Effect of Progesterone on the Invasive Properties and Tumor Growth of Progesterone Receptor-transfected Breast Cancer Cells MDA-MB-231[1]

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ABSTRACT

One of the potential therapeutic interventions to hormone-independent breast cancer would be to reactivate the expression of estrogen receptor or progesterone receptor (PR) in the tumor cells so as to render the tumor responsive to the hormones. We have reported previously that progesterone markedly inhibited cell growth and induced remarkable focal adhesions in PR-transfected MDA-MB-231 cells. The aim of this study was to determine the effects of progesterone on the invasive properties and *in vivo* tumor growth of PR-transfected MDA-MB-231 cells. It was found that progesterone has increased cell resistance to trypsin digestion and increased cell attachment to extracellular matrix proteins, especially laminin and fibronectin. *In vitro* invasion assays using modified Boyden chambers showed that progesterone increased cell migration through matrix protein-coated membranes. However, Northern blotting analysis demonstrated that progesterone strongly down-regulated (up to 60-fold) the gene expression of urokinase plasminogen activator and increased (up to 5-fold) the expression of tissue-type plasminogen activator in these cells. This pattern of gene regulation suggested an inhibition of cell invasiveness because numerous clinical studies have indicated that low levels of urokinase plasminogen activator and high levels of tissue-type plasminogen activator in breast cancer are associated with favorable prognosis. Furthermore, animal studies showed that progesterone strongly inhibited the tumor formation and growth in Scid mice. After 12 weeks of inoculation, the median weight of tumors in the progesterone-treated group was 25 mg compared with 203 mg in the placebo group ($P < 0.001$). These results suggest that progesterone may provide effective treatment for estrogen receptor- and PR-negative breast cancer if the PR expression were reactivated. Alternatively, activation of progesterone-mediated molecular pathways in hormone-independent breast cancer may achieve similar therapeutic effects.

INTRODUCTION

Estrogen and progesterone are critically involved in the development of breast cancers. Hormonal therapy is the front-line treatment for hormone-dependent breast cancers (1–3). The expression of ER[3] and PR is widely used as indicators of hormonal dependency (4–6). On the other hand, almost half of all breast cancer cases are ER- and PR-negative, and most of them do not respond to hormonal therapy (7, 8). These hormone-independent breast cancers are commonly presented with aggressive biological behaviors and often have poor prognosis.

Inactivation of ER and PR expression is characteristic of hormone-independent breast cancer. A potential therapeutic intervention for this type of breast cancer is to reactivate the expression of the ER or PR gene so as to restore the response of the cancer cells to hormonal treatment. Reactivation of ER expression by transfection in ER- and PR-negative breast cancer cells MDA-MB-231 restored responses of the cancer cells to estrogen treatment in terms of cell growth and induction of PR (9). Estrogen inhibited the growth of these ER-transfected MDA-MB-231 cells, although it normally stimulates the growth of ER-positive breast cancer.

We have reported recently (10) that reactivation of PR expression in MDA-MB-231 cells by transfection enables progesterone to inhibit markedly the growth of PR-transfected cells. Progesterone also induced remarkable cell spreading and focal adhesions (11), cellular processes known to be related to tumor invasion and metastasis. The findings are very interesting because two essential features of breast cancer are uncontrolled growth and metastasis. This study had been designed to address the question of whether the reactivation of PR gene expression enables progesterone to inhibit cancer development both in invasiveness and *in vivo* tumor growth. The effect of progesterone on cell migration was measured by *in vitro* invasion assay using modified Boyden chamber. The ability of the cells to attach to matrix protein-coated substratum and their resistance to protease (trypsin) digestion were used as indicators of cell cohesion. Expression of invasion-related genes such as uPA, tPA, and PAI-1 were analyzed as indicators for cell invasiveness

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; uPA, urokinase protease activator; tPA, tissue-type protease inhibitors; PAI-1, plasminogen activator inhibitor-1; DPBS, Dulbecco's PBS.

and metastatic potentials. Finally, the effects of progesterone on tumor growth of PR-transfected MDA-MB-231 cells were also investigated in Scid mice.

MATERIALS AND METHODS

Materials. MDA-MB-231 cells were obtained from the American Tissue Culture Collection in 1995 at passages 28. They were cloned using a 96-well plate by the method of single-cell dilution, and clone 2 (MDA-MB-231-CL2) was used for the study described herein. All of the tissue culture reagents were obtained from Life Technologies, Inc. Tissue culture plastic wares were from Corning. Extracellular matrix proteins were from Sigma Chemical Co., and Matrigel was from Becton Dickinson. [^{32}P]dCTP was from Amersham, Inc.

Cell Culture. All of the cells were routinely maintained in phenol red-containing DMEM, supplemented with 7.5% FCS, 2 mM L-glutamine, and 40 mg/liter gentamicin. For all of the assays, cells were grown in phenol red-free DMEM supplemented with 5% dextran charcoal-treated FCS to remove the endogenous steroid hormones that might interfere with the analysis. Cells were treated with progesterone from 1000-fold stock in ethanol. This gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only.

Transfection. MDA-MB-231-CL2 cells were transfected with PR expression vectors hPR1 and hPR2 coding for PR isoform B and isoform A, respectively, in pSG5 plasmid (12). Vector pBK-CMV (Stratagene) containing the neomycin-resistant gene was cotransfected with hPR1 and hPR2. Details of transfection were described previously (9). Eight PR-positive clones expressing both PR isoforms A and B were isolated and characterized. They showed similar responses to progesterone treatment. In this study, effects of progesterone on clone ABC28 that expressed ~660 fmol PR/mg protein were described in this report. Cell clone CTC15 stably transfected with both vectors pBK-CMV and pSG5 was used as a transfection control. Progesterone had no effect on CTC15 cells, as has been reported in previous studies (10, 11).

Measurement of Cell Attachment. Cells were treated with or without 1 nM progesterone for 48 h. Treated cells (2×10^5) were plated onto the 24-well plates that were precoated with either BSA (100 μg/ml), laminin (25 μg/ml), fibronectin (25 μg/ml), collagen I (100 μg/ml), or collagen VI (25 μg/ml). After incubation for 1 h at 37°C, the cells were washed gently 3 times with DPBS. The attached cells were stained with 0.5% crystal violet in 20% methanol and lysed with 1% SDS, and the absorbances were measured on a microplate reader at 595 nm. The results are the average of two independent experiments, each with 6–8 replications. Standard curves were obtained by plotting different numbers (0.5×10^4–10×10^4) of progesterone-treated or control cells against their respective absorbances. The number of attached cells was calculated from the standard curve using the absorbance values. The attachment of progesterone-treated cells was normalized with each of the controls coated with the same extracellular matrix protein.

Resistance to Trypsin Digestion. Cells (2×10^5) were plated onto 60-mm Petri dishes for 48 h before they were treated with either 1 nM progesterone or control vehicle for an additional 48 h in DMEM. After removing the growth medium and being washed with DPBS, the cells were incubated with either 0.005% trypsin/0.02% EDTA or 0.1% trypsin/0.02% EDTA for 10 min at room temperature. The number of detached and attached cells was then determined by counting. The results (mean ± SE) are percentages of cells attached after trypsinization and are, therefore, indications of resistance to protease digestion ($n = 5$–6).

***In Vitro* Invasion Assay.** Cells treated with or without 1 nM progesterone for 48 h were trypsinized and plated at 5×10^4/well on the 8-μm pore polycarbonate membranes of transwell chambers (Costar Corporation, Cambridge, Massachusetts) in phenol red-free DMEM containing 5% dextran charcoal-treated-FCS. The membranes had been precoated with either 0.1% BSA, mixtures of extracellular matrix proteins (laminin, 50 μg/ml; fibronectin, 50 μg/ml; collagen I, 200 μg/ml; and collagen VI, 50 μg/ml) or with Matrigel used at 1:10 dilution. After incubation for 16 h in a CO_2 incubator at 37° in the presence or absence of 1 nM progesterone, cells that had migrated to the undersides of the membranes and to the culture wells below were detached by trypsin, and the cell number was determined by counting. The migration is expressed as the percentage of 0.1% BSA-coated controls (mean ± SE; $n = 4$–6).

Northern Blotting Analysis. Cells at 60–70% confluency were treated with 0.1 μM progesterone or control vehicle for various time periods. Total RNA was isolated from the cells by lysing in guanidine thiocyanate followed by centrifugation in a cesium chloride gradient. Twenty μg of total RNA of each sample in duplicates were separated on a 1.2% Northern gel and transferred to nylon Hybond-N membrane (Amersham). ^{32}P-labeled uPA, ^{32}P-labeled tPA, and ^{32}P-labeled PAI-1 were generated by random priming reaction using PCR-amplified cDNA fragments of the individual genes.

The uPA probe was a 294-bp fragment located at nucleotides 1796–2062 of human uPA mRNA. The tPA probe was from a 370-bp cDNA fragment located between nucleotides 1411 and 1757 of the tPA mRNA. The 448-bp PAI-1 probe was located at nucleotides 700-1148 of the PAI mRNA. The probes were sequentially hybridized to the membrane using Ultrahyb solution of Ambion. Levels of specific mRNA expression were analyzed using Bio-Rad Molecular Image Analyzer.

Tumor Growth of PR-transfected-MDA-MB-231 Cells in Scid Mice. Female Scid mice of 6–7 weeks of age were used. Bilateral ovariectomy was performed 1 week before the inoculation. Each mouse received implantations with either a 90-day slow-release progesterone pellet (25 mg/pellet) or a placebo pellet 2 days before tumor inoculation. Cells (5×10^6) were suspended in 0.1 ml of DPBS and inoculated into both flanks using 23-gauge needle. The mice were examined weekly for tumor appearance and growth. They were sacrificed after 12 weeks of tumor inoculation, and the tumors were carefully dissected, measured, weighed, and finally fixed in formalin for histology examination.

Statistical Analysis. Differences between treatments in cell culture experiments were analyzed with the unpaired *t* test. The experiments of tumor growth in Scid mice were analyzed by the Mann-Whitney nonparametric test using the SPSS program for Windows, version 10.

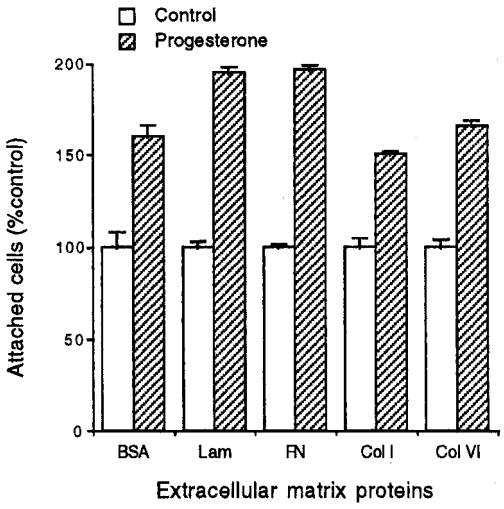

Fig. 1 The effect of progesterone on the attachment of ABC28 cells. Cells were treated with either 1 nM progesterone or control vehicle for 48 h before they were plated onto the 24-well plates. The plates were precoated with either BSA (100 μg/ml), laminin (*Lam*; 25 μg/ml), fibronectin (*FN*; 25 μg/ml), collagen I (*Col I*; 100 μg/ml), or collagen VI (*Col VI*; 25 μg/ml). One h after incubation at 37°C, the attached cells were stained with 0.5% crystal violet in 20% methanol and lysed with 1% SDS, and the absorbances were measured on a microplate reader at 595 nm. The results are the average of two independent experiments, each with six to eight replications. The attachment of progesterone-treated cells was normalized with each of the controls coated with the same extracellular matrix protein.

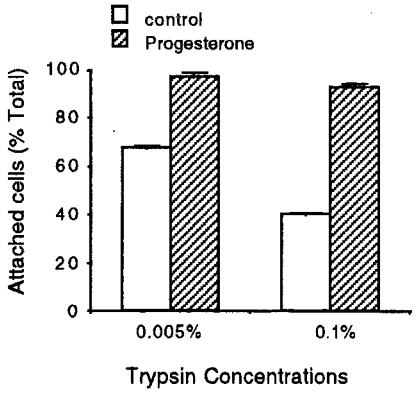

Fig. 2 The effect of progesterone on cell resistance to trypsin digestion. Cells (2×10^5) were plated onto 60-mm Petri dishes for 48 h before they were treated with either 1 nM progesterone or control vehicle for an additional 48 h in DMEM. After removing the growth medium and being washed with DPBS, the cells were incubated with either 0.005%/0.02% EDTA or 0.1% trypsin/0.02% EDTA for 8 min at room temperature. The number of detached and attached cells was then determined by counting. The results (mean ± SE) are percentages of cells attached after trypsinization ($n = 5–6$).

RESULTS

We have reported previously (10, 11) that progesterone induced growth inhibition and focal adhesion in PR-transfected MDA-MB-231 cells in a dose-dependent manner at concentrations from 10^{-12}–10^{-6} M. The magnitude of the effects increased with increasing concentrations of progesterone. Maximal inhibition of cell growth was achieved with 1 nM (10^{-9} M) progesterone. Thus, the concentration of progesterone used in this study was 1 nM.

Progesterone Increased the Attachment of ABC28 Cells on Extracellular Matrix Proteins. The effect of progesterone on cell attachment to extracellular matrix protein-coated substratum was tested. The results in Fig. 1 (Fig. 1) are the average of two independent experiments. Progesterone increased the attachment of ABC28 cells to all of the coatings tested, including the BSA by 50–100%. The increases were significantly higher ($P < 0.01$) in attachment to substratum coated with laminin and fibronectin than with others. On the other hand, there were no significant differences in cell attachment between collagen I- or collagen IV-coated substratum and 0.1% BSA-coated substratum, suggesting a nonspecific mechanism of the attachment to the collagens. Therefore, progesterone-stimulated cell attachment may be mediated through the specific receptors of laminin and/or fibronectin.

Progesterone-treated ABC28 Cells Are More Resistant to Trypsin Digestion than Vehicle-treated Controls. The initial step of metastasis is believed to be the detachment of tumor cells from the primary tumor mass when the cells lose the ability to attach (Fig. 2). The strength of the attachment depends on the bonding force of cells-cells and cells-matrix and the disrupting force such as proteases to break up the bond. In this study, the ability of one of the proteases, trypsin, to disrupt the cells-substratum bonding was measured. After 8 min of treatment with 0.005% trypsin, 93% of progesterone-treated cells were still attached, as compared with 70% of control cells ($P < 0.01$). When cells were treated with 0.1% trypsin for 8 min, 91% of progesterone-treated cells were still attached, as compared with 41% of the control cells ($P < 0.001$).

Progesterone Increased the Migration of ABC28 Cells through Matrix Protein-coated Membranes in Modified Boyden Chamber. The experiments were repeated twice, each with 2–4 replications. The results were similar, and Fig. 3 (Fig. 3) shows the average of two experiments. After 16 h of incubation, progesterone increased the cell mobility through membranes coated with mixtures of extracellular matrix proteins by 8-fold ($P < 0.001$). The increase was 3.5-fold ($P < 0.005$) through membrane-coated commercial Matrigel (Becton Dickinson) at 1:10 dilution. On the other hand, there was no significant difference ($P > 0.05$) in cell migration between progesterone-treated cells and the controls through 0.1% BSA coated-membranes. Therefore, progesterone has specifically increased the matrix protein-mediated mobility of ABC28 cells.

Progesterone Markedly Decreased the mRNA Levels of uPA, Increased the Level of tPA mRNA in PR-transfected MDA-MB-231 Cells. uPA, tPA, and PAI-1 are genes known to be related to cell invasiveness and metastasis potentials. Numerous clinical studies (13–16) have shown that high levels of uPA and PAI-1 are associated with early relapse and poor prognosis, whereas high levels of tPA are related to good prognosis of breast cancer patients. Fig. 4 (Fig. 4) shows the results of Northern blotting analysis of uPA and tPA gene expression at various time points after progesterone treatment. Each of the RNA samples was analyzed in duplicate. The

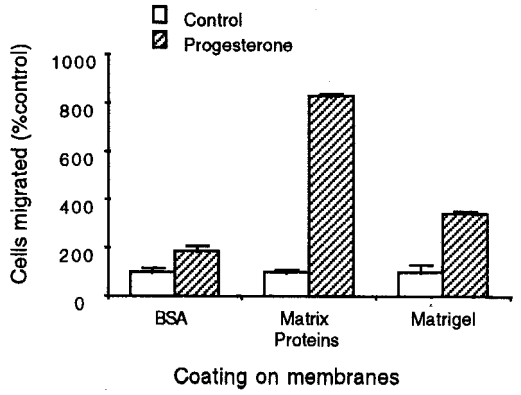

Fig. 3 The effect of progesterone on the migration of ABC28 cells through Matrigel-coated membranes. Cells treated with or without 1 nM progesterone for 48 h were plated at 5×10^4/insert on 8-μm pore membranes in transwell chambers. The membranes had been coated either with 0.1% BSA, mixtures of extracellular matrix proteins, or with commercial Matrigel used at 1:10 dilution. After incubation for 16 h in a CO_2 incubator, the cells that had migrated to the undersides of the membranes were detached by trypsin and the cell number was determined by counting. Results are cells migrated in percentage of controls (mean ± SE; $n = 4–6$).

average variation between the duplicates was 7%, which is an indication of reproducible loading and even transfer. Progesterone markedly down-regulated the gene expression of uPA beginning as early as 1 h after treatment (Fig. 4, *A* and *B*). This marked effect of inhibition persisted throughout the 72-h period studied. The decrease of uPA expression was as much as 60-fold after 72 h of progesterone treatment. In contrast, the effect of progesterone on the expression of tPA gene was significantly increased by progesterone (Fig. 4, *A* and *B*). The increase of tPA mRNA began after 3 h of progesterone treatment. After 72 h of treatment, mRNA levels of tPA were increased to five times of the control. Progesterone had no significant effects on the expression of PAI-1 mRNA (data not shown).

Progesterone Significantly Inhibited the Growth of PR-transfected MDA-MB-231 Tumors in Scid Mice. Tumor cells were injected into both flanks of each mouse (Fig. 5). The appearance and the growth of the tumor were monitored weekly. Because the sizes of the tumor were small, it was not possible to measure tumor dimensions in live animals. Twelve weeks after cell inoculation, the mice were killed and tumor weight and size were recorded. There was no significant difference in tumor growth between the right and the left flanks. Therefore, the data of the right and the left tumors were pooled together for analysis. In the progesterone-treated group, three of six inoculations gave rise to tumors, as compared with 100% tumor growth in placebo groups. The value of zero was assigned to the data points with no tumor. The data were analyzed by the Mann-Whitney nonparametric test. Endogenous progesterone was not sufficient to inhibit the growth of the tumor because the volumes and weights of the tumors were not significantly different between ovariectomized mice and nonovariectomized mice. Progesterone significantly inhibited the tumor growth in Scid mice. The median weight of tumors in progesterone-treated mice was 25 mg, as compared with 203 mg in the ovariectomized placebo group ($P < 0.001$) and with 299 mg in the nonovariectomized placebo group ($P < 0.005$; Fig. 5*A*). Similarly, the median volume of the tumors was significantly lower ($P < 0.005$) in the progesterone-treated group (6 mm^3) than that in the ovariectomized placebo group (255 mm^3) and in nonovariectomized controls (343 mm^3). There was no gross metastasis of the tumors in the mice studied. Histology examination confirmed that all of the tissue mass analyzed was tumor tissue.

DISCUSSION

Cancer is a disease of genetic alterations. In hormonal-independent breast cancer, ER and PR genes are inactivated as a result of genetic alterations. We (10, 11) and others (9) have shown that reactivation of ER or PR expression in the breast cancer cells restored the response of the cells to hormonal treatment. Particularly, reactivation of PR expression in MDA-MB-231 cells by transfection rendered the ability of progesterone to inhibit remarkably the growth of the transfected cells (10). This study examined the effect of progesterone on invasive properties and tumor growth of these PR-transfected cells *in vivo*.

The initial step of tumor metastasis is the detachment of tumor cells from the primary tumor mass when the cells lose the ability to attach (17). The experiments have revealed that progesterone was able to increase cell resistance to trypsin digestion and increase cell attachment to extracellular matrix proteins, especially laminin and fibronectin. These properties may make it difficult for the tumor cells to detach from the primary tumor mass.

Accumulating evidence suggests that the components of the plasminogen-plasmin system are critically involved in invasion and metastasis in breast cancer (18–21). uPA converts an enzymatically inactive plasminogen into an active plasmin. Metalloproteases, which are activated by plasmin or by uPA directly, degrade the extracellular matrix surrounding cancer cells and enable the cells to invade. tPA is the other form of plasminogen activator. Numerous clinical studies (13–15, 18, 20, 22, 23) indicated that low levels of uPA and high levels of tPA in breast cancer tissue are associated with overall disease-free survival and a lower relapse rate. In our study, progesterone drastically inhibited the expression of uPA mRNA by as much as 60-fold in PR-transfected cells. Progesterone also markedly increased the gene expression of tPA by 5-fold. Because uPA and tPA are invasion-related gene products, the findings suggest that progesterone may reduce the invasiveness of PR-transfected MDA-MB-231 cells.

This study is the first to demonstrate the direct effect of progesterone in the regulation of the plasminogen-plasmin system in breast cancer cells. It suggests a direct role of progesterone in the regulation of cancer cell invasion and metastasis. Estrogen and its analogues have been shown to inhibit the expression of uPA and PAI-1 in ER-transfected MDA-MB-231 cells (24, 25). On the other hand, the effects of estrogen on tPA expression are inhibitory in these ER-transfected cells, which is opposite to the effect of progesterone in PR-transfected cells. Progesterone had no significant effect on the expression of PAI-1 in PR-transfected MDA-MB-231 cells, as opposed to the inhibitory effect of estrogen on PAI-1 in ER-transfected cells. It

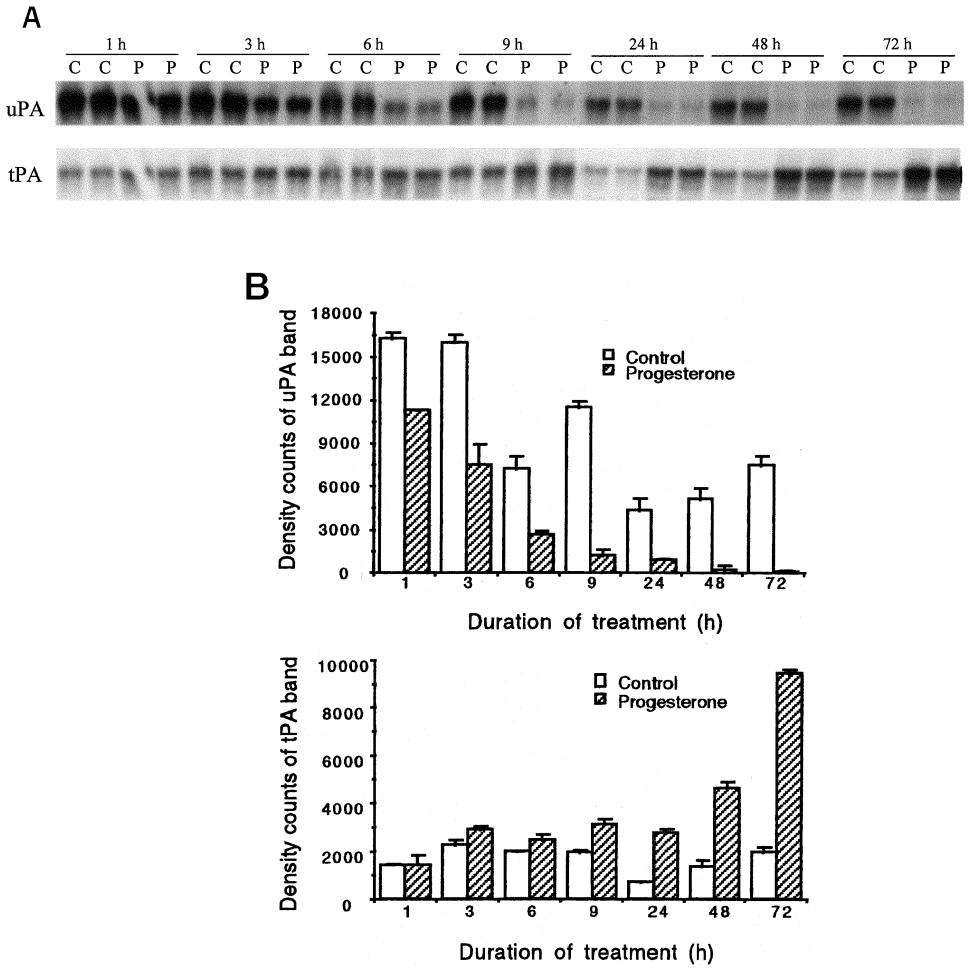

Fig. 4 The effect of progesterone on the mRNA levels of uPA and tPA in PR-transfected MDA-MB-231 cells. Cells at 60–70% confluence were treated with 0.1 μM progesterone (*P*) or control vehicle (*C*) for various time periods. Total RNA was extracted from the cells, and 20 μg of total RNA from each sample in duplicates were separated on a Northern gel and transferred to nylon membrane. ^{32}P-labeled uPA and ^{32}P-labeled tPA probes were hybridized to the membrane sequentially after striping. Levels of specific uPA and tPA mRNA expression were analyzed using Bio-Rad Molecular Image Analyzer. *A*, Northern blots showing specific uPA and tPA bands. *B*, quantitative analysis of uPA and tPA expression by densitometer.

has long been recognized that progesterone acts in synergy with estrogen to regulate the function of the target cells. There is probably a coordinated regulation of the plasminogen-plasmin system by the two hormones in the tissue to achieve the desired physiological concentration. The levels of uPA and tPA proteins were not measured in the present study. However, it has been shown that changes in levels of uPA and tPA proteins correlate well with their mRNA expression in MDA-MB-231 cells (25).

This study revealed that progesterone not only inhibits the growth of the PR-transfected cells in culture but also strongly inhibits tumorigenesis of PR-transfectant cells in Scid mice. On the other hand, *in vitro* invasion studies using transwell chambers showed that progesterone increased cell migration through Matrigel-coated membranes. This confounds the results of uPA and tPA expression study and of Scid mice study. There have been a number of reported studies (26–28) that *in vitro* invasion assays did not correlate with stage of malignancies or *in vivo* tumorigenicity in breast cancer cells. Using six cell lines from human breast cancer, Le Marer and Bruyneel (26) demonstrated that benign (242A) and primary carcinoma cell lines (341 and 531E) were more invasive than cells from late stages of malignancies (ZR75-1 and T47D) in Matrigel barrier. Madsen and Briand (27) also studied two nonmalignant human breast epithelial cell lines (HBL100 and HMT3522) and six malignant cell lines (MCF-7, T47D, ZR-75-1, CAMA-1, BT-20, and HMT-3909) for correlation between tumorigenicity, *in vitro* invasiveness, and uPA production. They found that cell invasion *in vitro* by the embryonic chick heart assay was correlated neither to tumorigenicity nor to the secretion of uPA. Therefore, *in vitro* invasion assays may not reflect a true *in vivo* situation, and the results should be interpreted with caution.

In summary, two important findings are reported here. First, progesterone strongly inhibited tumor growth of PR-transfected ER and PR-negative MDA-MB-231 cells in Scid mice. Second, progesterone markedly decreased the expression of uPA and increased the expression of tPA in these cells. Clinical studies have revealed that low uPA and high tPA levels are associated with good prognosis. Progesterone may, therefore, decrease the invasiveness and metastatic potentials of the cells by regulating the expression of invasion-related genes. Because two essential features of breast cancer are uncontrolled growth and metastasis, the findings suggest that progesterone may provide effective treatment for ER- and PR-negative breast cancer if the PR expression were reactivated. Alternatively, activation

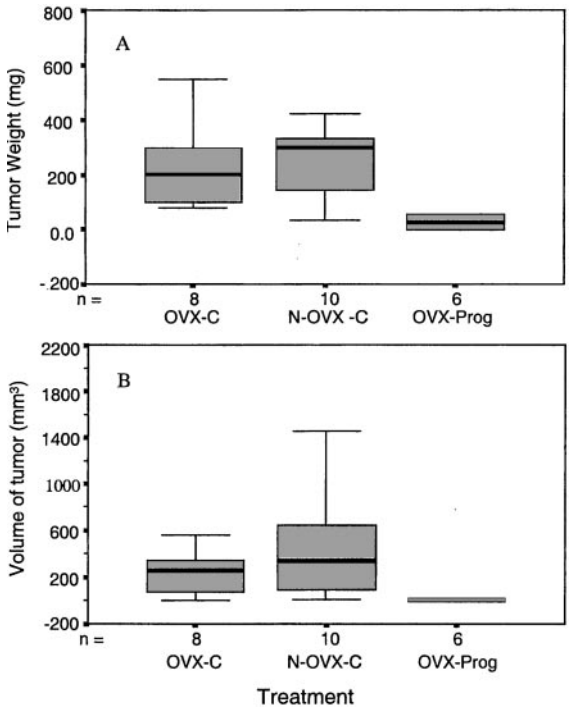

Fig. 5 The effect of progesterone on tumor growth of PR-transfected MDA-MB-231 cells in Scid mice. Each mouse received implantations with either a 90-day slow-release progesterone pellet (25 mg/pellet) or a placebo pellet 2 days before tumor inoculation. Cells (5×10^6) were suspended in 0.1 ml of PBS and inoculated into both flanks. The mice were sacrificed after 12 weeks of tumor inoculation. The tumor weight (*A*) and tumor size (*B*) of different treatments were plotted as box plots that show the distribution of tumor weight and volume by quartiles. *OVX-C*, ovariectomized mice receiving placebo; *N-OVX-C*, nonovariectomized mice receiving placebo; *OVX-Prog*, ovariectomized mice receiving progesterone pellet; $n = 6–10$.

of progesterone-mediated molecular pathways in hormone-independent breast cancer may achieve similar therapeutic effects. The study also suggests potential significance of progestins in the treatment of breast cancer.

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