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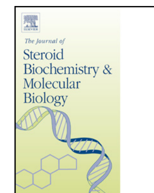

Progesterone-induced stimulation of mammary tumorigenesis is due to the progesterone metabolite, 5α-dihydroprogesterone (5αP) and can be suppressed by the 5α-reductase inhibitor, finasteride

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ABSTRACT

Progesterone has long been linked to breast cancer but its actual role as a cancer promoter has remained in dispute. Previous *in vitro* studies have shown that progesterone is converted to 5α-dihydroprogesterone (5αP) in breast tissue and human breast cell lines by the action of 5α-reductase, and that 5αP acts as a cancer-promoter hormone. Also studies with human breast cell lines in which the conversion of progesterone to 5αP is blocked by a 5α-reductase inhibitor, have shown that the *in vitro* stimulation in cell proliferation with progesterone treatments are not due to progesterone itself but to the metabolite 5αP. No similar *in vivo* study has been previously reported. The objective of the current studies was to determine in an *in vivo* mouse model if the presumptive progesterone-induced mammary tumorigenesis is due to the progesterone metabolite, 5αP. BALB/c mice were challenged with C4HD murine mammary cells, which have been shown to form tumors when treated with progesterone or the progestin, medroxyprogesterone acetate. Cells and mice were treated with various doses and combinations of progesterone, 5αP and/or the 5α-reductase inhibitor, finasteride, and the effects on cell proliferation and induction and growth of tumors were monitored. Hormone levels in serum and tumors were measured by specific RIA and ELISA tests. Proliferation of C4HD cells and induction and growth of tumors was stimulated by treatment with either progesterone or 5αP. The progesterone-induced stimulation was blocked by finasteride and reinstated by concomitant treatment with 5αP. The 5αP-induced tumors expressed high levels of ER, PR and ErbB-2. Hormone measurements showed significantly higher levels of 5αP in serum from mice with tumors than from mice without tumors, regardless of treatments, and 5αP levels were significantly higher (about 4-fold) in tumors than in respective sera, while progesterone levels did not differ between the compartments. The results indicate that the stimulation of C4HD tumor growth in BALB/c mice treated with progesterone is due to the progesterone metabolite 5αP formed at elevated levels in mammary cells as a result of the 5α-reductase action on progesterone. The results provide the first *in vivo* demonstration that stimulation of breast cell tumorigenesis and tumor growth accompanying progesterone treatment is due to the progesterone metabolite 5αP, and that breast tumorigenesis can be blocked with the 5α-reductase inhibitor, finasteride.

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1. Introduction

Breast cancer is the most frequent cancer among women worldwide, with nearly 1.4 million new cases and more than 450,000 deaths per year [1]. Progesterone has long been linked to breast cancer but its direct role is not clear. Some *in vitro* studies have shown that progesterone or progesterone-like analogs stimulate breast cell proliferation and cell cycle progression [2–4] and some human trials suggest that incidence of breast cancer is higher in women treated with estrogens plus progestins

Abbreviations: 5αP, 5α-dihydroprogesterone; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; MAPK, mitogen-activated protein kinase; MPA, medroxyprogesterone acetate; P, progesterone; PR, progesterone receptor; RIA, radioimmunoassay.

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than with estrogen alone [5–11]. Our previous studies have shown that breast tissues [12] and cell lines [13] readily convert progesterone to 5α-pregnanes such as 5α-pregnane-3,20-dione (5αP) and that 5αP significantly stimulates proliferation and detachment of human breast cells *in vitro* [12,14] as well as tumorigenesis and tumor growth *in vivo* [15], leading to the hypothesis that the breast cancer stimulating actions attributed to progesterone may be due to 5αP resulting from *in situ* metabolism of progesterone. Supporting the hypothesis are findings that incubated tumor breast tissues [12] and breast cancer cell lines [13] produce higher levels of 5αP than normal breast tissues and non-tumorigenic cell lines, due to increased levels of expression of 5α-reductase [13,16]. Moreover, recent *in vivo* studies [15] using a xenograft model involving immunosuppressed mice inoculated with human breast cells showed that the concentrations of 5αP were significantly higher in serum from mice with tumors than from tumor-free mice, and in tumors than in serum, whereas progesterone levels did not differ significantly. *In vitro* proof of principle that the pro-cancer actions attributed to progesterone may be due to raised levels of 5αP in the breast microenvironment, was provided by experiments in which 5α-reductase activity was blocked with a 5α-reductase inhibitor [17]. In this model, increases in proliferation and detachment of progesterone-treated human breast cell lines were suppressed by 5α-reductase inhibition which effectively blocked the conversion of progesterone to 5α-pregnanes. In turn, the suppression due to the 5α-reductase inhibitor was abrogated by concomitant treatment with 5αP, showing that the effects were due to the 5α-reduced metabolite 5αP, not to progesterone.

The objective of the current studies was to determine if *in vivo* progesterone-induced mammary tumorigenesis results from progesterone itself or from the metabolite 5αP. For this purpose we employed the 5α-reductase inhibitor finasteride along with 5αP in the well characterized model of hormonal tumorigenesis in which either progesterone [18] or the synthetic progestin, medroxyprogesterone acetate (MPA) [19–21], induces C4HD murine cells to form mammary adenocarcinomas in virgin female BALB/c mice.

Here we show for the first time that initiation of C4HD tumorigenesis and stimulation of tumor growth observed in progesterone-treated mice is dose-dependently suppressed by the 5α-reductase inhibitor, finasteride, and that the suppression is abrogated by concomitant treatment with 5αP. The studies provide the first *in vivo* proof-of-principle that the progesterone metabolite, 5αP, not progesterone, is the tumorigenic factor in mammary carcinoma resulting from progesterone treatment. The findings also demonstrate for the first time that 5α-reductase inhibition can block onset and growth of progesterone-sensitive breast tumors.

2. Materials and methods

2.1. Reagents and supplies

5α-Pregnane-3,20-dione (5αP), progesterone and finasteride were purchased from Steraloids Inc. (Newport, RI, USA). Fetal calf serum (FCS) was purchased from Gen (Buenos Aires, Argentina), [^{3}H]thymidine (methyl-^{3}H-thymidine; 20 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Other chemicals and solvents were of appropriate grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. C4HD tumor

The hormone-dependent ductal tumor line C4HD originated in BALB/c mice treated with 40 mg of MPA every 3 months for 1 year and was maintained by serial transplantation in animals treated with 40 mg of MPA given subcutaneously in the flank opposite to the tumor inoculums as previously described [20,22,23]. The C4HD tumor line is of ductal origin and expresses PR and ER and overexpresses ErbB-2 [21,23]

2.3. In vitro *proliferation studies*

For the *in vitro* studies, primary cultures of epithelial cells obtained from C4HD tumors were grown for 24–48 h in culture flasks with DMEM:F12 (without phenol red) containing 10% FCS. The cells were then trypsinized and plated in a 96-well plate at 10^4 cells/well in test medium (DMEM:F12 containing 5% charcoal-stripped fetal calf serum) and treated with vehicle (control; 0.01% ethanol in test medium) or vehicle containing 10^{-6} M test substances (progesterone, 5αP, finasteride). Cell proliferation was assessed by hemocytometer counts and by incorporation of [^{3}H]-thymidine (1 μCi/well) which had been previously demonstrated to correlate with the number of cells/well [24].

2.4. In vivo *experiments*

Experiments were carried out on virgin female BALB/c mice raised at the Institute of Biology and Experimental Medicine (IBYME) of Buenos Aires. All animal studies were conducted in accordance with the highest standards of animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals and these procedures were approved by the IBYME Animal Research Committee. Mice were inoculated subcutaneously (s.c.) into the left flank with a fragment of C4HD tumor (1 mm^3) and were injected with a suspension of either 5αP or progesterone, alone or in combination with finasteride. The suspensions were prepared under sterile conditions in sterile-filtered vehicle (0.9% NaCl in double distilled H_2O, containing 0.1% double distilled ethanol and 0.05% Tween 80) [15]; they were stored at 4 °C prior to use and were administered s.c. (150 μl/injection) in the right flank using a 1.0 cc syringe with gauge #23 needle. Animals were monitored for general health, tumor development and body weight variation. Tumor length (*L*) and width (*W*) were measured three times a week with a Vernier caliper and tumor volume (mm^3) was calculated as (LxW^2)/2. Tissues were fixed in 10% buffered formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Proliferation was assessed by counting mitotic figures per high power field.

2.5. Immunohistochemistry and immunofluorescence

Sections (10 μm) of formalin-fixed paraffin-embedded tumors were incubated in 10 mM sodium citrate buffer pH 6 for 50 min at 92 °C for antigen retrieval, and treated as described [25]. Briefly, they were incubated with primary antibodies of progesterone receptor (PR; H-190, dilution 1:200) and estrogen receptor α (ERα; MC-20, dilution 1:1000) or with control rabbit immunoglobulin G overnight at 4 °C. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sections were subsequently incubated with the polydetector HRP system (Bio SB, Santa Barbara, CA, USA) and developed in 3-3′-diaminobenzidine tetrahydrochloride. Immunostainings were run with known positive and negative tumor controls and were blindly evaluated by two pathologists. ER and PR were scored as described [26]. Localization of ErbB-2 was basically as described [25]. Briefly, sections were subjected to antigen retrieval as above and then were incubated with rabbit polyclonal (C-18) and goat anti-rabbit IgG-Alexa 488 (Molecular Probes, Eugene, OR, USA) as secondary antibodies. Negative controls were carried out using PBS instead of primary antibodies, or 5X competitive peptide (Santa Cruz Biotechnology) and nuclei

were stained with propidium iodide. Sections were viewed using a Nikon Eclipse E800 confocal laser microscopy system. ErbB-2 was scored according to the American Society of Clinical Oncology/ College of American Pathologists' (ASCO/CAP) guidelines [27].

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from primary cultures of epithelial cells of C4HD tumors [20] using TRIzol® reagent (Invitrogen) following the manufacturer's protocol. Complementary DNA (cDNA) was obtained from 1.0 μg of total RNA using 200 U Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen) in a total volume of 20 μl following the manufacturers protocol. To exclude amplification of genomic DNA, experiments included conditions in which the reverse transcriptase enzyme was omitted. The transcribed cDNA fragments were amplified in a 50 μl reaction volume with 100 μmol/l each dNTP, 0.4 μmol/l each primer, and 1 U Taq DNA polymerase (TaqUBA, BIONAC, Buenos Aires, Argentina). Primers for mouse 5αR1 were as follows: (F) 5′-CCT GGT GTG TCC TGA AAG GT-3′ and (R) 5′- GGG CAG TTT GGT CCT TCA TA-3′ and for mouse 5αR2 were: (F) 5′-TTT CCT GGG CGA GAT TAT TG-3′ and (R) 5′- TGA ATG GAA TGA GGG CTT TC-3′. Amplification was performed in an Applied Biosystems Veriti thermal cycler (Applied Biosystems, Austin, TX, USA). The PCR products were separated by electrophoresis on a 1.5% or 2.5% agarose gel, and visualized by SYBR safe (Life Technology).

2.7. Measurements of hormones

Synthesis of [^{3}H]-5αP and 5αP-BSA conjugate and preparation of antisera. [9,11,12-^{3}H]5αP was prepared by oxidation of [9,11,12-^{3}H]5α-pregnan-3α-ol-20-one as described [28]. Purification of ^{3}H-labeled 5αP was by HPLC (C_{18} column and methanol: water, 3:1) and TLC (Fisherbrand silica gel GF; three runs in hexane:ethyl acetate, 5:2). Preparation of 5α-pregnane-3,20-dione-11α-hemisuccinate-BSA (5αP-BSA conjugate) was as described [15] and purity of the conjugate was confirmed by HPLC in the solvent system acetonitrile:H_2O:trifluoroacetic acid (45:55:0.1) using a Vydac C_4 column (4.6 × 250 mm) for protein with particle size 5 μm and pore diameter 300 A. Preparation of 5αP and progesterone antisera for RIAs was as described [15].

Steroid extraction and chromatographic separation. Steroids were extracted from mouse sera and tumors and separated by thin layer chromatography as described [15].

Radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). The RIAs for progesterone and 5αP were basically as described [15] using specific antisera and scintillation spectrometry (Beckman-Coulter LS 6500 Scintillation Counter). 5αP levels in some serum and tumor samples were also measured by a 5αP-ELISA and all progesterone determinations were by ELISA (Diagnostics Biochem Canada, London, Ontario, Canada). For comparison purposes, concentrations were standardized to ng/ml for serum and ng/g for tumors and it was assumed that these two measures represent a reasonable equivalence [15].

2.8. Statistical analyses

Statistical analyses were carried out with GraphPad Instat software (Graph-Pad Software, Inc.,-San Diego, CA, USA). Results are presented as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* multiple comparison tests, or by unpaired Student's *t*-test, as appropriate, with $P < 0.05$ considered statistically significant.

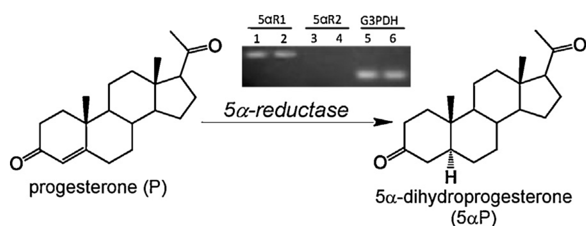

Fig. 1. Conversion of progesterone (P) to 5α-dihydroprogesterone (5αP) by the actions of 5α-reductase. Gel shows expression of 5α-reductase type 1 (5αR1; lanes 1 and 2), undetected expression of 5α-reductase type 2 (5αR2; lanes 3 and 4) and glycerol-3-phosphate dehydrogenase (G3PDH; lanes 5 and 6; internal control) in C4HD cells.

3. Results

3.1. C4HD cells express 5α-reductase type 1.

Conversion of progesterone to 5αP is catalyzed by 5α-reductase. RT-PCR assays showed that 5α-reductase type 1 is expressed in C4HD cells, whereas expression of type 2 was not observed (Fig. 1).

3.2. In vitro *inhibition of progesterone-induced cell proliferation by finasteride is abrogated by 5αP*

To determine the *in vitro* response of C4HD cells to progesterone, 5αP and the 5α-reductase inhibitor, finasteride, proliferation assays were performed using cell counts (Fig. 2A) and [^{3}H] thymidine incorporation (Fig. 2B). C4HD proliferation was significantly stimulated by either progesterone or 5αP; the progesterone-induced stimulation was significantly suppressed by finasteride and the suppression was abrogated by concomitant treatment with 5αP.

3.3. 5αP stimulates C4HD tumorigenesis and tumor growth

To determine the potential of the progesterone metabolite 5αP to stimulate tumorigenesis, mice inoculated with C4HD cells were treated (on day 0) with 5αP at several doses (Fig. 3). The results showed that 5αP had a dose-dependent stimulatory effect on tumor development. At termination (day 30) no tumors had developed in the vehicle-treated mice, small tumors (20–30 mm^3) were present in two out of six (2/6) mice treated with 4 mg 5αP, tumors at 214 ± 36 mm^3 were present in five out of six (5/6) mice treated with 10 mg 5αP, and tumors at 456 ± 81 mm^3 were present in six out of six mice treated with 40 mg of 5αP. At termination of experiments, mice from all treatment groups appeared to be in good body condition, including normal locomotion, weight gain, general health, and no evidence of kidney, heart or lung toxicity. Histopathological analyses of tumors from 5αP treated mice showed ductal mammary carcinomas (Fig. 4A-i and ii) with high anisokaryosis and active proliferation with a count of mitotic figures per high powered field between 3 and 5 (Fig. 4A–iii), in line with a high proliferative growth rate tumor. Examination of the liver revealed the presence of localized metastases (Fig. 4A–iv). Immunohistochemistry showed that C4HD tumors had high levels of ER (95–98%, Alred Score 8, Fig. 4B–i) and PR (90–95%, Alred Score 8, Fig. 4B–ii) and over-expressed ErbB-2, at 30% of cells with intense staining (Fig. 4C).

3.4. In vivo *inhibition of progesterone-induced tumorigenesis by finasteride is abrogated by 5αP*

To determine the effect of 5α-reductase inhibition on progesterone-induced tumorigenesis, C4HD-implanted mice were treated with either vehicle, progesterone or progesterone plus

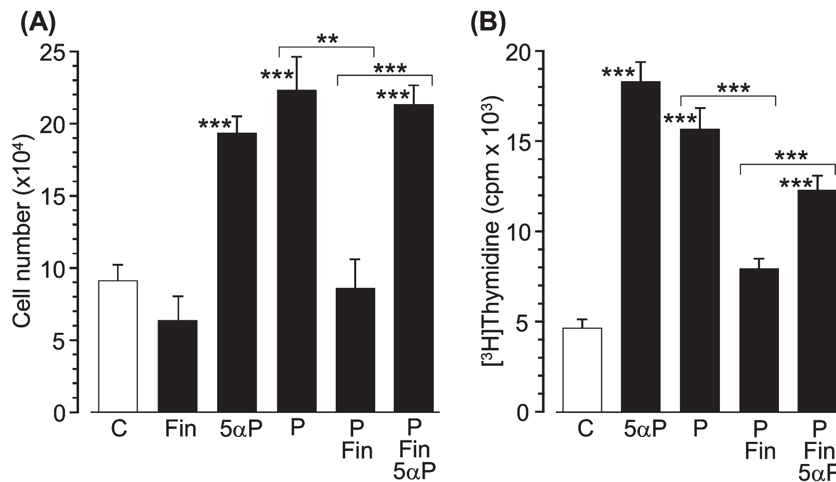

Fig. 2. *In vitro* effects of progesterone (P), 5αP and finasteride (Fin) on proliferation of C4HD cells as determined by (A) cell counts and (B) [^{3}H]thymidine incorporation. Cells were allowed to attach for 24 h and then were treated for 96 h (A) or 48 h (B) without (C; control) or with 10^{-6} M of either Fin, P, 5αP, P + Fin or P + Fin + 5αP. Data are from one of two sets of separate experiments with essentially similar results and are presented as the means and SE of 6 replicates. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control and between the indicated comparisons.

finasteride (Fig. 5A). Progesterone resulted in significant stimulation of tumorigenesis and the progesterone-induced stimulation was significantly suppressed by finasteride in a dose-dependent manner. To determine if 5αP can abrogate the finasteride-induced suppression of tumorigenesis and tumor growth, mice were treated with either vehicle, progesterone, progesterone + finasteride, or progesterone + finasteride + 5αP (Fig. 5B). The results showed that stimulation of tumorigenesis by progesterone was significantly suppressed by the 5α-reductase inhibitor, and that concomitant treatment with 5αP abrogated the suppression and restored tumor stimulation to levels that were not significantly different from those of progesterone without finasteride.

3.5. 5αP concentrations are high in tumors

Hormone measurements at the end of experiments showed that 5αP levels were significantly higher in tumors than in respective sera and in serum from tumor-bearing mice than from tumor-free mice (Fig. 6A). The progesterone concentrations did not differ significantly between serum and tumors (Fig. 6A and B).

4. Discussion

Progesterone has long been linked to breast cancer but its direct role is not clear. Our studies have shown that human breast cell lines and tissues can convert progesterone to 5αP by the action of 5α-reductase [12,13,17] and that 5αP acts as a breast cancer promoter hormone [12,14,15,29,30]. *In vitro* studies using a 5α-reductase inhibitor [17] had shown that stimulation in proliferation and detachment of human breast cells treated with progesterone is due to the progesterone metabolite, 5αP. The current studies using a syngeneic mouse-mammary cell model provide the first *in vivo* evidence that the metabolite, 5αP, rather than progesterone, is responsible for the stimulation of tumorigenesis when mammary cells/tissues are exposed to progesterone. In addition, the findings provide the first *in vivo* evidence that 5α-reductase inhibitors can inhibit breast tumorigenesis. The 5αP-induced C4HD tumors are highly proliferative, PR/ER-positive, ErbB-2 overexpressing tumors, comparable to MPA-induced tumors [21,23,25], suggesting that they could serve as a model for human luminal PR/ER-positive, ErbB-2-expressing breast cancers.

The enzyme responsible for the conversion of 4-ene steroids to 5α-reduced steroids is 4-ene-steroid 5α-reductase, known as 5α-reductase [31]. There are two isoforms of 5α-reductase, namely type 1 (encoded by the *SRD5A1* gene) and type 2 (encoded by the *SRD5A2* gene) [31,32]. Type 2 isoform is considered to be the predominant form in human prostate, epididymis and hair follicles [33], and to be primarily responsible for catalyzing reduction of testosterone to 5α-dihydrotestosterone. On the other hand, both isoforms are responsible for the reduction of progesterone to 5αP [33], and in human breast tissues [16,34] and in human breast cell lines [13] 5α-reductase type 1 has been demonstrated to be the main isoform. Also, women with steroid 5α-reductase type 2 deficiency have normal concentrations of 5αP [35], implying that 5αP may be derived principally from 5α-reductase type 1 action. The present study also showed higher levels of expression

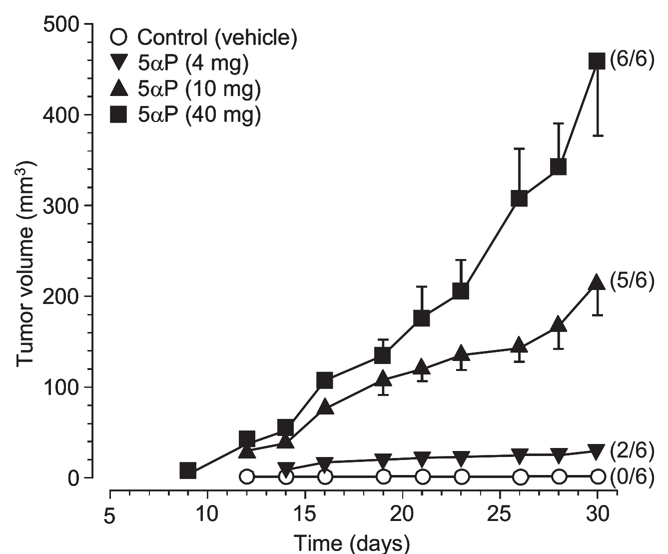

Fig. 3. Effect of 5αP on C4HD tumorigenesis and tumor growth. Four groups of 6 mice each were implanted with C4HD cells and were treated with either vehicle (control) or 5αP (4, 10, or 40 mg) on day 0. Values in brackets denote number of mice, out of six, with tumors for each treatment group. Data points and error bars show the mean tumor volumes and SE.

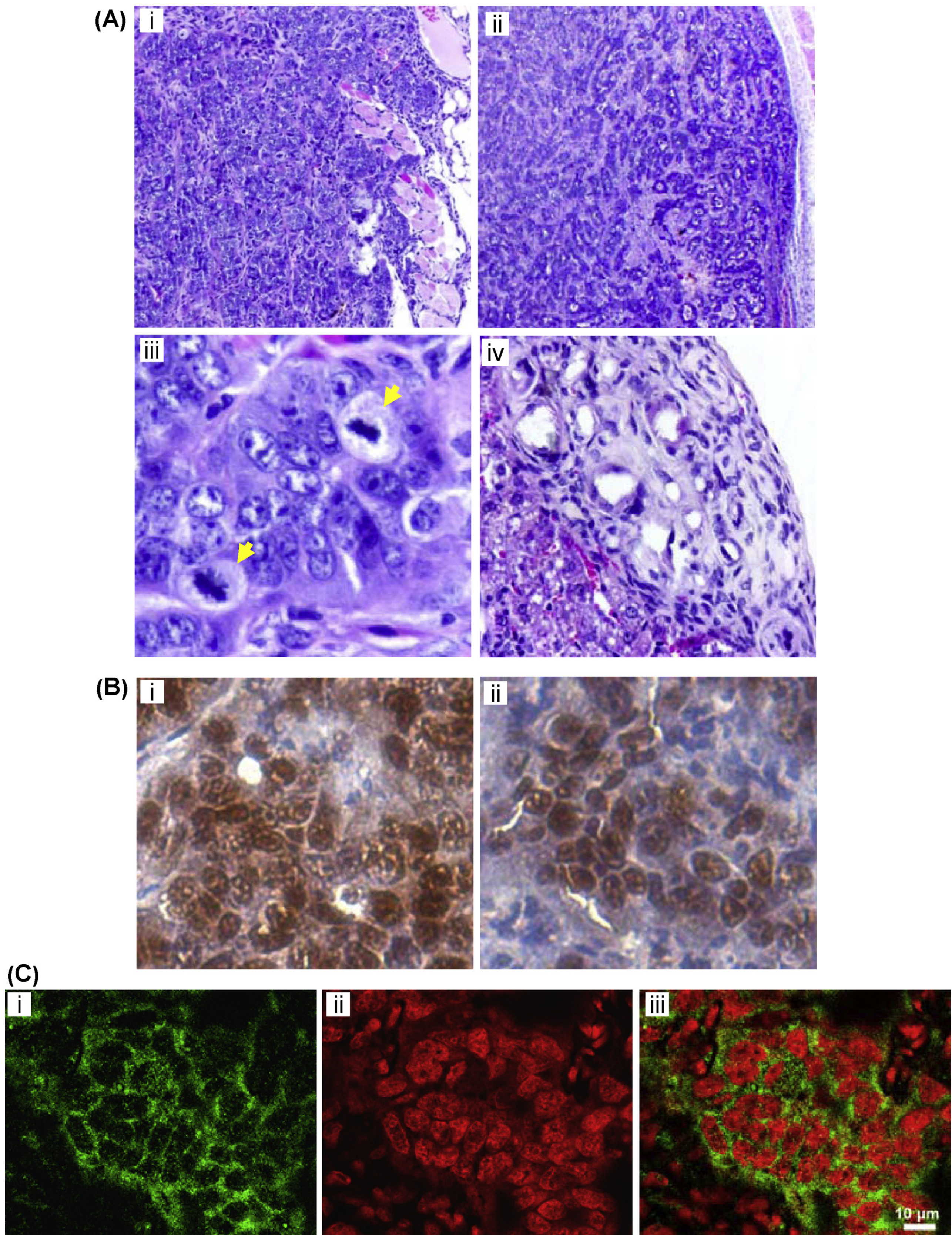

Fig. 4. Histopathology and immunohistochemistry of 5αP-induced C4HD tumors. (A-i) and (A–ii) solid undifferentiated and moderately differentiated mammary tumors, respectively (original magnification ×100), (A–iii) mitotic figures (arrows; original magnification ×400), (A–iv)) metastasis to liver (original magnification x100). Immunohistochemical demonstration of presence of ER (B–i) and PR (B–ii) (original magnification ×400). (C) Localization of ErbB-2 by immunofluorescence and confocal microscopy in a tumor section: (C–i) ErbB-2 (green), (C–ii) nuclei (red; propidium iodide stain), (C–iii) composite of C–i and C–ii.

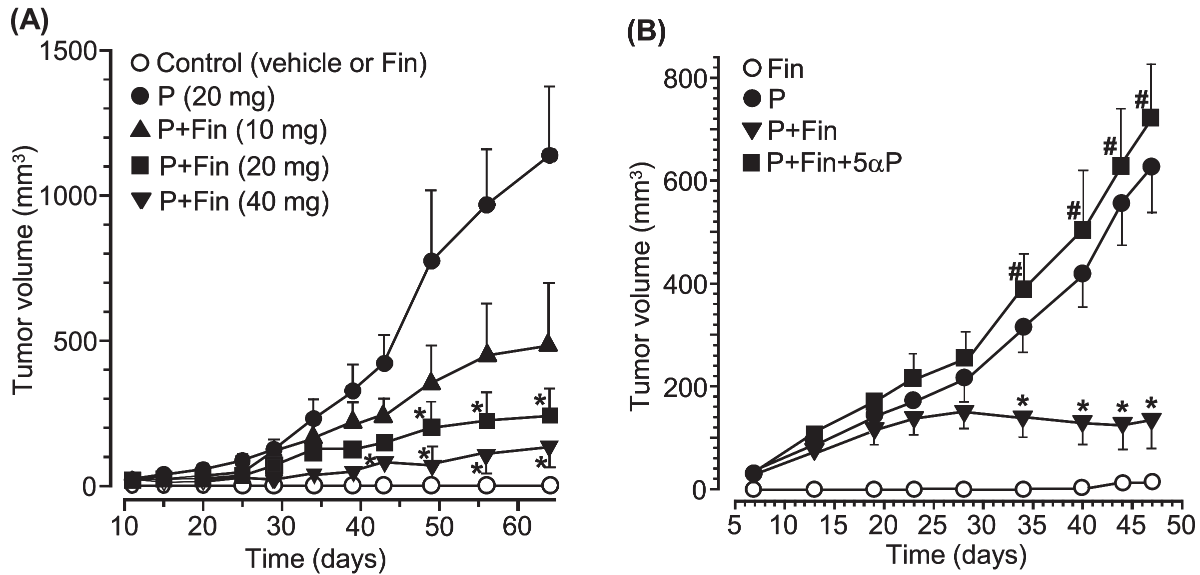

Fig. 5. Effects of progesterone (P), finasteride (Fin) and 5αP on C4HD tumor growth. (A) Effect of finasteride on progesterone-induced C4HD tumorigenesis and tumor growth. Five groups of 6 mice each were implanted with C4HD cells (day 0) and received a single treatment of either vehicle or Fin (20 mg; control), P (20 mg), or P (20 mg) + Fin (at 10, 20, or 40 mg) on day -1. Data points and error bars show the mean tumor volumes and SE. *, significantly different from progesterone alone on the respective days at $P < 0.05$. (B) Effect of 5αP on finasteride-induced suppression of tumor growth. Four groups of 7 mice each were implanted with C4HD cells and were treated with either Fin (10 mg), P (10 mg), P + Fin (10 mg each), or P + Fin + 5αP (10 mg each) on days 0, 15, and 29. Data points and error bars show the mean tumor volumes (mm^3) and SE. (From one of three experiments with similar results). *, Significantly smaller than progesterone alone on the respective days at $P < 0.05$. #, significantly greater than P + Fin on the respective days at $P < 0.05$.

of 5α-reductase type 1 than type 2 in C4HD murine mammary cells.

Finasteride is a 4-azasteroid and analogue of testosterone that acts as a competitive inhibitor of 5α-reductase types 1 and 2 isoforms [36]. While the mechanism of action of finasteride in human male tissues is based on its preferential inhibition of the type 2 isozyme [33], both isoforms of 5α-reductase are inhibited by finasteride in rodents [37]. The current studies, showing that finasteride inhibits stimulation of proliferation (*in vitro*) and tumorigenesis (*in vivo*) of murine C4HD cells treated with progesterone, indicate effective 5α-reductase inhibition by finasteride in our C4HD-mouse model. The inhibition of C4HD tumors by finasteride is the first *in vivo* demonstration suggesting that PR-positive progesterone-sensitive human breast tumor formation and/or growth can be blocked by 5α-reductase inhibitors.

Studies of progesterone metabolism have demonstrated that significantly more 5αP is produced by tumorous breast tissues and tumorigenic human breast cell lines than by non-tumorous tissues and non-tumorigenic cells lines [12,13], and that these higher levels of 5αP are due to significantly elevated levels of 5α-reductase mRNA expression [13,16]. In the current studies, measurements of hormone levels at termination (about 4–10 weeks after treatment) show that 5αP levels were significantly higher in tumors than in respective sera, and in serum from mice with tumors than in serum from mice without tumors. The elevated 5αP levels were evident at termination in mice with tumors regardless of prior treatment, indicating that the tumors are a primary source of 5αP in the mammary microenvironment. The observations that progesterone levels were about the same in tumors and serum and did not differ significantly between serum from mice with tumors and without tumors, indicate a constant supply of progesterone for conversion to 5αP. Similar results have been observed in xenograft studies [15] which showed that 5αP levels were significantly higher in human breast cell line tumors than in serum, as well as significantly higher than the progesterone levels, regardless of prior treatment. In addition, the dose-dependent finasteride-induced inhibition of tumor development indicates a correlation between tumorigenesis and degree of 5α-reductase-catalyzed conversion of progesterone to 5αP.

The cancer-promoting action mechanisms of 5αP have been investigated in human breast cell lines. Receptors for 5αP (5αPR) have been identified in plasma membranes of both ER/PR-positive (MCF-7) [28] and ER/PR-negative (MCF-10A) [38] human breast cell lines, with no evidence of 5αP binding in nuclear and cytosolic fractions. Plasma membrane-bound 5αP is not displaced by progesterone, estradiol, androgens or corticosteroids, indicating high specificity of the 5αPR. The 5αP binding sites exhibit high affinity with dissociation constants (K_d) of 4.5 and 19 nM and receptor densities of about 486 and 682 fmol/mg membrane protein for MCF-7 and MCF-10A cells, respectively [28,38]. Progesterone and estradiol bound to their respective receptors

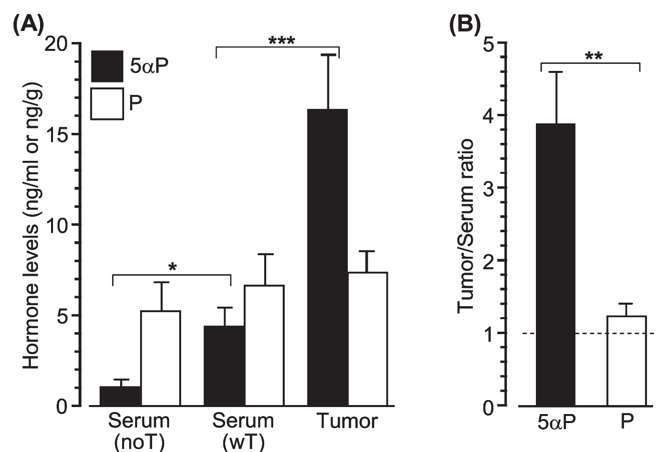

Fig. 6. 5αP and progesterone (P) levels in serum and tumors. (A) 5αP and P levels in serum from mice without (noT) and with (wT) tumors, and in the tumors. Hormone levels are presented as ng/ml for serum (noT, $n = 6$; wT, $n = 15$) and as ng/g for tumors ($n = 12$). (B) Tumor:serum ratio of 5αP and P concentrations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for the indicated comparisons.

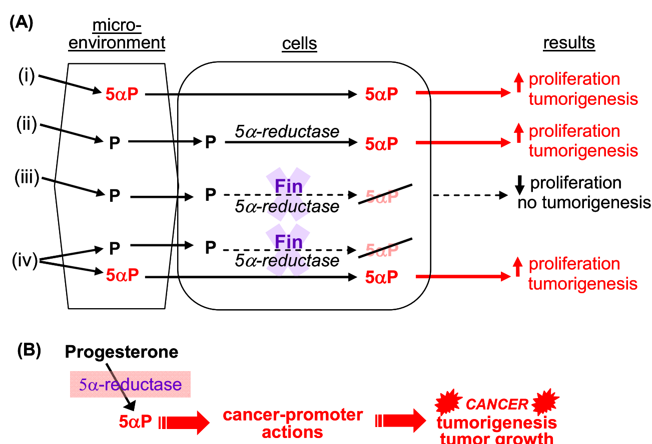

Fig. 7. Explanation of treatment results and general scheme showing that progesterone (P) acts as pre-hormone and the metabolite, 5αP, is the active cancer-inducing hormone. (A) Diagrammatic explanation of treatments and results: (i) treatment with 5αP results in stimulation of C4HD proliferation and tumorigenesis; (ii) treatment with progesterone provides more substrate for conversion to 5αP by the C4HD cells, resulting in increased levels of 5αP in the microenvironment and, in turn, 5αP-induced stimulation of proliferation and tumorigenesis; (iii) administration of the 5α-reductase inhibitor, finasteride (Fin), blocks conversion of administered progesterone to 5αP, and in the absence of 5αP cell proliferation and tumorigenesis are suppressed; (iv) administration of 5αP in the presence of progesterone + Fin obviates cellular conversion of progesterone to 5αP and provides a direct supply of 5αP which restores the stimulation. (B) General scheme showing that cancer-inducing actions of progesterone treatment are due to the progesterone metabolite, 5αP, which can act as a cancer-promoter hormone by mechanisms explained in [15,29].

are not displaced by 5αP, indicating that the actions of 5αP are not conveyed *via* PR or ER. Additional evidence that 5αP action is not *via* PR (or ER) comes from observations that 5αP-induced stimulation of cell proliferation and detachment is essentially the same in PR/ER-negative (MCF-10A, MDA-MB-231), progesterone/estrogen-unresponsive human breast cells as in PR/ER-positive (MCF-7, T47D) cells [12,14,38]. In terms of mechanisms of action, studies with human breast cell lines have shown that binding of 5αP to its receptor results in activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk1/2) [[29]; and unpublished results], increased Bcl-2/Bax expression ratio [14] and actin depolymerization [30], and decreased expression of adhesion plaque associated vinculin [30], leading to decreased apoptosis and increased mitosis and cell detachment [14,30]. Studies with the C4HD cell model have shown that MPA-induced stimulation of proliferation and tumorigenesis likewise involves activation of the MAKP/Erk1/2 pathway [24,39,40]. We suggest that 5αP may act in a similar manner on C4HD cells, and it would be of interest to conduct studies to determine presence of 5αP receptors and 5αP effect on MAPK signaling in C4HD cells.

Although previous studies [18] had shown that progesterone induces mammary carcinoma in BALB/c female mice, the majority of studies with the C4HD cells-BALB/c mouse model have involved the synthetic progestin MPA and have shown that MPA stimulates proliferation and tumorigenesis. In light of the current findings that the progesterone metabolite 5αP is the stimulatory tumorigenic factor in progesterone-treated C4HD cells *in vitro* and *in vivo*, how might the previous tumorigenic effects of MPA be interpreted? No metabolites of MPA with a 3-keto, 5α-reduced A ring configuration similar to 5αP have been identified [41] and our data do not permit the suggestion that the action of MPA on C4HD cells, in inducing tumorigenesis, is *via* 5αP. However, it is conceivable that MPA, or one or more of its metabolite(s), may act as ligand(s) for 5αP binding sites, and/or another as yet unidentified receptor, linked to a signaling pathway similarly to 5αPR in human breast cell lines.

5. Summary and conclusions

Fig. 7 summarizes the current findings and provides a general scheme explaining that 5αP is responsible for breast cancer induction, and that 5α-reductase inhibition can block presumptive progesterone-induced breast tumorigenesis.

In conclusion, this study provides the first *in vivo* evidence that progesterone-associated stimulation of breast tumorigenesis is not due to progesterone itself but to the metabolite 5αP. Using the PR/ER-positive and ErbB-2-positive murine breast tumor model, C4HD in BALB/c mice, we showed that tumor initiation/growth in progesterone treated mice can be blocked by the 5α-reductase inhibitor, finasteride. The evidence indicates that 5α-reductase inhibitors might be used to prevent onset and/or growth of human breast cancers in general and ErbB-2-positive and hormone receptor-positive breast cancer in particular. In addition, the observed high concentrations of 5αP in tumors suggest potential new biometric diagnostics for the early detection of breast cancers. Overall, the findings have added to our understanding of the role of progesterone metabolites in breast tumorigenesis, and suggest new approaches to breast cancer diagnostics and therapeutics.

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