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Report

Progesterone receptor expression in medroxyprogesterone acetate-induced murine mammary carcinomas and response to endocrine treatment

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Summary

Using medroxyprogesterone acetate (MPA) as a carcinogen, we were able to induce in BALB/c female mice, several progestin-dependent mammary ductal carcinomas that regress completely with estrogen or antiprogestins and are maintained by serial transplantations in syngeneic mice. Progestin-independent variants were subsequently generated or appeared spontaneously. Based on their response to estrogen or antiprogestins, we subdivided them into responsive progestin-independent (R-PI) variants which regress completely and unresponsive progestin-independent (UR-PI) carcinomas which are resistant to both families of compounds. In this study we have investigated progesterone receptor (PR) expression in six responsive progestin-dependent, six R-PI, and three UR-PI tumors. Progestin-dependent and R-PI tumors disclosed a higher expression of the PR_A isoform as compared with PR_B, as well as an additional band of 78 kDa that was not detected in uterine tissue; all were down-regulated by progestins. UR-PI tumors expressed lower levels of all bands in western blots, but were highly reactive by immunohistochemistry. PR RNA expression was detected in both, UR-PI and R-PI tumors. PR binding was comparable in progestin-dependent and R-PI tumors. In the three UR-PI tumors, only 29/61 (48%) of the samples evaluated showed low binding levels, the rest were negative. This report is the first to describe in an experimental model of breast cancer the expression of PR isoforms and their distribution. Our results suggest the expression of functionally altered isoforms in a subgroup of mammary carcinomas, which may explain their lack of hormone response.

Introduction

Steroid hormones, and specially estrogens, have been associated for years with the etiology of breast cancer [1]. Antiestrogen treatments, such as tamoxifen therapy, remain a central and successful approach in the treatment of this disease. However, the emergence of hormone resistance, associated with failure to respond to the treatment and eventual disease progression, remains a major setback [2]. Among several steroid hormones, the role of the estrogen/estrogen receptor (ER) system in both breast cancer etiology as well as in the transition from a hormone-dependent to a hormone-independent phenotype has extensively been studied [3]. The role of progestins in the origin of breast cancer has recently been underlined by several clinical studies [4] and, among them, specially striking are the findings of the WHI trial [5], in which an increase in breast tumor incidence was significantly higher in the estrogen plus medroxyprogesterone-treated patients as compared to the placebo group.

Progesterone receptors (PR), members of the steroid hormone receptor family, are ligand-activated nuclear transcription factors. When bound to progesterone, PR dissociate from chaperone proteins, dimerize, and bind to specific DNA sequences [6], enhancing transcription of target genes [7, 8]. Two isoforms have been described in human cells, PR_B

and PR_A [9], with molecular weights of 114–120 and 94 kDa, respectively. PR_A can dominantly inhibit PR_B and other members of the steroid receptor family [10], and regulate genes known to be involved in breast cancer or mammary gland development [11]. Unlike human breast cancer cell lines in culture in which the isoforms ratio has been analyzed and found to be approximately equimolar [12], a quantitative analysis of PR-positive human breast tumors indicates that an important proportion disclosed a significant excess of PR_A [13]. Another study showed that most of the tumors expressed PR_A levels equal or higher than those of PR_B [14], and that poorly differentiated phenotypes and higher tumor grades were correlated with an excess in PR_A.

Mouse mammary tissues from different developmental stages express both isoforms, PR_B and PR_A, with a molecular weight of 115 and 83 kDa, respectively [15]. PR_A:PR_B ratio in normal murine uterus and mammary gland has been reported to be 3:1 [16]. In transgenic mice, overexpression of PR_A results in extensive epithelial hyperplasia and excessive ductal branching [17], while PR_A null mice exhibit normal mammary gland development [18]. To our knowledge there is no information regarding PR_A:PR_B ratios in mouse mammary tumors.

In this paper, we have explored a possible relation between response to hormone treatment and PR expression, using a unique mouse mammary tumor model to analyze hormone-dependent and hormone-independent tumors derived from the same primary lesion. With progestins alone, medroxyprogesterone acetate (MPA) [19–21] or progesterone (Pg) [22], we developed a series of experimental models of mammary carcinogenesis in BALB/c female mice. The tumors induced by MPA are ductal carcinomas and express high levels of ER and PR [21]. These primary tumors have been maintained for several years by syngeneic transplantation in MPA-treated mice, since they behave as progestin-dependent. When transplanted in untreated or ovariectomized animals, they start to grow slowly after 2 or 6 months, respectively [23]. Progestin-independent variants that may retain high levels of ER and PR expression were generated after a certain number of passages, and they grow similarly in treated and untreated animals [24].

Since PR expression is a valuable marker for tumor prognosis [25], and considering that our model is a reliable tool to investigate the role of PR in breast cancer progression, we decided to study PR isoform expression in different mouse mammary tumors with different hormone responsiveness.

We classified tumors as progestin-dependent or independent according to their ability to grow in progestin-treated or untreated mice. Progestin-independent tumors were also classified as responsive (R-PI) if they regressed after 17-β-estradiolβ (E_2) or antiprogestin treatment or unresponsive (UR-PI) if they were resistant. In progestin-dependent and R-PI tumors we demonstrated by western blot a higher expression of PR_A than PR_B, and a new band of 78 kDa. Very low levels of PR were detected with binding techniques in 29/61 (48%) of the samples of the three UR-PI tumors. By western blot a lower expression of PR isoforms was also observed in this tumor type; however, no significant differences between both types of progestin-independent tumors were detected by immunohistochemistry. PR RNA expression was also confirmed by RNase protection assay in UR-PI tumors. These observations suggest the expression of functionally altered PR isoforms in unresponsive mammary carcinomas.

Materials and methods

Animals

Two-month-old BALB/c female virgin mice were used in all experiments. The animals were fed *ad libitum* and kept in air-conditioned rooms at 20 ± 2°C with a 12 h light-dark period. Animal care and manipulation was in agreement with institutional guidelines, which are in accordance with the Guide for the Care and Use of Laboratory Animals [26].

Tumors

MPA-induced mammary ductal carcinomas were used in all the experiments [27, 28]. Tumors were maintained by serial transplantation in BALB/c virgin female mice. Progestin-dependent tumors were transplanted simultaneously with MPA (20 mg depot sc) in the contralateral flank. A group of animals was left untreated to control progestin-dependence. Occasionally, tumors started to grow similarly in untreated and in MPA-treated mice. The tumor was considered a progestin-independent variant and was henceforth maintained by syngeneic transplantation in untreated mice. Samples of the progestin-dependent tumors are kept in liquid nitrogen. To continue to work with the parental progestin-dependent tumor, samples are thawed and transplanted again in MPA-treated mice.

In this study we used six progestin-dependent tumors and their derived nine progestin-independent

variants. In every passage, fragments of each tumor (2–3 mm^2) were transplanted subcutaneously to four virgin BALB/c female mice, when the tumors reached a size of 100 mm^2, the mice were killed and the lesions excised. Samples were immediately stored in liquid N_2 for western blot, binding and RNA studies, or fixed in 10% buffered formalin for immunohistochemistry, or used to perform the next consecutive passage in other four female virgin mice. Samples from 25 consecutive passages of each tumor were used in this study.

Reagents

MPA depot (Medrosterona) was a gift from Gador Laboratories, Buenos Aires. ZK 98299 (onapristone) was kindly provided by Schering AG, Berlin. RU 38486 (mifepristone) was a gift from Roussel Uclaf, Romainville, France. The reagents used in western blots were purchased from Gibco BRL, New York. Methanol was purchased from Merck Química Argentina. Molecular weight markers are Rainbow prestained molecular weight markers (Amersham Life Science, Buckinghamshire, UK). [^{3}H]-R5020, [α^{32}P]CTP and R5020 were purchased from NEN, Boston MA; KCl from Anedra, Buenos Aires. Dithiothreitol, EDTA, sucrose, protease inhibitors and E_2 were purchased from Sigma, St. Louis, MO. Activated charcoal was from Mallinckrodt Chemical Works, New York.

In vivo *experiments*

Tumor regression was studied in all progestin-independent tumors. Mifepristone and onapristone stock solutions were prepared in ethanol and diluted 1:100 in NaCl solution immediately before use. Daily sc injections of mifepristone (6.75 mg/kg body weight) or onapristone (10 mg/kg body weight) were administered to groups of three to four animals. E_2 was administered as one 5 mg silastic pellet implanted sc in the back of the animal. Tumor size was measured with a Vernier caliper (length × width) every 2 days and hormone treatments began when their size was within the range 25–50 mm^2.

Tissues and tumors used for PR binding, western blot and RNA studies

Uteri from wild type (wt) and PR knockout (PRKO) [29] adult mice were used as positive and negative controls, respectively. Different passages of each tumor (at least two samples of each passage) were taken when they reached approximately 50–100 mm^2, and immediately frozen in liquid N_2. To evaluate possible PR down-regulation by long-term exposure to progestins, MPA depot was surgically removed 10 days before the dissection of progestin-dependent tumors or inoculated sc 10 days prior to excision of progestin-independent tumors.

Preparation of whole cell extracts

Tissues and tumors were homogenized in a polytron at setting 50 with three bursts of 5 s in a 1:4 proportion tissue: buffer TEDG. The buffer was 20 mM Tris–HCl pH 7.4, 1.5 mM EDTA, 0.25 mM dithiothreitol, 20 mM Na_2MoO_4, 10% glycerol. Protease inhibitors (0.5 mM PMSF, 0.025 mM ZPCK, 0.025 mM TLCK, 0.025 mM TPCK, 0.025 mM TAME) were added to the buffer immediately before use. The homogenate was sonicated at medium frequency for 10 s (tubes were always kept on ice) and centrifuged for 45 min at 40,000 rpm, 4°C. The supernatant was immediately frozen in liquid nitrogen and stored at −70°C until later use in western blot assays. Protein concentration was determined according to Lowry et al. [30].

PR binding assays

Binding of [^{3}H]-R5020 to PR was performed on cytosolic and nuclear fractions. Briefly, the tissues were homogenized in buffer TEDG + 0.25 M sucrose (TEDGS) and the homogenate was centrifuged at 1,000 rpm, 10 min at 4°C. The supernatant was collected and centrifuged at 12,000 rpm, for 20 min at 4°C. The resulting supernatant is the cytosolic fraction. The 1,000 rpm pellet was resuspended in buffer TEDGS + 0.4 M KCl, kept on ice for approximately 1 h and then centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant is the nuclear fraction. The binding reaction was performed on both fractions. PR were labeled by incubating duplicate aliquots of the extracts with 30 nM [^{3}H]-R5020 for 3 h either alone or with 3 μM R5020.

Western blot analysis

A total of 82 samples belonging to 6 progestin-dependent, 71 samples from 6 R-PI and 56 samples from 3 UR-PI tumors were studied.

The samples (100 μg total protein/lane) were separated on 7.5% discontinuous polyacrylamide gels (SDS-PAGE) using the Laemmli's buffer system [31]. The proteins were dissolved in sample buffer (6 mM Tris pH 6.8, 2% SDS, 0.002% bromophenolblue, 20%

glycerol, 5% mercaptoethanol) and boiled for 4 min. After electrophoresis they were blotted onto a nitrocellulose membrane and blocked overnight in 5% dry skimmed milk dissolved in PBST 0.1% (0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% KH_2PO_4, pH 7.4, 0.1% Tween 20). The membranes were washed several times with PBST and then incubated with PR Ab-7/hPRa 7 (Ab7) (Neomarkers, Union City, CA) at room temperature for 2 h, at a concentration of 2 μg/ml in PBST. This monoclonal antibody was generated using purified PR from a human endometrial carcinoma as the antigen [32] and has been used to detect PR_B and PR_A in mouse [15] and human tissues [33]. The polyclonal anti-mouse PR antibody Ab-1 AB (Ab1) [34] was used under the same experimental conditions. In this case, rabbit antiserum was prepared against a synthetic peptide corresponding to amino acid residues 376–394, selected from the amino-terminal half of the mouse PR sequence [34]. Blots were probed with sheep anti-mouse or donkey anti-rabbit Ig, horseradish peroxidase-conjugated whole antibody (Amersham Life Science, Buckinghamshire, UK). The luminescent signal was generated with ECL western blotting detection reagent kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the blots were exposed to a medical X-ray film (Curix RP1, Agfa Argentina) for 10 s to 5 min. Band intensity was not quantified if the film was saturated.

Immunohistochemistry

Tumor samples as well as normal mammary glands and PRKO mice tissues, were fixed in 10% buffered formalin and embedded in paraffin. Five micrometer sections were dewaxed in xylene, rehydrated through graded ethanols, treated with 1% triton X-100 in phosphate buffer saline (PBS) for 20 min at room temperature and then washed with PBS, three times, 5 min each. The slides were incubated 30 min at room temperature with 3% H_2O_2 in distilled water to quench endogenous peroxidase activity, washed extensively with PBS and incubated in 3% albumin or normal horse serum in PBS for 20 min. The sections were then reacted with Ab1 or PR (C-20) (rabbit polyclonal IgG specific for progesterone receptor, Santa Cruz Biotechnology, CA) diluted 1:100 in PBS for 48 h at 4°C [35]. This antibody was raised against a peptide corresponding to amino acids 545–564 mapping at the carboxy terminus of the human PR (identical to the mouse sequence). The slides were washed with PBS and successively incubated for 30 min at room temperature with anti-rabbit biotin-conjugated immunoglobulins (Vector Labs, San Francisco, CA) diluted 1:250 in PBS, and with the ABC complex, prepared according to the manufacturer's directions (Vector Labs). The slides were thoroughly washed with PBS, and developed under microscopic control with 3,3′-diaminobenzidine, 0.06% in PBS, and H_2O_2 at a final concentration of 0.1%. After rinsing in distilled water to stop the reaction, the slides were stained with methyl green, air dried, cleared with xylene and mounted in synthetic medium. Primary or secondary antibodies were omitted to control specificity.

RNA isolation

Total RNA was extracted from 0.2 g of frozen tissue using the guanidine thiocyanate method of Chomczynski and Sacchi [36]. The final RNA pellet was dissolved in 0.1% SDS and stored at −70°C.

Construction of PR A&B riboprobe and RNase protection analysis

The riboprobe was designed to detect expression levels of the two PR isoforms. Using the mouse PR cDNA (mPR17) [37], a fragment of 356 bp, corresponding to nucleotides −7 to +349 was subcloned into pBluescript (Stratagene). This region comprises the amino terminal regions of PR_B and the 5′ untranslated region of PR_A. Antisense riboprobes were generated using Maxiscript *in vitro* transcription kit (Ambion, TX, USA) by linearizing the plasmid by digestion with *Xmn*I and then transcribing with T3 RNA polymerase in the presence of [α^{32}P]CTP. The riboprobe was a full-length transcript of 420 bp, of which 356 bp hybridized fully to PR_B and PR_A, corresponding to nucleotides −7 to +349. Assays were carried out on 10 μg total RNA extracted from tumors and uteri, using the RPAII ribonuclease protection assay kit (Ambion). The riboprobe was added in excess, to ensure linearity of the test. The protected hybridization products were purified by extraction in phenol:chloroform:isoamyl alcohol (25:24:1) and analyzed by polyacrylamide gel electrophoresis, followed by autoradiography. Expression levels of the two PR isoforms were quantified using image analysis with Image Quant® software

(Molecular Dynamics, Version 3.3), and normalized to β-actin.

Statistical analysis

ANOVA and Tukey multiple post-test were used to study differences in tumor size between control and treated animals. PR content in the cytosol and nucleus was compared using the Student's *t*-test for paired data. Western blot band intensity was quantified using the Image Quant® software; only samples separated in the same gel were compared. Differences between PR_A/PR_B ratios of tumors from animals treated or not with MPA were compared using *t*-test for paired data. Optical density in RNase protection assay was normalized to β-actin and differences between samples were evaluated with Student's *t*-test.

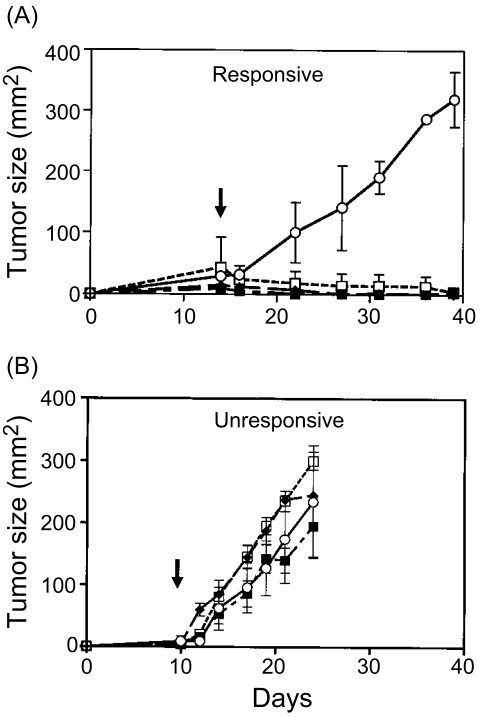

Figure 1. Response of progestin-independent tumors to E_2 and antiprogestins *in vivo*. Representative examples of responsive (R-PI) (A) and unresponsive (UR-PI) (B) tumors are shown. Six of the nine tumors regressed with the treatments and the remaining three did not respond. The tumors were inoculated sc in the right inguinal flank. Treatments were initiated when they had an approximate size of 25–50 mm^2 (arrow). Daily sc injections of mifepristone 6.75 mg/kg (♦) or onapristone 10 mg/kg (■) were administered sc in the left inguinal flank; E_2 (□) was supplied as a 5 mg silastic pellet implanted sc in the back of the animal. Control animals (◯) remained untreated.

Results

Effects of hormones on tumor growth

We evaluated the *in vivo* responses of progestin-independent tumors to E_2 and two different antiprogestins, mifepristone and onapristone. Six out of nine progestin-independent tumors growing in syngeneic animals regressed completely after endocrine treatment while the remaining three did not regress (Figure 1). Based on this behavior we classified them into responsive progestin-independent (R-PI) or unresponsive progestin-independent (UR-PI) tumors.

Binding analysis

Nuclear and cytosolic receptors at single saturating points were evaluated in samples from progestin-dependent, R-PI and UR-PI tumors. In pooled data from progestin-dependent ($p < 0.05$) and R-PI tumors ($p < 0.001$) cytosolic PR contents were higher than in the nuclear fraction. Both nuclear and cytosolic levels were down-regulated by MPA ($p < 0.01$). Receptor levels were similar in untreated progestin-dependent and R-PI tumors. Low levels of PR could be detected in 29/61 (48%) of the samples evaluated

Table 1. PR content and cellular distribution in progestin-dependent and progestin-independent tumors

Tumor responsiveness	Protein (fmol/mg) ($X \pm$ SE)		*n*
	Cytosol	Nucleus	
Progestin-dependent (+) MPA	$128 \pm 18^{a,b}$	$78 \pm 19^{a,c}$	27/27
Progestin-dependent (−) MPA	231 ± 39^{b}	191 ± 48^{c}	17/17
Responsive progestin-independent (R-PI)	$323 \pm 42^{d,e}$	139 ± 22^{d}	48/48
Unresponsive progestin independent (UR-PI)	$35 \pm 11^{e,f}$	90 ± 19^{f}	29/61[g]

[a] Progestin-dependent tumors treated with MPA – cytosol versus nucleus: $p < 0.05$.
[b] Progestin-dependent tumors treated (+) or depleted (−) of MPA – cytosol versus cytosol: $p < 0.01$.
[c] Progestin-dependent tumors treated (+) or depleted (−) of MPA – nucleus versus nucleus: $p < 0.01$.
[d] Responsive progestin-independent tumors – cytosol versus nucleus: $p < 0.05$.
[e] Responsive and unresponsive progestin-independent tumors – cytosol versus cytosol: $p < 0.01$.
[f] Unresponsive progestin-independent tumors – cytosol versus nucleus: $p < 0.01$.
[g] Only positive samples were evaluated in statistical analysis.

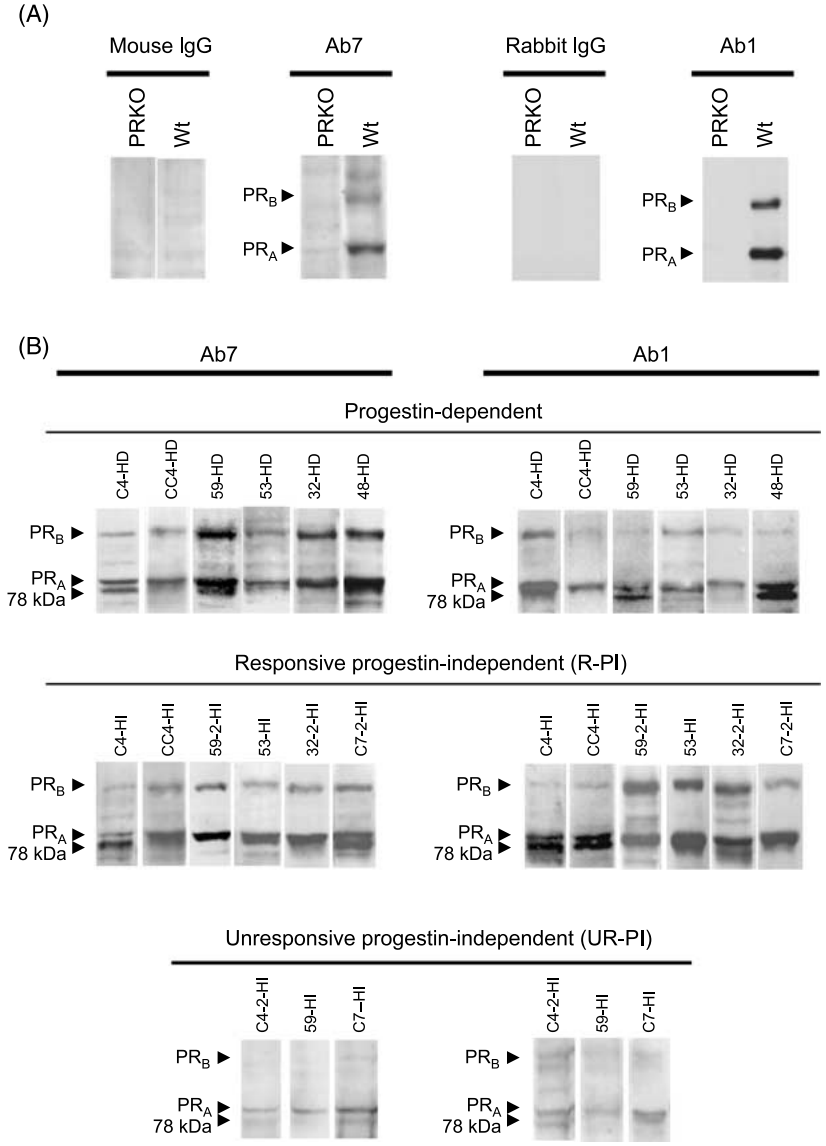

Figure 2. Representative western blots of PR isoform expression in progestin-dependent and independent tumors. Whole cell extracts (100 μg/lane) were resolved by 7.5% SDS-PAGE, blotted onto nitrocellulose and immunodetected with Ab7 or Ab1; the signal was visualized by a chemoluminescent reaction. (A) PR detection in Wt but not in PRKO uterus. The specificity of the second antibodies used is shown to the left of each primary antibody. (B) One sample representative of each of the six progestin-dependent, six responsive progestin-independent (R-PI) and three unresponsive progestin-independent (UR-PI) carcinomas evaluated are shown.

from the three different UR-PI tumors, and PR were located mainly in the nuclear compartment ($p < 0.01$) (Table 1).

Western blot studies

PR isoform expression was evaluated in samples from the six progestin-dependent and nine progestin-independent tumors. Whole cell extracts were separated in 7.5% SDS-PAGE and the blots probed with Ab7 or Ab1 antibodies. PR_A and PR_B were detected in wt uterus, but no signal was observed in PRKO uterus. These results indicate the suitability of the antibodies to evaluate mouse tissues (Figure 2(A)). Mammary gland expressed lower levels of both isoforms (not shown).

Representative samples of each of the six progestin-dependent, six R-PI and three UR-PI tumors

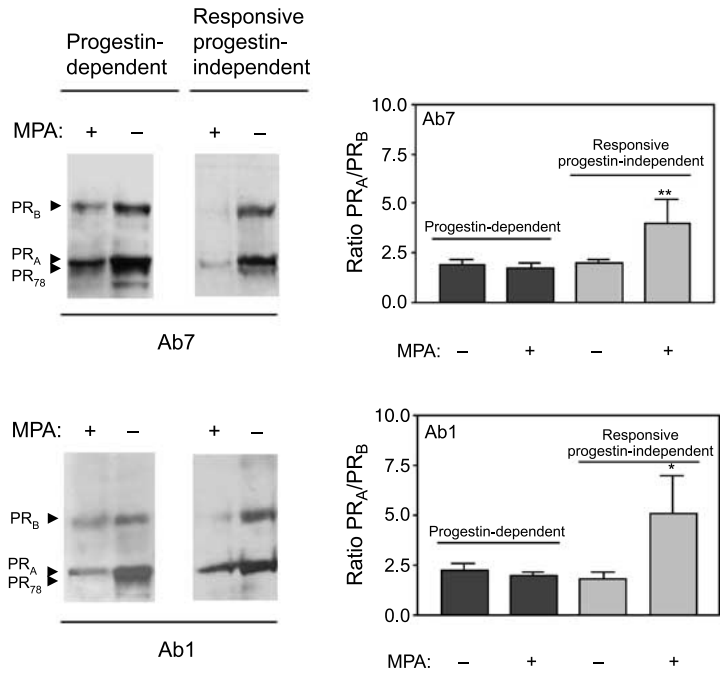

Figure 3. PR regulation by MPA. Total cellular extracts were prepared from tumors growing in MPA treated (+) or untreated (−) mice. The 100 μg of total proteins/lane were separated on 7.5% SDS-PAGE and the blots were probed with Ab7 or Ab1. The intensity of PR_A and PR_B signal was quantified for each sample and the ratio PR_A/PR_B was compared within the same tumor type. Western blots using Ab7 – 6 progestin-dependent tumors: 52 samples MPA (+), 32 samples MPA (−); 6 R-PI tumors: 6 samples MPA (+), 65 samples MPA (−). Western Blots using Ab1 – 6 progestin-dependent tumors: 8 samples MPA (+), 9 samples MPA (−); 6 R-PI tumors: 4 samples MPA (+) and 12 MPA (−). $^{*}p < 0.01$; $^{**}p < 0.05$.

are shown in Figure 2(B) (at least eight samples of each tumor were evaluated). Progestin-dependent and R-PI tumors expressed PR_A and PR_B. An additional protein of 78 kDa was observed in 33/82 (40%) of progestin-dependent and 47/71 (66%) of R-PI samples. In all the samples from the three UR-PI tumors the level of intensity of these isoforms was much lower, in fact PR_B was detected only in 26 of the 56 samples studied.

Down regulation of all isoforms in MPA-treated animals including the 78 kDa band was observed in progestin-dependent and R-PI tumors (Figure 3, left panels). The ratio of intensity of PR_A/PR_B isoforms was in all cases significantly higher than 1. In progestin-dependent tumors MPA down-regulated equally both isoforms. The PR_A/PR_B ratio was similar in treated and untreated animals. In R-PI tumors, MPA down-regulated preferentially PR_B; the PR_A/PR_B ratio was significantly higher in MPA-treated animals (Figure 3, right panels). The PR isoforms were not down-regulated by MPA in UR-PI tumors (Figure 4(A)).

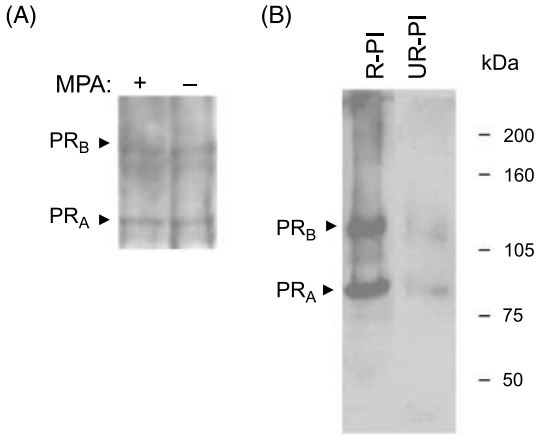

Figure 4. PR expression and regulation by MPA in unresponsive progestin-independent (UR-PI) tumors. (A) The 100 μg total protein/lane were resolved in a 7.5% SDS-PAGE and the blots developed with Ab7. Two samples from the same passage of one UR-PI tumor treated (+) or not (−) with MPA for 10 days. These results are representative of four independent experiments. (B) The 100 μg total protein/lane were separated in a 10% SDS-PAGE and the blots developed with Ab1. The results shown are representative of five different tumor samples from each type of tumor.

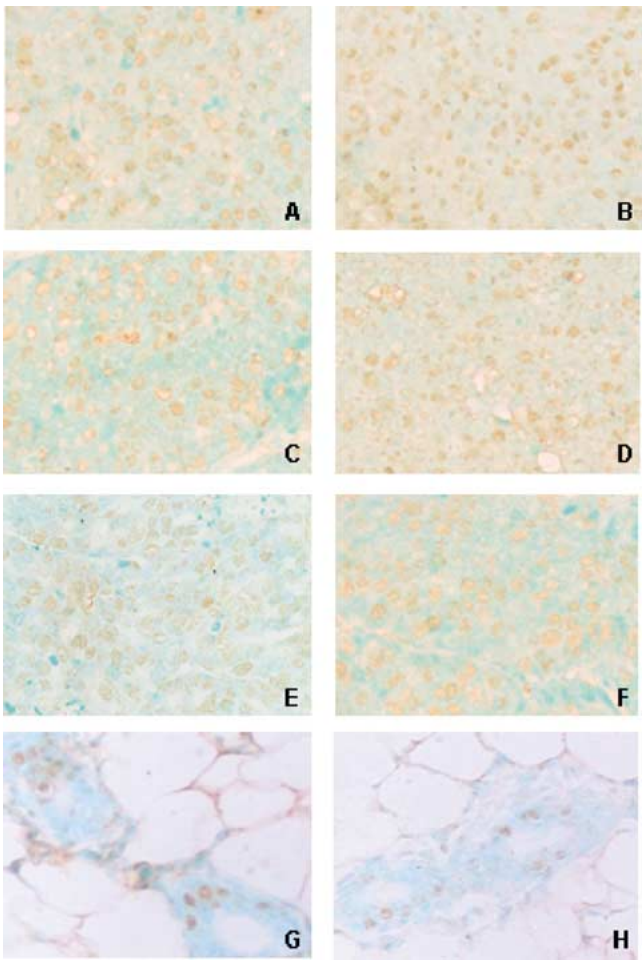

Figure 5. PR expression by immunohistochemistry in tumors and mammary gland. Representative tumors of each tumor type reacted with anti-PR antibodies Ab1 (right panels) and PR C-20 (left panels). (A) and (B) Progestin-dependent tumor; (C) and (D) responsive progestin-independent (R-PI); (E) and (F) unresponsive progestin-independent (UR-PI); (G) and (H) normal mammary ducts. Although the tumors showed variable degrees of reactivity, usually more than 50% of the cells disclosed positive, moderate to strong nuclear staining for the antibodies (immunohistochemistry 40×). Normal ductal structures show positive nuclear staining with both antibodies.

Bands of 105, 90 (between PR_B and PR_A), and of 67 kDa were also detected with variable intensity in most samples, regardless of the tumor type studied (Figures 2 and 3). These bands seem to be also down-regulated by MPA treatment in progestin-dependent and R-PI tumors (Figure 3) and are probably due to proteolytic cleavage; these bands were also detected in some samples of normal uterus.

To evaluate the possible presence of lower molecular weight bands due to proteolytic degradation or to alternative splicing, which could explain the lower levels of PR isoforms observed in UR-PI tumors, the whole cell extracts were separated in a 10% SDS-PAGE. The blots were developed with the polyclonal antibody Ab1. No differences were observed between the pattern of band expression of both tumor types studied (Figure 4(B)).

Immunohistochemistry

A specific nuclear signal was observed in all progestin-dependent and independent tumors evaluated, regardless of their hormone responsiveness. Representative images are shown in Figure 5. No differences in staining pattern were observed between UR-PI and R-PI tumors. Normal mammary glands from BALB/c mice also showed positive nuclear staining with both antibodies in the glandular cells lining the ductal structures (Figure 5(G) and (H)). No reactivity was detected in PRKO tissues (not shown).

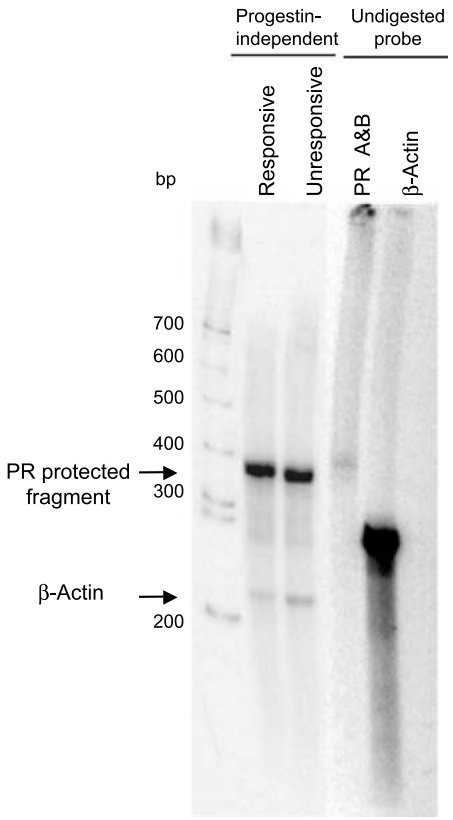

Figure 6. RNase protection assay was performed on responsive (R-PI) and unresponsive progestin-independent (UR-PI) tumors. PR RNA was detected from total RNA preparations and the signal was normalized to β-actin content. High levels of PR RNA were observed in both tumor types.

These results indicate that PR are still expressed in UR-PI tumors, although these receptors are less reactive in western blots and bind hormones less efficiently.

RNase protection assay

To corroborate the expression of PR in UR-PI tumors and to further validate our previous results, we decided to study PR expression at the RNA level. High PR RNA levels were detected in samples of both R-PI and UR-PI tumors ($n = 2$, Figure 6).

Discussion

In this study we have classified a series of MPA-induced mammary carcinomas in mice, on the basis of their hormone responsiveness to estrogen and antiprogestins. We demonstrated in this experimental model, that progestin-dependent and progestin-independent tumors that regress after hormone treatment expressed higher levels of PR_A than PR_B, as well as an additional 78 kDa band, all of which were down-regulated by MPA. On the other hand, progestin-independent tumors refractory to endocrine therapy failed to bind hormones to the same extent as the responsive ones, and expressed significantly less PR as determined by western blots. MPA did not down-regulate PR isoforms in these tumors. However we found no differences in PR expression by immunohistochemistry between responsive or unresponsive tumors; and by RNase protection assay, we detected high levels of expression in both tumor types. We hypothesize that the lack of endocrine response in UR-PI tumors may be due to the presence of receptors with altered functionality.

The differences between western blot and immunohistochemical studies are of difficult interpretation and they may not be merely due to differences in assay sensitivity. *In vivo* post-translation modifications such as sumoylations or phosphorylations [38], have been shown to play a role in modulating the stability, or transactivation functions of PR and could be responsible for possible changes in western blot immunoreactivity. The protein modifications induced by formalin fixation/paraffin embedding may have altered the structure of combinations of epitopes, leading to an increased exposure of specific antigenic determinants and to the consequent increase in immunoreactivity in immunocytochemistry, as compared with western blot. Interestingly, in the clinical setting, should had immunohistochemistry been the only study performed, these tumors would have been labeled as PR positive, and treated accordingly, although PR were poorly detected by western blots and binding techniques, and the tumors were unresponsive to endocrine therapy. This observation is of particular relevance if we consider that failure to bind the ligand in otherwise immunoreactive proteins has been reported in human breast cancers [39]. Castoria et al. [40] had also reported previously that from 34 human mammary cancers with significant amounts of a 67 kDa ER, 8 showed relatively low levels of estradiol binding, and that these receptors could be switched to hormone binding forms by *in vitro* treatment with ATP-calmodulin. In another study on GR murine mammary tumors the same authors conclude that loss of ER through the syngeneic passages could be due to decreased phosphorylation levels on ER [41].

PR expression is positively regulated by estrogens, through the ER pathway. We have studied ERα expression by western blot in all the tumors of the MPA-induced carcinoma model (unpublished observations), the intensity of the expression varied within a wide range and we found no association between the level of expression and the hormone-responsiveness, suggesting that less PR content in the UR-PI tumors may not be due merely to lower levels of ER expression.

The fact that the three unresponsive tumors studied had the same PR dysfunction and that, in all of them, lack of response to antiprogestin was associated with estrogen unresponsiveness suggests that PR may be also involved in E_2-induced tumor regression.

There are several reports in relation to the inhibitory effects of estrogens in breast cancer. They have been used as successfully as tamoxifen in the treatment of this disease but they were replaced due to their higher side effects [42]. In addition, the human breast cancer tumor T61, transplanted in nude mice regresses after E_2 pellet implantation [43] and human breast cell lines overexpressing PKCα, inoculated in nude mice [44] also regress in the presence of E_2. Moreover in many ER transfected cells estrogens exert inhibitory effects [45, 46]. Thus, the classical concept of estrogens acting uniquely as promoters of cell proliferation in breast should be revisited.

There are few reports regarding the PR isoform distribution in human breast cancer [13, 14]. Most reports associate an excess of PR_A expression with a more aggressive behavior. Graham et al. [13] reported the presence of a 78 kDa band in 25% of breast cancer samples and suggested that it may correspond to a truncated PR_A form. Yeates et al. [33] ruled out the possibility that it is a product of proteolytic cleavage. Although a functional role for this protein in the progesterone signaling pathway remains to be established, it seems to be preserved across species, as we have been able to identify it in mouse tissue. Interestingly, in a paper dealing with breast tumors in dogs, a similar 78 kDa band is clearly seen in the western blots shown, although the authors do not specifically referenced it in the text [47]. Interestingly its expression is highly regulated by MPA. Its apparent absence from non-neoplastic tissues suggests an application as a tumor marker.

It has been demonstrated that an imbalance in the expression of PR_A and PR_B may have important consequences in mouse normal mammary development. Transgenic mice overexpressing the PR_A isoform develop ductal hyperplasia, suggesting that an aberration in the mechanisms regulating the differential expression of the two isoforms can have major implications to mammary carcinogenesis [17]. In a recent study, Richer et al. [11] reported that 65 of the 94 progesterone-regulated genes are uniquely regulated by PR_B, only 4 uniquely by PR_A, and 25 by both isoforms. Also, an important set of progesterone-regulated genes have been related to mammary gland development and/or implicated in breast cancer such as the anti-apoptotic gene *Bcl-*X_L uniquely upregulated by PR_A.

All these data suggest that hormone independence, defined by the acquisition by a tumor of the ability to grow in the absence of previously required hormone, is independent of the ability to respond to endocrine therapy. These results are in agreement with a previous report of our laboratory in which PR are involved in hormone-independent growth [48]. We found no differences in PR receptor expression between progestin-dependent and responsive progestin-independent (R-PI) tumors, except for the fact that MPA preferentially down-regulated PR_B in the latter. The possible significance of this result remains to be elucidated. The lack of response to endocrine treatment in some progestin-independent tumors correlates with the expression of PR that poorly bind the ligand, suggesting genetic alterations or post-translational modifications. The possibility that these PR might be mutated or constitutively activated is the topic of ongoing research that will help to understand mechanisms related with hormone resistance.

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