacement was similar either in the presence or absence of unlabeled cortisol (data not shown). Similar results were obtained using cytosol samples from primed uteri, indicating that the binding properties of PR in this mammary tumor model are similar to those of normal tissue.

With these studies we demonstrate that PI variants express similar levels of immunoreactive ER and PR, with the same affinities, as the parental PD tumor line.

3.3. Androgen and glucocorticoid binding sites

Similar results were obtained with ^{3}H-R5020 or ^{3}H-R1881, even in the presence of 1000X triamcinolone; no binding sites were evident when ^{3}H-DHT was used at experimental conditions in which prostate tissue gave positive results. These results suggest that R1881 is not suitable for the determination of androgen receptors in mice. Levels lower than 3 fmol/mg prot were observed when ^{3}H-dexa was used, confirming the results obtained in the previous experiments in which the addition of F did not displace MPA binding.

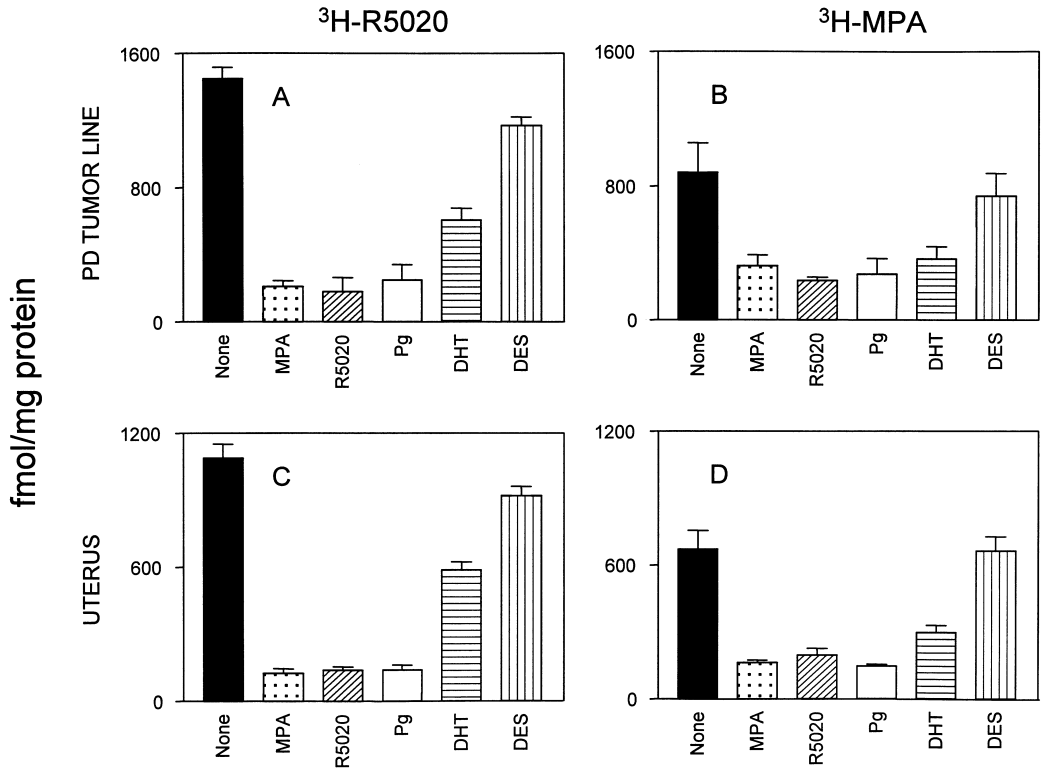

Fig. 4. Displacement of ^{3}H-R5020 (a and c) or ^{3}H-MPA (b and d) binding by 200 fold excess of unlabeled MPA, R5020, Pg, DHT, or DES at saturation in mouse uterus (C and D) and in one representative PD tumor line (A and B). Similar results were obtained in the presence of 200 fold molar excess of F.

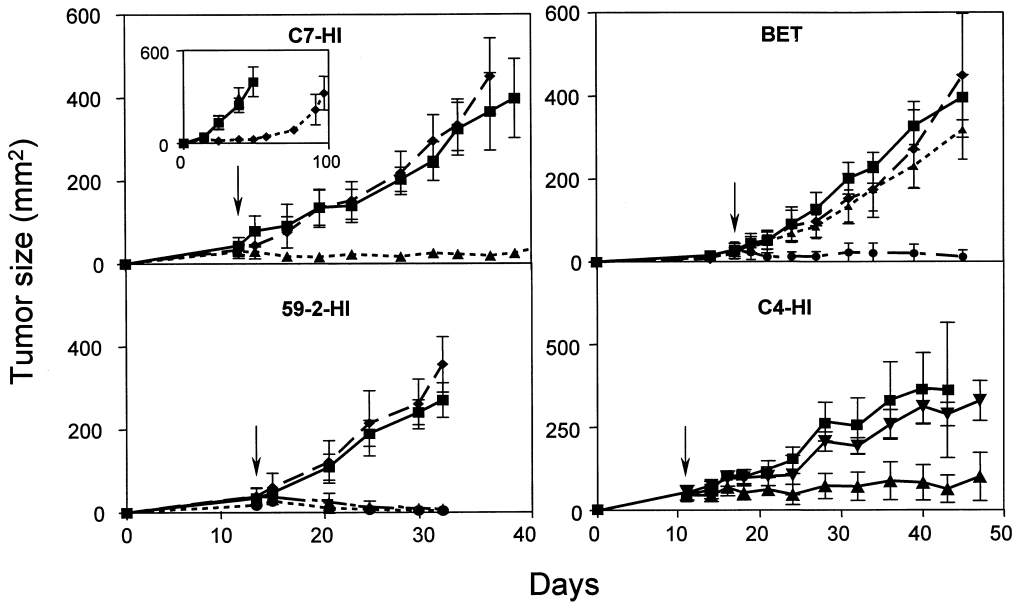

Fig. 5. Tumor growth of several PI tumor lines treated with: onapristone (1 mg/kg/day) (▼), onapristone (10 mg/kg/day) (▲), E_2 (pellet 5 mg) (●), flutamide (pellet 5 mg) (◆) or control (■). Treatments started when tumor transplants reached approximately 50 mm^2; animals were followed for one month. Inset: In this group onapristone treatment was followed for 70 days. Results are expressed as mean±S.D.

These results rule out the possibility that MPA could be exerting its proliferative effect using AR or GR.

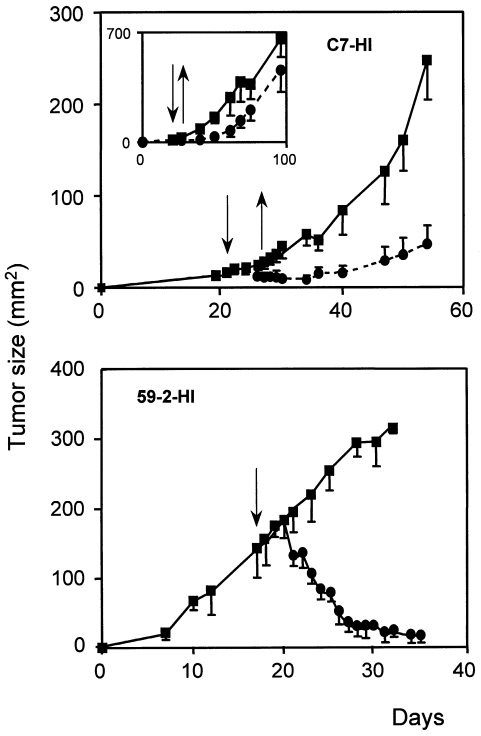

Fig. 6. Effect of mifepristone on tumor growth of two PI tumor lines. C7-HI was treated for 11 days with mifepristone (4.5 mg/kg/day). 59-2-HI was treated for 30 days starting when tumors were larger than 100 mm^2. (●) mifepristone, (■) control. Results are expressed as mean±S.D.

3.4. Effect of mifepristone, onapristone, flutamide and E_2 on PI tumors

To investigate the role of PR on tumor growth, mice bearing PI tumors (50 mm^2) were treated with two antiprogestins: onapristone and mifepristone. Estrogen treatment was also performed since we have previously demonstrated that E_2 was able to inhibit tumor growth [15]. Flutamide pellets (antiandrogen) were also implanted as controls to corroborate previous results that indicate the absence of AR in these tumors. Onapristone at concentrations of 10 mg/kg inhibited tumor growth in 3 of the 4 tumor lines studied, inducing complete remissions in 2 (C7-HI and 59-2-HI) tumors followed for 60 days (Fig. 5). In only one case (BET), tumor regression was observed with estrogens and not with the antiprogestin. Concentrations of 1 mg/kg were less effective (Fig. 5). Flutamide did not modify tumor kinetics. Similar responses were obtained when silastic pellets of onapristone were used (data not shown). The effect of mifepristone was evaluated in two tumor lines. To be able to evaluate the reversibility of hormone treatment, mifepristone was administered for only 11 days to animals carrying the C7-HI tumor line. The antihormone induced a significant decrease in tumor size that reverted several days after mifepristone treatment was interrupted (Fig. 6). For the 59-2-HI line tumors larger than 100 cm^3 were chosen to obtain a steep regression curve (Fig. 6).

Similar levels of PR were detected in the four tumor lines studied (14 and submitted), suggesting that the

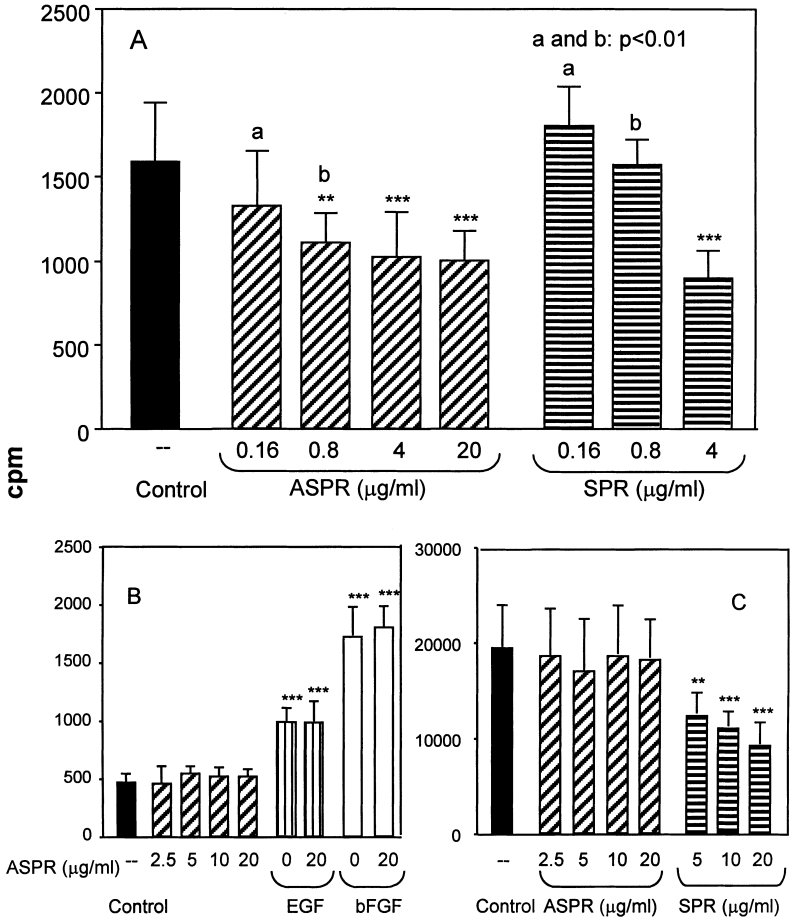

Fig. 7. Effect of ASPR on [^{3}H]-thymidine uptake in primary cultures of a PI variant (59-2-HI). The results shown are representative of a series of three experiments. (A) Cells were incubated with ASPR or SRP in the presence of DMEM-F12 without phenol red and 2.5% chFCS. After 72 h cells were incubated with fresh experimental medium and 1 μCi/ml of ^{3}H-thymidine, and harvested 24 h later. The results are expressed as mean cpm±S.D. Fibroblasts from the stroma of the same tumor line (B) and MCF-7 cells (C) were used as controls. ASPR had no affect on fibroblastic cell growth, even when stimulated with 10 ng/ml EGF or 100 ng/ml bFGF or on MCF-7 cells.

differential response of BET to antiprogestins is not due to a lower level of PR expression. With these analyses we demonstrate that (a) onapristone, a type I antiprogestin, and (b) mifepristone, a type II antiprogestin may induce complete remissions of PI tumors, (c) antiprogestins and E_2 might exert their inhibitory effect using different pathways, (d) resistance to antiprogestins is not associated to lack of PR expression. These results suggest that antiprogestins induce tumor regression through a blockade of the PR or that they might exert an antiproliferative effect per se using the PR or other signaling pathway.

3.5. Effect of antisense oligodeoxynucleotides of PR on ^{3}H-thymidine uptake of primary cultures of a PI tumor line

To ascertain the specific role of PR in tumor growth, primary cultures of one of the autonomous variants were set up (59-2-HI) and the role of PR in cell growth was studied blocking PR synthesis using ASRP. A significant inhibition of ^{3}H-thymidine uptake was observed at concentrations higher than 0.8 μg/ml (Fig. 7). Using SRP an inhibition was obtained at concentrations higher than 4 μg/ml. To further confirm the specificity of ASRP effects, it was also tested in a similar range of concentrations in two different cell lines: 3T3 and MCF-7 and in primary cultures of stromal cells of the same tumor incubated with EGF or bFGF. ASPR did not inhibit cell proliferation in any of the concentrations tested in these three cases; SRP inhibited cell growth in a nonspecific manner (Fig. 7). ScASRP was tested in similar experiments; no inhibition was observed even at concentrations of 20 μg/ml (data not shown). The ability of ASPR to inhibit PR synthesis was tested in primary cultures of the C4-HD tumor line. With binding techniques, 59.3±14% ($n=3$) of inhibition in ^{3}H-R5020 binding was observed when the cells were incubated in the presence of 20 μg/ml of ASPR.

Since ASPR specifically inhibited ^{3}H-thymidine uptake in the 59-2-HI tumor line in a dose dependent manner, and this inhibition correlated with a significant decrease in PR, it can be concluded that the PR pathway is involved in cell proliferation in the absence of progestins, even in tumors which have acquired an apparent hormone-independent pattern of growth.

4. Discussion

In this study we demonstrate that the PR pathway is involved in progestin-independent tumor growth in our experimental model of MPA-induced mouse mammary carcinogenesis. We have also characterized the binding properties of the PR from PD and PI tumor variants. The transition from tumors unable to grow in the absence of the trophic hormone, to apparently fully independent tumors, involves in our model a series of steps suggestive of a progressive acquisition or development of alternate mechanisms of growth control, in which no obvious change in the PR was evident. The possibility arose then that the progestin-independent behavior was only apparent, and that the tumors were now expressing a different type of PR that had acquired the ability to use very low levels of the ligand. Exhaustive analysis of the binding parameters of PR from different PD and PI variants revealed no significant difference in K_d or in the number of receptors. To evaluate if MPA, in addition to PR, was binding to other receptors, we performed Scatchard plots with ^{3}H-MPA instead of ^{3}H-R5020, and no differences were detected. The presence of androgen and/or glucocorticoid receptors was ruled out with self-displacement analysis and using ^{3}H-Dexa and ^{3}H-DHT; flutamide had no effect in tumor cell growth. The described approach demonstrated that, regardless of hormone-related growth pattern, the tumors expressed the same type of receptor, and suggested that the mechanisms of growth stimulation that had now taken over in the absence of significant serum quantities of the proliferative hormone, were no longer influenced by this hormone. Interestingly, PRs were constantly present in PI variants, a fact that suggested that they might still be playing a role in tumor growth. The administration of two antiprogestins with different mechanisms of action [26] to animals bearing PI carcinomas induced significant tumor regression.

Complete regressions were achieved in some cases, a fact that rules out the possibility of PI tumors being composed of a significant percentage of hormone-independent cells, as suggested by other authors [9]. This is further supported by the fact that hormone resistant tumors arising occasionally during antihormone treatment still express similar levels of steroid receptors (Montecchia et al., submitted). The antiproliferative effects of several progesterone antagonists have been demonstrated in different experimental models. Mifepristone significantly delays the appearance of mammary tumors in the DMBA carcinogenesis model in the rat [27] and it also blocks the proliferation induced by progesterone in tumors that had been suppressed with tamoxifen [28]. The same antihormone can prevent the growth of already established DMBA-induced mammary carcinomas [29]. Onapristone completely inhibits tumor growth in the MXT-transplantable tumor model and mifepristone behaves similarly, an effect comparable to that of ovariectomy [30,31]; in this model, the antiprogestins effectively antagonized the proliferative effects of equimolar concentrations of MPA or estradiol. Some clinical trials using mifepristone in postmenopausal breast cancer patients with or without metastatic disease have delivered promising results, although they involved a relative small number of patients [26,32,33].

In vitro studies using ASPR confirmed that the inhibition of PR had a crucial negative effect in cell growth, suggesting that in our model the PR pathway may be an important step in cell proliferation of both PD and PI tumors. This biological event is not exclusive of our model, and it is similar to what happens in human breast cancer, in which hormone-dependent tumors arise in post-menopausal women whose estrogen levels are extremely low and, even though they respond to antiestrogen treatment [1]. Even in these cases anti-hormone resistance is only occasionally associated with a selection of ER negative cells [34]. In ongoing experiments we are now characterizing the isoforms involved in each type of tumors in order to look out for differences in receptor variants. It has recently been demonstrated that substances other than steroids, with no structural or chemical relation, are able to use the steroid hormone receptor pathway to stimulate transcription of genes under the control of steroid receptors [4,35,36]. An attractive hypothesis to explain the acquisition of hormone-independence (hormone-independence defined as the possibility to grow without need of hormone administration) may be that tumors using their steroid receptors, in the absence of ligands, may use other signaling pathways to increase cell growth.

The role of ER in our model is still obscure since estrogens have shown to exert an inhibitory effect [15]. On the other hand high levels of PR are detected in tumors from ovx animals suggesting that although the expression of PR may be increased in estrogen-treated animals there is a constitutive expression of PR [37].

An interesting finding is that in the presence of 1000X triamcinolone, which is commonly used to evaluate the presence of AR, ^{3}H-R1881 can bind to PR [38]. In our experimental tumors and in mouse

uteri, assays were nearly indistinguishable when performed with ^{3}H-R5020 or ^{3}H-R1881 and displaced with any of the two ligands at saturation levels. Thus, ^{3}H-R1881 may not be adequate to measure androgen receptors using single saturating assays in this model.

To summarize, in this report we have confirmed our hypothesis that PR are involved in PI tumor growth. We have also (a) characterized the PR binding parameters of PI variants and demonstrated that are similar to those of the parental PD tumor, (b) confirmed the presence of ER and PR by immunocytochemical methods, (c) demonstrated the absence of AR and GR by binding techniques, (d) demonstrated that antiprogestins may induce complete tumor regressions. The results obtained using the ASRP also suggest the involvement of PR in in vitro PI cell proliferation. The fact that PR are still expressed in hormone-resistant variants suggests that this effector system seems to represent a key node in the proliferation control into which several positive and negative modulators converge, offering another entry point to manipulate the growth of cell populations, which may not be primarily under the control of progestins. The fact that impressive tumor regressions can be obtained using antiprogestins suggest that in vivo, the specific blockage of PR, may induce a cascade of events in which other mechanisms in addition to growth arrest, may be involved.

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