Progestin-Dependent Progression of Human Breast Tumor Xenografts: A Novel Model for Evaluating Antitumor Therapeutics

Yayun Liang,1 Cynthia Besch-Williford,2 Rolf A. Brekken,4 and Salman M. Hyder1,3

1Dalton Cardiovascular Research Center, 2Veterinary Pathobiology, and 3Department of Biomedical Sciences, University of Missouri, Columbia, Missouri; and 4Hamon Center for Therapeutic Oncology Research and Department of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas

Abstract

Recent clinical trials indicate that synthetic progestins may stimulate progression of breast cancer in postmenopausal women, a result that is consistent with studies in chemically-induced breast cancer models in rodents. However, progestin-dependent progression of breast cancer tumor xenografts has not been shown. This study shows that xenografts obtained from BT-474 and T47-D human breast cancer cells without Matrigel in estrogen-supplemented nude mice begin to regress within days after tumor cell inoculation. However, their growth is resumed if animals are supplemented with progesterone. The antiprogestin RU–486 blocks progestin stimulation of growth, indicating involvement of progesterone receptors. Exposure of xenografts to medroxyprogesterone acetate, a synthetic progestin used in postmenopausal hormone replacement therapy and oral contraception, also stimulates growth of regressing xenograft tumors. Tumor progression is dependent on expression of vascular endothelial growth factor (VEGF); growth of progestin-dependent tumors is blocked by inhibiting synthesis of VEGF or VEGF activity using a monoclonal anti-VEGF antibody (2C3) or by treatment with PRIMA-1, a small-molecule compound that reactivates mutant p53 into a functional protein and blocks VEGF production. These results suggest a possible model system for screening potential therapeutic agents for their ability to prevent or inhibit progestin-dependent human breast tumors. Such a model could potentially be used to screen for safer antiprogestins, antiangiogenic agents, or for compounds that reactivate mutant p53 and prevent progestin-dependent progression of breast disease. [Cancer Res 2007;67(20):9929–36]

Introduction

The role of natural and synthetic progestins in the proliferation of breast tumor cells is controversial (1, 2). Initial studies suggested that progestins have antiproliferative effects in breast cancer (3). However, those results contradict recent in vivo and in vitro studies suggesting that progestins stimulate proliferation of normal and neoplastic breast cells in various species (4–7). It is likely that the growth-stimulating effects of exogenous progestins are mediated by the progesterone receptor (PR), which is required for growth of rodent mammary gland and 7,12-dimethylbenz(a)anthracene (DMBA)-induced tumors (7, 8). Several human clinical trials show that the risk of breast cancer is higher in women on combined estrogen-progestin hormone replacement therapy (HRT) than in women on HRT that only contains estrogen (9–11). These results have stimulated additional research into the mechanism by which progestins might stimulate growth of human breast tumors. However, the mechanistic basis of this effect has not yet been resolved, at least in part because there are few relevant animal models suitable for addressing this question. Furthermore, the studies conducted in model systems to date, mostly in rodents, have yielded conflicting results (1–3). Consequently, there is an urgent need for an improved animal model involving human breast cancer cells for studying progestin-dependent proliferation of human tumor cells.

This study proposes a human-mouse hybrid model system based on progestin-dependent growth of human tumor xenografts in nude mice. This model uses human T47-D and BT-474 breast cancer cells, which express PR and mutant p53, to generate xenograft tumors in nude mice supplemented with exogenous hormones. Tumor cells in most models are cojected with Matrigel and require exogenous estrogen for optimal growth. However, in this model, tumor cells are injected in the absence of Matrigel but in the presence of exogenous estrogen, and these tumors rapidly regress after initial growth. They regain the capacity to proliferate in the presence of exogenous progestin that can be blocked by inclusion of the antiprogestin RU–486. We have previously shown that progestins induce the vascular endothelial growth factor (VEGF) in breast cancer cells that contain progesterone receptor and mutant p53 protein but not in cells that contain the wild-type p53 protein (12, 13). This model can be used to test the efficacy of antitumor therapeutic agents in blocking progression of progestin-dependent breast tumors. A proof-of-principle experiment conducted here yielded positive results: RU–486, an anti-VEGF antibody (2C3), or PRIMA-1 (p53-dependent reactivation and induction of massive apoptosis), a small-molecular-weight compound that reactivates mutant p53 protein into an active protein (14) and blocks the production of progestin-induced VEGF (12), prevented progestin-stimulated growth of the xenograft tumors. Thus, this study provides evidence that the xenograft tumor model described here is suitable for testing antiprogestins or antiangiogenic compounds for their ability to block progression of hormone-dependent human breast cancer.

Materials and Methods

Cell lines and culture. The estrogen and progesterone receptor containing breast cancer cell lines BT-474, T47-D, and MCF-7 were from the American Type Culture Collection (ATCC) and grown in phenol-red-free DMEM:F12 medium (Invitrogen Corporation and Life Technologies) supplemented with 10% fetal bovine serum (JRH Biosciences) in
100 × 20-mm tissue culture dishes. Cells were harvested using 0.05% trypsin-EDTA (Invitrogen Corporation and Life Technologies).

Reagents. Sixty-day release pellets containing 17-β-estradiol (1.7 mg), progesterone (10 mg), medroxyprogesterone acetate (MPA; 10 mg), milepiristine (RU486; 25 mg), and placebo pellets were from Innovative Research of America. Anti-VEGF antibody 2C3 that prevents binding of VEGF to VEGF receptor 2 (VEGFR2, KDR/flk-1) was raised against recombinant human VEGF as described previously (15). C44, a mouse isotype-matched control monoclonal antibody specific for colchicine was from ATCC. PRIMA-1 (p53-reactivation and induction of massive apoptosis) was from Tocris.

Progestin-dependent growth of human breast xenograft tumors. Female athymic nu/nu nude mice, 5 to 6 weeks old (18–22 g), were purchased from Harlan Sprague-Dawley, Inc. Mice were housed in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current federal regulations and standards. Nude mice were inoculated with 17-β-estradiol pellets 24 to 48 h before implantation of BT-474 or T47-D cells. Cells were harvested by trypsinization and washed twice with DMEM/F12 medium, and cell pellets were resuspended (1 × 10^6 cells in 0.15 mL) in DMEM/F12 medium and injected (s.c.) into the left and right flanks of each mouse. Tumor volume was measured every 3 days using a digital caliper and calculated using the formula (L × W × H) / 2 as previously described (16). Tumors began to regress after reaching 60 to 100 mm^3 (in size (~6–10 days). When tumor volume had decreased >50%, mice were inoculated with a progesterone or MPA pellet. When desired, progesterone or MPA pellets were removed under anesthesia after tumor volume was restored or nearly restored to pre-regression size.

Inhibition of progestin-dependent breast tumor growth. Tumor-bearing mice, whose tumors had undergone regression as described above, were inoculated with progesterone pellets and assigned to four groups of six mice each: control (placebo), progesterin alone, progesterin + anti-VEGF 2C3, and progesterin + control antibody C44. Treatment with either 2C3 or C44 antibodies (100 µg/mouse, ip) or 0.2 mL PBS was thrice a week. Tumors were measured every 3 days with a digital caliper, and tumor volumes were calculated using the formula (L × W × H) / 2 (ref. 16). At the end of the treatment or experiment, animals were sacrificed, and tumors were harvested and weighed. Fresh tumor tissue was immediately placed in 4% paraformaldehyde for immunohistochemical analysis (IHC) or frozen in liquid nitrogen for future analysis.

PRIMA-1 treatment followed the same procedure, except that tumor-bearing mice were treated with 50 mg/kg PRIMA-1 via tail vein injection for the indicated number of days (see figure legends; ref. 14). Tumor tissue was excised and analyzed by IHC as described below.

Immunohistochemical analysis. Immunohistochemical analysis was carried out for VEGF, factor VIII, ERα, and PR. Tumor tissue was fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. Sections of 5 µm were mounted onto ProbeOn Plus microscope slides (Fisher Scientific Inc.), stained with H&E, and examined for cellularity by light microscopy. For immunohistochemical analysis, sections were dehydrated in xylene, rehydrated through graded concentrations of ethanol, rinsed in distilled water, and, if necessary, stored in PBS at 4°C until use. Sections were subjected to heat-induced epitope retrieval in 10 mmol/L citrate buffer (pH 6.0; VEGF, ERα, PR) or proteinase K [20 µg/mL in TE buffer (pH 8.0); factor VIII antigen]. Slides were treated with 3% hydrogen peroxide in absolute methanol (to inactivate endogenous peroxidase activity), washed in 3 × PBS, incubated in blocking buffer with 5% bovine serum albumin for 20 min, and treated with polyclonal antibody at room temperature for 60 min. Antibodies and dilutions were as follows: anti-VEGF antibody [1:200 dilution of a rabbit anti-VEGF polyclonal antibody (sc-152); anti-ERα [1:300 dilution of a rocket anti-ERα polyclonal antibody (sc-542); Santa Cruz Biotechnology, Inc.]; anti-PR [1:50 dilution of a rabbit PR polyclonal antibody (A0098)]; and anti-factor VIII antibody [1:400 dilution of a rabbit anti-factor VIII polyclonal antibody (A0082); DAKO]. Sections were washed and sequentially incubated with secondary antibody (biotinylated swine anti-mouse IgG or biotinylated swine anti-rabbit IgG; DAKO) and streptavidin-linked horseradish peroxidase product (DAKO) for 30 min, also at room temperature. Alternatively, some sections were incubated with EnVision+, a horseradish peroxidase–labeled polymer conjugated with anti-rabbit antibodies (DAKO). Bound antibodies were visualized with 3,3′-diaminobenzidine tetrahydrochloride (0.05% with 0.015% H2O2 in PBS; DAKO). Sections were counterstained with Mayer’s hematoxylin, dehydrated, cleared, and coverslipped for microscopic examination.

Vessel density. For blood vessel enumeration, sections from two to four tumors from each treatment group were labeled with anti–factor VIII and examined at 100× magnification. Total number of vessels were counted in each of the two to three fields (each field represents ~4,000 µm²). Vessel counts are expressed as vessel density per square millimeter.

Statistical analysis. Statistical significance was tested using one-way ANOVA or t test with repeated measure over time. The assumption of ANOVA was examined and nonparametric measure based on ranks was used, as needed. Values were reported as mean ± SE. When ANOVA indicated significant effect (F ratio, P < 0.05), the Student-Keuls multirange test was employed to compare the means of individual groups using Sigma Stat Software (Sigmastat Software Inc.).

Results

Progesterone stimulates growth of BT-474 and T47-D xenograft tumors in nude mice. This study exploits a model system based on the growth properties of BT-474 and T47-D human breast cancer cells in nude mice. These cells contain mutant p53 protein and form xenograft tumors in estrogen-supplemented nude mice when they are injected s.c. in the presence or absence of Matrigel. The xenograft tumors grow aggressively when coimplanted in the presence of Matrigel; however, in the absence of Matrigel, the tumors reach a volume of 50 to 100 mm^3 in 6 to 10 days and then regress (Fig. 1A and B; also ATCC data sheet). However, regressing tumors resume growth when the host animal is treated with exogenous progesterone (i.e., by implantation of a progesterone pellet). In this model, chronic dosing with progesterone is required for the xenograft tumors to continue to grow. As shown in Fig. 1A, xenografts obtained with BT-474 regressed after 6 days following tumor cell injection and initial growth phase. Progesterone pellets were implanted when tumors were ~50% of their initial volume (day 12), inducing tumor proliferation. Tumors did not proliferate in animals receiving placebo. Tumors in animals exposed to progesterone were much larger than those in controls, with respective volumes 260% and 350% of those in placebo controls 3 and 4 weeks postprogesterone administration. To show progestosterone dependence in this model, we removed the hormone pellet on day 42, leading to a 3-fold reduction in tumor volume after 2 weeks compared with tumors exposed to progesterone throughout the same period (Fig. 1A). We next determined the ability of progesterone to rescue xenografts obtained from T47-D cells. As shown in Fig. 1B, T47-D xenografts were unable to sustain their initial growth phase following implantation of an estrogen pellet and regressed after 9 days. Tumor volume declined to 75% of the original volume in about 20 days, at which point animals were again exposed to progesterone and tumors grew as previously described with BT-474 xenografts (Fig. 1B). Removal of the progesterone pellet on day 45 led to loss of tumor growth, confirming the dependence of the T47-D xenografts on progesterone. T47-D xenografts did not grow as aggressively as BT-474 cells.

Progesterone was unable to bring about tumor progression if animals were pretreated with the antiprogestin RU-486, indicating that progesterone receptors are likely required for tumor cell
proliferation (Fig. 1C). In contrast to BT-474 and T47-D cells, tumors generated from wild-type p53 containing MCF-7 cells were not stimulated by progesterone (data not shown). The studies described below involve xenograft tumors generated by injecting nude mice with highly aggressive BT-474 cells to establish this cell line as a model that can be used to study progesterone dependent progression of breast cancer xenografts.

**Progesterone-dependent rescue of tumor growth is associated with altered expression of VEGF, ER, and PR.** Because VEGF expression is associated with tumor growth and expansion (17, 18), we sought to determine whether VEGF expression was altered during various phases of tumor growth following tumor cell injection (Fig. 1A–C). BT-474 cells were injected into nude mice and tumor xenografts collected (a) after 6 days (before tumor regression), (b) during tumor regression when tumors were ~50% of their pre-regression volume, and (c) 1 week after implantation of progesterone pellets, when the initial signs for resumption of tumor growth were recorded. As shown in Fig. 1D, regressing tumors expressed lower levels of VEGF compared with tumors at other stages of development, with the highest levels of VEGF expressed following progesterone administration. Thus, in this model, VEGF expression correlates with tumor growth patterns and is most likely under progesterone regulation, as shown previously in vitro (19).

Because placement of an estrogen pellet is essential for initial tumor uptake and subsequent tumor growth, we wondered whether tumor cells might have lost their steroid receptors, resulting in tumor regression. Both ER and PR were expressed highly in tumors before regression, but their expression was reduced during the regression phase, and levels remained low during the progesterone-induced regrowth phase within 1 week in which the tumor samples were analyzed.

**Progesterone-dependent rescue of tumor growth requires VEGF-VEGFR2.** Previous studies showed that progesterone stimulates secretion of VEGF by human breast cancer cells, including BT-474 cells that contain mutant p53 protein but not by cells that contain wild-type p53 protein (12). Secreted VEGF is strongly proangiogenic and promotes endothelial cell survival and proliferation and stimulation of tumor cell proliferation (13, 20). It is therefore possible that progesterone might rescue regressing...
tumors through VEGF-dependent angiogenesis. With this in mind, we inhibited VEGF function with 2C3, a monoclonal antibody that blocks the interaction between VEGF and VEGFR2. Figure 2A shows that 2C3 antiserum, although not control antibody (C44), prevented progesterone-dependent growth of regressing xenograft tumors. By the end of the experiment, tumor volumes were suppressed 4-fold in 2C3 and placebo-treated animals, compared with tumors in progesterone or C44-treated animals (Fig. 2A). Furthermore, tumors exposed to 2C3 had reduced vascularity and were characterized by a white appearance (Fig. 2B). In contrast, tumors from animals treated with C44 seemed larger and bloody. Figure 2C shows that at the end of the study, average tumor weight from animals treated with 2C3 was significantly lower (4–5-fold) than tumor weight from animals treated with C44 or progesterone and was not significantly different from tumor weights observed in the control group. This result is consistent with a specific anti-VEGF-induced inhibition of tumor growth. Average animal body weight was unaffected in the two treatment groups, except at late time points when tumor weight likely contributed significantly to total body weight (Fig. 2D).

PRIMA-1 blocks progesterone-dependent tumor growth and causes tumor regression. PRIMA-1 is a low-molecular-weight compound that reactivates mutant p53 and inhibits progestin-induced in vitro secretion of VEGF from tumor cells (14). Based on the results shown in Fig. 2 and previously published in vitro studies (12), it seemed possible that PRIMA-1 might also inhibit VEGF secretion in xenograft tumors and, thus, inhibit progesterone-dependent tumor growth. Figure 3A shows that PRIMA-1 treatment of animals with progesterone-rescued tumors inhibited progesterone-dependent growth of xenograft tumors. Two weeks after PRIMA-1 treatment, tumor volume decreased by 75% compared with animals treated with vehicle alone. In some PRIMA-1–treated animals, tumors regressed completely, whereas in other cases, PRIMA-1–treated tumors showed marked loss of vascularity (Fig. 3B). As observed above for animals treated with 2C3, PRIMA-1 caused significant reduction in tumor weight.

![Figure 2](image-url)

**Figure 2.** Inhibition of VEGFR2 activation prevents progesterone-dependent growth of xenograft tumors. A, BT-474 cells were injected, tumor growth was monitored, and P pellets were given as described in Fig. 1. When mean average tumor volume reached ~100 mm³, animals were injected with 2C3, which disrupts VEGF-VEGFR2 interaction, or an isotyped matched control antibody (C44; broken arrow) as described in Materials and Methods. Animals were sacrificed and tumors excised and characterized. *, P < 0.05, ANOVA; significantly different from P and P + C44 group. B, images of representative tumors are shown. C, average tumor weight at sacrifice. *, P < 0.05, ANOVA; significantly different from C group; **, P < 0.05, ANOVA; significantly different from P and P + C44 group.
(≈ 80%) compared with tumor weight in control animals (Fig. 3C), without causing a decrease in total body weight or other signs of toxicity (Fig. 3D). It is likely that both PRIMA-1 and 2C3 antibody are antiangiogenic, preventing progesterone-stimulated secretion of VEGF by BT-474 cells or blocking the action of secreted VEGF. This was confirmed by estimating blood vessel density in xenograft tumors from mice treated with or without PRIMA-1 or 2C3 antiserum. The results (Fig. 4B) are consistent with the hypothesis that PRIMA-1 and 2C3 inhibit angiogenesis in our model system.

**MPA stimulates growth of xenograft tumors in nude mice.** Recent clinical trials show that synthetic progestins could increase the risk of breast cancer in women on HRT (9–11). Because the progestin in most HRT protocols is MPA and because our previous studies show that MPA induces VEGF in BT-474 breast cancer cells in vitro (12), the effect of MPA was tested in the model system described here. We found that MPA and progesterone had very similar effects on BT-474 xenografts (Fig. 5), a similarity that extended to attenuation of MPA-dependent growth of xenograft tumors by PRIMA-1 and 2C3. Tumor weights were influenced comparably by both means of inhibition, and neither showed animal toxicity (data not shown).

**Discussion**

This study describes an animal model system for studying progestin-dependent progression of human breast cancer. The model exploits xenograft tumors derived by injecting BT-474 and T47-D cells into estrogen-supplemented nude mice. Both cell types express PR and mutant p53 protein, and tumor development is progesterone dependent. We mainly focused on the development of the BT-474 cells as a suitable progestin-dependent model in this study, and we describe experimental proof that this system is a suitable model for the in vivo assessment of the
efficacy of agents which inhibit progestin-dependent tumor growth or cause tumor regression. Such agents might act via multiple direct or indirect mechanisms, including reactivation of mutant p53, inhibition of angiogenesis, stimulation of apoptosis, and interference with hormone-induced signaling pathways. In addition, using this model, we show that a synthetic progestin used for HRT in postmenopausal women and associated with increased risk of breast cancer (9–11) can also increase VEGF and tumor growth.

This model exploits the fact that BT-474 and T47-D cells form transient tumors that regress spontaneously when injected into estrogen-supplemented nude mice in the absence of Matrigel. Furthermore, regressing tumors are rescued by chronic dosing with natural or synthetic progesterone via a mechanism that requires secretion of VEGF by the regressing tumor. This system requires inoculation of estrogen-supplemented nude mice with tumor cells in the absence of Matrigel, which would normally be used to provide the growth and survival factors essential for tumor cell proliferation. Previous efforts to develop such a system may have failed because these requirements were not met or because ovariectomized (but estrogen-supplemented) animals were used (21). Wild-type p53 containing MCF-7 cells do not elaborate increased VEGF levels in response to progestins in vitro (12) and fail to show progestin-dependent tumor growth (data not shown).

It is important to emphasize that the initial growth of tumor cells in the xenograft models studied here are completely dependent on estrogen. A possible explanation for estrogen dependence of these tumors is that the steroid may be required to support continued expression of PR (22), although we cannot exclude additional effects of estrogen, such as stimulating the expression of other factors required for tumor growth (23). It is interesting to note that sections taken from tumors before regression showed high levels of VEGF, ER, and PR compared with regressing tumors. Because animals contain an estrogen pellet throughout the course of the study, it would seem that estrogen-induced effects are not sufficient to sustain growth in this model in the absence of any

Figure 5. MPA rescues growth of regressing xenograft tumors in nude mice. BT-474 cells were injected into estrogen-supplemented intact nude mice, and tumor growth was monitored. Once tumors began to regress, an MPA pellet was implanted (arrow). When mean average tumor volume reached ~100 mm³, animals were injected with 2C3, C44, or PRIMA 1 (broken arrow). *, P < 0.05, ANOVA; significantly different from MPA group. **, P < 0.05, ANOVA; significantly different from P group.
Matrigel, or that tumors begin to synthesize angiogenic factors that prevent tumor progression. The reason for reduced levels of ER and PR during the regression phase remains to be determined, although it might be explained by the absence of required growth factors normally present in Matrigel. Because PR is under ER regulation (22, 24), reduced levels of PR could be due to a loss of ER within tumor tissues. However, once progesterone pellets are in place, tumor cells are once again able to sustain VEGF production, thereby creating a milieu conducive to angiogenesis and tumor expansion. Under these conditions, levels of ER and PR remain low although not absent. Functional PR seems to be a vital component of the growth process because RU-486 overcomes the stimulatory effects of progestins; furthermore, it has been shown that VEGF is under PR regulation in human breast cancer cell lines (12, 19). We cannot, however, rule out the involvement of ER in the regulation of VEGF in breast cancer cells, although this remains controversial (25).

Based on a number of observations, we can conclude that VEGF is critical for progesterone-dependent progression of xenograft tumors: (a) tumor growth is suppressed by 2C3, an antibody that blocks the interaction between VEGF and VEGFR2; (b) PRIMA-1 inhibits progesterone-induced secretion of VEGF and reactivates mutant p53 (12); (c) both BT-474 and T-47-D cells express VEGFR2 (13). It is likely that VEGF acts directly as an angiogenic agent in this system, because tumors from animals treated with 2C3 or PRIMA-1 are less vascularized than tumors from control animals. Additional mechanisms involved in the antiangiogenic effects of 2C3 and PRIMA-1 may involve recruitment of endothelial precursor cells or inhibition of other pathways essential for tumor growth. Additional experiments are required to explore these possibilities. Because levels of VEGF correlate with poor response to chemotherapy and disease-free survival rate in breast cancer patients (26, 27), this model provides a means by which we may assess antiangiogenic compounds such as PRIMA-1, as well as angiobased therapies that might prevent the progression of progesterone-dependent breast cancers.

During the conduct of the studies reported herein, we observed that the rescue of tumor progression by progesterone depended on the degree of regression before implantation of the progesterone pellet. When the degree of initial regression was about 40% to 50% of peak tumor volume, tumor growth was rapidly rescued following re-exposure to progesterone, and large tumor masses quickly developed (e.g., Fig. 2A). On the other hand, if the BT-474 xenografts regressed more than 50%, a far greater period was required for progesterone-induced rescue and a resumption of tumor growth (e.g., Fig. 1A). Nevertheless, it is important to note that all tumors did eventually resume their growth. A number of explanations might account for the resumption of growth following exposure to progesterone. The reduced number of tumor cells present following regression would presumably require longer to produce threshold levels of VEGF essential for triggering angiogenesis. It is also possible that regression eliminates potential stem cells which might be required for rapid growth following progesterone signaling. However, we cannot rule out that variations in tumor growth might also arise as a consequence of using different batches of cells or inherent variations in the batches of animals used. These parameters require further study.

In the present study, we describe an animal model system which is suitable for screening potential therapeutic agents for their ability to prevent or arrest the growth of progesterone-dependent breast human tumors. The model is appropriate for screening anti-progestins, progestins mimetics, and antiangiogenic compounds and will, we believe, enhance the future development of safer progestins and their antagonists, as well as compounds capable of activating mutant p53 and suppressing angiogenesis.

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References


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