[CANCER RESEARCH 64, 7886–7892, November 1, 2004]

Reduction of Human Metastatic Breast Cancer Cell Aggressiveness on Introduction of Either Form A or B of the Progesterone Receptor and Then Treatment with Progestins

Tomoki Sumida,[1,2] Yoko Itahana,[1] Hiroyuki Hamakawa,[2] and Pierre-Yves Desprez[1]

[1]California Pacific Medical Center, Cancer Research Institute, San Francisco, California; and [2]Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, Ehime, Japan

ABSTRACT

The sex steroid hormone progesterone (Pg) is critically involved in the development of the mammary gland, and it also is thought to play a role in breast cancer progression. However, the effect of Pg on malignant phenotypes is not fully understood in breast cancer. We previously reported that in Pg receptor (PR)–positive T47D breast cancer cells, Pg was able to counterbalance the stimulatory effect of estrogen or serum on proliferation and on expression level of Id-1, which generally stimulates cell proliferation and inhibits differentiation. Conversely, metastatic MDA-MB231 breast cancer cells lack PR and express high levels of Id-1 constitutively, and Pg showed no effect on Id expression, proliferation, and invasion in these cells. However, after introducing PR (either PR-A or PR-B) into MDA-MB231 cells, Pg inhibited the expression of Id-1 mRNA drastically. PR-transfected MDA-MB231 cells exhibited less proliferative activity after Pg treatment than parental or control MDA-MB231 cells, an effect which correlated well with reduction of Id-1 mRNA. This inhibitory effect on proliferation was accompanied by p21 up-regulation and c-*myc* down-regulation. Moreover, Pg-treated PR transfectants showed significant morphologic change, appearing more flattened and spread out than control ethanol-treated cells. Boyden chamber invasion assay revealed that PR-transfected MDA-MB231 cells also lost most of their invasive properties after Pg treatment. Zymographic analysis revealed that Pg drastically inhibited matrix metalloproteinase-9 (MMP-9) activity in cells transfected with either PR-A or PR-B. To determine whether Id-1 could act as a key mediator of the effects of Pg, we prepared cells transfected with Id-1 and PR. The morphologic change and p21 up-regulation still were observed after Pg treatment. However, c-*myc* down-regulation was not observed; the proliferative and invasive activities were mostly recovered; and MMP-9 down-regulation could not be detected anymore. From these observations, we conclude that either form of the PR is sufficient to reduce the malignant phenotypes on treatment with Pg and that Id-1 plays an important role as a mediator of the effects of Pg on breast cancer cell proliferation and invasion.

INTRODUCTION

The development of malignant neoplasms is a multistep process that involves many genetic and epigenetic alterations. Identifying these alterations is essential to understand the mechanisms of cancer progression and to develop more effective methods of diagnosis and management. In the case of the reproductive organs, hormonal stimulation also is critically involved in carcinogenesis. The sex steroid hormones, estrogen and progesterone (Pg), play an important role in normal mammary gland development, and it is believed that breast cancer progression is influenced by them and their receptors (1, 2).

The level of these steroid hormone receptors is a strong prognostic factor for a patient with breast cancer and has been used in clinical management as an indicator of endocrine responsiveness (3, 4). Many advanced breast cancers are negative for Pg receptor (PR) and estrogen receptor (ER) and fail to respond to endocrine therapy (5, 6). Although progestins currently are used for the management of advanced breast cancer (7), their effect on the malignant phenotype still is controversial. Depending on the tissue, Pg is classified as a proliferative or differentiative sex steroid hormone (8, 9). In breast cancer cells, Pg often acts as an inhibitor of cell growth, but in other reports, it acts as a growth stimulator (10–12). The mechanism of hormone-dependent cancer progression generally still is poorly understood.

We have shown that Id (inhibitors of DNA bindings) proteins, negative regulators of basic helix-loop-helix (bHLH) transcription factors, play an important role not only in normal development of mammary epithelial cells but also in invasive and proliferative phenotype in breast cancer cells (13–18). The bHLH proteins typically form heterodimers with other bHLH proteins and direct many developmental and differentiation processes (19). Products of *Id* genes lack the basic DNA binding domain of the bHLH transcription factors, and when they heterodimerize with bHLH proteins, the complexes are inactive. Therefore, the Id proteins block the formation of transcriptionally active complexes. High levels of expression of *Id* genes have been identified in cell lines derived from a wide variety of different tumors and also in tumor tissues, and recent reports implicate Id proteins in cancers of many organs (20).

In most nonaggressive breast cancer cell lines we examined, we could detect only low levels of Id-1 expression that were absent when the cells were cultured in a serum-free medium. Conversely, most aggressive breast cancer cell lines possessed high levels of constitutive Id-1 expression that were maintained in serum-free conditions (13). We also have shown that Pg is able to counterbalance the stimulatory effect of estrogen or serum on proliferation and on the level of Id-1 expression in PR-positive T47D cells, whereas Pg did not have any effect on these parameters in PR-negative MDA-MB231 (13).

Therefore, the relationship between Pg biology and Id protein expression appears to be critical to understand the hormonal regulation of breast cancer development. Specifically, because *Id* genes appear to be key regulators of breast cancer progression, we hypothesize that there is a strong relationship between Pg signaling and Id regulation. To obtain more insight into this relationship, we have transfected PR isoform (PR-A and/or PR-B) into metastatic MDA-MB231 cells and determined the effect of treatment with Pg on their aggressive phenotype and expression of *Id* gene. Our findings provide support for the conclusion that Pg acts as a suppressor of malignant phenotype via PR by regulating some proteins that are associated with proliferative and invasive properties. We specifically show that Id-1 plays a crucial role as a mediator of the effects of Pg on breast cancer cell proliferation and invasion.

Received 3/31/04; revised 7/27/04; accepted 8/26/04.

Grant support: Japanese Ministry of Education, Culture, Sports, Science and Technology (Japanese Government Oversea Study Program) to T. Sumida; and National Cancer Institute (RO1 CA82548) and California Breast Cancer Research Program (5IB-0111 and 7WB-0026) to P. Y. Desprez.

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Requests for reprints: Pierre-Yves Desprez, California Pacific Medical Center, Cancer Research Institute, Stern Building, 2330 Clay Street, San Francisco, CA 94115. Phone: 415-600-1760; Fax: 415-600-1390; E-mail: pdesprez@cooper.cpmc.org.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines T47D and MDA-MB231 were obtained from the American Tissue Culture Collection (Manassas, VA). The cells were routinely maintained in RPMI 1640 supplemented with 10% dextran charcoal-treated fetal bovine serum and 5 μg/mL insulin at 37°C in the presence of 5% CO_2. For serum-free conditions, fetal bovine serum was omitted from the medium.

Transfection with Progesterone Receptor and Id-1. Vectors hPR1 and hPR2 contained human PR cDNA coding for PR-B and PR-A, respectively, in pSG5 plasmid (gifts of Professor Pierre Chambon, Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France; ref. 21). Vector pBK-CMV (Stratagene, La Jolla, CA) containing the neomycin-resistant gene was cotransfected with hPR1 and/or hPR2 into MDA-MB231 cells using LipofectAMINE PLUS Reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. Neomycin-resistant clones were selected in medium containing G418. The cells cotransfected with empty pSG5 and pBK-CMV were used as control cells. We isolated 34 clones containing both PR isoforms, 32 clones with only PR-A, 25 clones with only PR-B, and 6 control clones. After determination of PR-A/PR-B protein content, we selected the population of MDA-MB231 cells expressing the highest levels of PR-A and PR-B (MDA-PR-AB), the highest levels of only PR-A (MDA-PR-A), and the highest levels of only PR-B (MDA-PR-B). For the retroviral infection experiments, vectors were packaged in TSA-54 cells (Cell Genesis, Foster City, CA). Full-length human Id-1 cDNA in a sense orientation was cloned into a pBabe vector. MDA-PR-AB cells, which had abundant expression of PR-A and PR-B, were infected with pBabe empty vector (PR-AB-CTL) and pBabe-Id-1 vectors (PR-AB-Id-1), and puromycin-resistant cells were selected in medium containing puromycin. We also produced MDA-MB231 cells infected with pSG5, pBK-CMV, and pBabe empty vector (CTL) as control cells.

Chemicals. Pg was obtained from Sigma Chemical Co. (St. Louis, MO) and was used at a concentration of 10 nmol/L. Pg compound was added from the 1000-fold stock in ethanol. This gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only. For the thymidine incorporation assays, the cells were treated with ethanol or Pg once daily, and for other assays, they were treated twice daily, respectively.

RNA Extraction and Northern Blot Analysis. The cells cultured in 10% serum were treated with either ethanol or Pg for 2 hours before harvesting for RNA extraction. Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi (22). Fifteen micrograms were separated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N; Amersham Biosciences, Piscataway, NJ). The membrane was hybridized to a ^{32}P-labeled human Id-1 (23), Id-2 (15), or Id-3 cDNA probe and washed and exposed to XAR-5 film (Kodak) for autoradiography as described previously (13). Id-3 probe was generated by reverse transcriptase PCR using a primer set of 5′-CATGAAGGCGCTGAGCCCGGT-3′ and 5′-CCACCTGGCTAAGCTGAGTGC-3′. Matrix metalloproteinase-9 (MMP-9) probe was generated by reverse transcription-PCR using a primer set of 5′-CACTGTCCACCCCTCAGAGC-3′ and 5′-GCCACTTGTCGGCGATAAGG-3′. Ribosomal 28S and 18S RNA were used to control for RNA integrity and quantitation.

Western Blot Analysis. The cells were lysed in 2× Laemmli buffer (24) and stored at −70°C. Protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Samples (30 μg) were separated by SDS-PAGE and were transferred to a polyvinylidene difluoride membrane (Hybond P; Amersham) by standard methods (24). The membrane was blocked for 1 hour at room temperature with 20 mmol/L Tris base, 137 mmol/L NaCl, 3.8 mmol/L HCl, and 0.1% Tween 20 containing 10% nonfat milk and was incubated with a rabbit polyclonal antibody specific for Id-1 (Z-8; Santa Cruz Biotechnology, Santa Cruz, CA), p27 (M-197; Santa Cruz Biotechnology), PR-A or PR-B (C-20; Santa Cruz Biotechnology), p21 (187; Santa Cruz Biotechnology), c-*myc* (9E10; Santa Cruz Biotechnology), or actin (C4; Chemicon International, Temecula, CA) for 1 hour. The membrane was washed and incubated with secondary antibody (goat antirabbit or antimouse IgG–horseradish peroxidase; Santa Cruz Biotechnology) and then washed again and developed for enhanced chemiluminescence using the Amersham ECL or ECL-plus kit according to the supplier's instructions.

[^{3}H]Thymidine Incorporation Assay. The cells were cultured on coverslips in 1% serum, and either ethanol or Pg was added 48 and 24 hours before harvesting. [^{3}H]Thymidine (10 μCi/mL; 60 to 80 Ci/mmol; Amersham) was included in the last 16 hours, and cells were fixed with a 1:1 methanol/acetone (v/v). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole diluted 1:10,000 in PBS. [^{3}H]Thymidine labeling was developed as described previously (17, 25, 26). The percentage of labeled nuclei was calculated by comparing the number of [^{3}H]thymidine-labeled nuclei with the number of 4′,6-diamidino-2-phenylindole–stained nuclei in a given field using light and fluorescence microscopy.

Anchorage-Dependent Growth Assays. Assays were performed as described previously (18). Briefly, liquefied 2% agarose was mixed with an equal volume of 2× DMEM/F12 growth medium lacking serum and supplemented with insulin (10 μg/mL) and gentamicin (100 μg/mL; 2× medium). One milliliter of the mixture was layered onto 35-mm dishes to create a 1% agarose base. Liquefied 0.6% agarose was mixed with an equal volume of 2× medium, and 10 mL of this solution were mixed with 1 mL of growth medium containing 1×10^5 MDA-MB231–transfected cells to yield 1×10^4 cells/mL in 0.27% agarose; 1 mL of this cell suspension was layered on top of the 1% agarose base, and 1 mL of DMEM/F12 containing 10% fetal bovine serum was added. The cells were incubated for 2 weeks in the presence of either ethanol or Pg (10 nmol/L). Counts were performed according to the size of the colonies.

Matrigel Invasion Assay. Invasion assays were performed in modified Boyden chambers with 8-mm pore filter inserts for 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). Filters were coated with 12 μL of ice-cold Matrigel (7.5 mg/mL protein; Becton Dickinson Labware). Cells (5×10^4 per well), which were pretreated with ethanol or Pg for 48, 72, and 96 hours, were added to the upper chamber in 200 μL of the appropriate medium containing 5 μg/mL insulin. The lower chamber was filled with 300 μL of NIH–mouse fibroblast cell-conditioned medium. After a 16-hour incubation, cells were fixed with 2.5% glutaraldehyde in PBS and were stained with 0.5% toluidine blue in 2% Na_2CO_3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy. Assays were performed in duplicate or triplicate, and the results were averaged.

Zymography and MMP-9 Western Blot Analysis. Proliferating cells (10^6 in 100-mm-diameter dishes) were shifted to serum-free medium for 2 days, after which they were given 8 mL of fresh serum-free medium. Forty-eight hours later, after ethanol or Pg treatment twice a day (total, four times), the conditioned medium was collected and concentrated 10- to 15-fold by using Vivaspin protein concentration filters (Fisher Scientific, Hampton, NH). The concentrated medium was analyzed on gelatin substrate gels as described by Talhouk *et al.* (27). Briefly, gels consisted of 10% polyacrylamide and 1 mg of gelatin (Sigma)/mL. Fifteen to 20 μL of concentrated conditioned medium were mixed with nonreducing Laemmli sample buffer and incubated at 37°C for 15 minutes. After electrophoresis, the gel was incubated for 1 hour in 2.5% Triton X-100 at room temperature, followed by 16 hours in substrate buffer [100 mmol/L Tris-HCl (pH 7.4) and 15 mmol/L $CaCl_2$]. The gels were stained with Coomassie blue for 30 minutes and were destained with 30% methanol and 10% acetic acid. Gelatinase activities were visible as clear bands, indicative of proteolysis of the substrate protein. Polyacrylamide gel electrophoresis also was performed by loading the same amount of the conditioned medium and served as a control for the protein loading. To confirm the existence of MMP-9, Western blot analysis also was performed using the same amount of the concentrated conditioned medium. A mouse monoclonal antibody specific for MMP-9 (Ab-2; Calbiochem, San Diego, CA) was used for the primary antibody incubation. Other procedures were the same as mentioned previously.

RESULTS

Progesterone Reduces Proliferation and Suppresses Id-1 Expression in PR-Transfected MDA-MB231 cells. As described in Materials and Methods, MDA-MB231 metastatic breast cancer cells were transfected with either both forms of the human PR (PR-A and PR-B) or only one form (PR-A or PR-B). Using Western blot analysis, we screened all of the clones isolated from the three different types of transfected cells (AB containing both forms, A containing only the A

form, and B containing only the B form). We selected one cell population from each group that expressed the highest levels of PR-A and PR-B (MDA-PR-AB cells), only PR-A (MDA-PR-A), or only PR-B (MDA-PR-B; Fig. 1*A*). As control, we used the T47D cells, which express both forms of the human PR, and MDA-MB231 control cells, which express none.

To investigate the relation between PR isoform expression and proliferative activity of these cells, we determined the rate of proliferation by thymidine incorporation assay (Fig. 1*B*). A significant reduction of the proliferative activity was observed after Pg treatment of the transfectants, including those with only one form of PR. The extent of this reduction was comparable with the reduction observed in PR-positive T47D cells. We also determined that the ability of the MDA-PR-AB cells to grow in an anchorage-independent growth assay was strongly reduced on treatment with Pg (Fig. 1*C*). There was no difference in the number of small, medium, and large colonies between the MDA-PR-CTL cells treated with ethanol or Pg, whereas Pg-treated MDA-PR-AB cells showed significantly fewer numbers of colonies than ethanol-treated MDA-PR-AB cells.

Pg treatment reduced Id-1 mRNA levels in most of the different cell populations, whereas Pg did not affect Id-1 mRNA expression in control vector MDA-MB231–transfected cells (CTL cells; Fig. 1*D*). Pg treatment did not affect Id-2 expression, which was not expressed in parental MDA-MB231 cells (18). In nonaggressive T47D cells, Id-2 was up-regulated after treatment with Pg, which is in agreement with the hypothesis that Id-2 is a differentiating agent in breast cancer cells. Id-3 mRNA was expressed in each cell population and also was down-regulated by Pg similarly to Id-1, although to a lesser degree.

Morphologic Change of PR-Transfected MDA-MB231 after Progesterone Treatment. Following Pg treatment, PR transfectants showed drastic morphologic change, appearing more flattened and spread out than the MDA-MB231 control or parental cells (Fig. 2). The morphologic changes began ~8 hours after treatment, were completed 16 to 24 hours after treatment, and continued as long as Pg was added. The PR transfectants treated with vehicle alone did not present any morphologic change (Fig. 2*C, E,* and *G*) and did not present any detectable difference with ethanol-treated control cells (Fig. 2*A*), suggesting that there is no major ligand-independent effect in the PR-overexpressing cells. Pg had no effect on the cells transfected with control empty vector (Fig. 2*A* and *B*). PR-B–expressing cells exhibited the same morphologic change as cells expressing both receptors, presenting an elongated bipolar shape (Fig. 2*D* and *H*). In contrast, cells expressing only PR-A exhibited a more flattened, stellate, and multipolar shape (Fig. 2*F*).

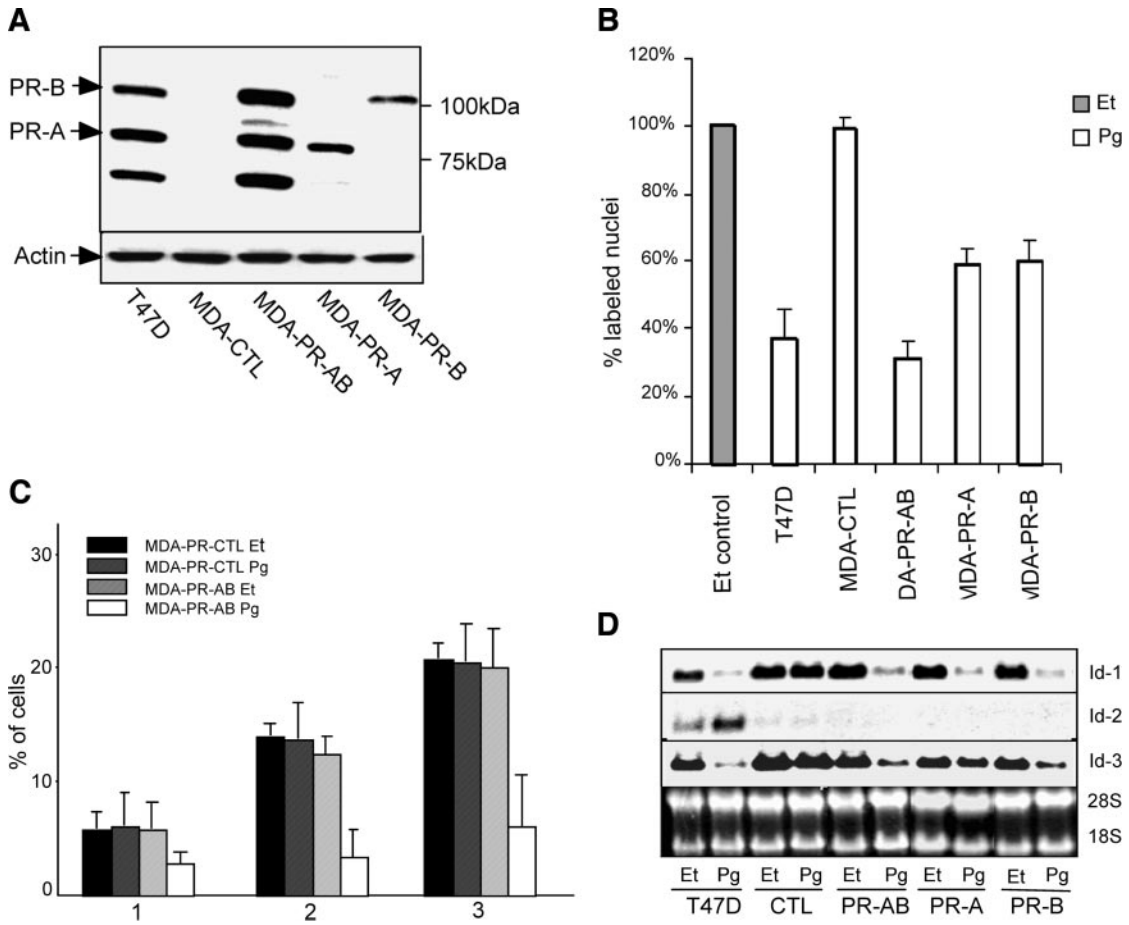

Fig. 1. *A,* expression of PR isoforms in T47D, control MDA-MB231, and PR-transfected MDA-MB231 cell populations. Proteins were extracted, and PR was analyzed by Western blot analysis as described in Materials and Methods. PR was detected using a polyclonal antibody against human PR-A and PR-B. Position of the two forms and molecular weight markers are indicated. Actin was used as control for protein integrity and quantitation. *B,* Pg reduces growth in PR-transfected cells. Cells were cultured in 1% serum with either ethanol (*Et*) or Pg for 48 hours. [^{3}H]Thymidine was included in the last 16 hours. The mean thymidine incorporation of labeled nuclei of all of the cells treated with ethanol was defined as 100%. Other values are given relative to this level. MDA-PR-CTL cells were MDA-MB231 cells transfected with an empty vector. MDA-PR-AB expressed both forms of PR. MDA-PR-A expressed only PR-A, and MDA-PR-B expressed only PR-B. *C,* Pg reduces anchorage-independent growth in PR-transfected cells. MDA-PR-CTL cells were either treated with ethanol (*black bars*) or with Pg (*dark gray bars*), and MDA-PR-AB cells were either treated with ethanol (*light gray bars*) or Pg (*white bars*). Colonies formed were classified as follows: large colonies (>50 μm diameter), column 1; medium colonies (from 0.25 to 0.5 μm diameter), column 2; and small colonies (<25 μm diameter), column 3. *D,* effect of Pg treatment on Id expression in PR-transfected cells. T47D, empty vector–transfected control MDA-MB231 cells, and PR-transfected MDA-MB231 cells were treated with ethanol (Et) or Pg for 2 hours, and then RNA was extracted and analyzed by Northern blot analysis.

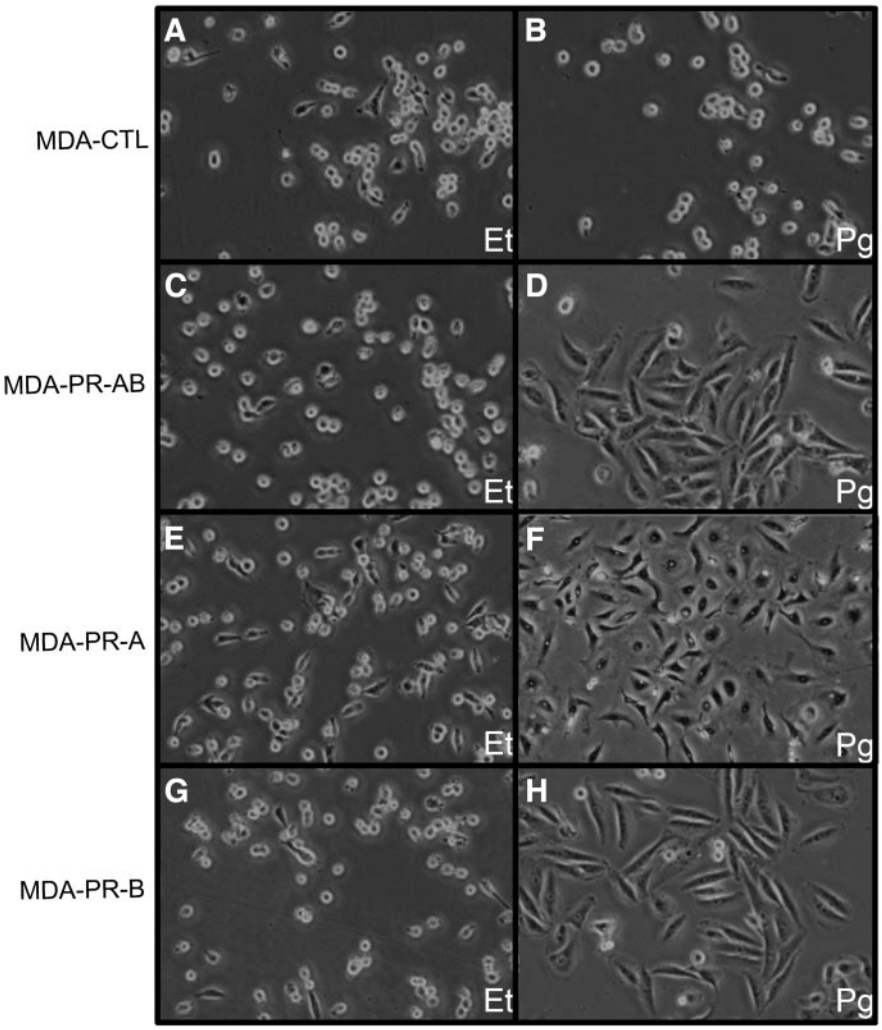

Fig. 2. Morphologic changes of PR-transfected cells in the presence of Pg. Cells cultured in 10% serum were photographed after treatment with either ethanol (*Et*) or Pg for 24 hours. *A* and *B,* CTL (control) cells; *C* and *D,* MDA-PR-AB cells, which expressed PR-A and PR-B; *E* and *F*, MDA-PR-A cells, which expressed only PR-A; and *G* and *H,* MDA-PR-B cells, which expressed only PR-B.

Progesterone Inhibits Cell Cycle by Down-Regulation of Id-1 and c-*myc* and Up-Regulation of p21. The inhibitory effect of Pg on proliferative activity in MDA-MB231 cells transfected with both forms of PR (MDA-PR-AB cells) was investigated by extraction of cell protein at different time points and Western blot analysis. As shown in Fig. 3, expression of Id-1 and c-*myc* was down-regulated in a time-dependent manner after Pg treatment. Conversely, p21 expression level was up-regulated after treatment, whereas p27 remained unchanged. The p21 up-regulation was observed within 24 hours after treatment started and continued in the presence of Pg.

Progesterone Inhibits PR-Transfected MDA-MB231 Cell Invasion and Reduces the Levels of MMP-9 Expression. To investigate the effects of Pg on invasive properties, cells were plated in Matrigel invasion chambers. After 48-hour pretreatment with Pg, MDA-PR-AB cells, containing both PR isoforms, did not show significant reduction of invasive capacity compared with ethanol-treated cells (Fig. 4*A*; $P = 0.1936$). However, after pretreatment for $\geq$72 hours, the invasive capacity of MDA-PR-AB cells was markedly inhibited. Pg also showed significant inhibitory effect in MDA-MB231 cells transfected with only one receptor type (either A or B). There was no Pg effect on invasiveness in cells transfected with the control empty vector (CTL cells).

To determine some of mechanisms by which Pg reduces invasion of PR-transfected MDA-MB231, we determined the level of MMP expression. Parental MDA-MB231 cells have abundant expression of MMP-9 but little expression of MMP-2 (data not shown). Zymographic analysis revealed that 48 hours of Pg treatment drastically reduced the amount of MMP-9 secreted into the medium by MDA-PR-AB cells (Fig. 4*B, top*). Western blot analysis of MMP-9 using the same concentrated conditioned medium used for zymography and Northern blot analysis using total RNA confirmed these results (Fig. 4*B, middle* and *bottom*).

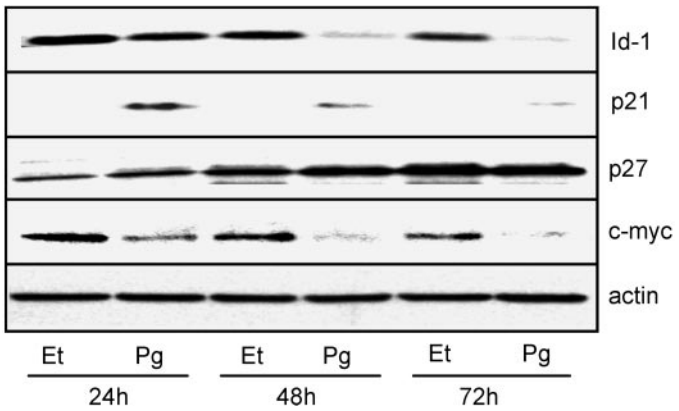

Fig. 3. Effect of Pg on expression of proteins associated with cell growth and cell cycle in MDA-PR-AB cells. Cells were cultured for the indicated times in the absence or presence of Pg and analyzed for Id-1, p21, p27, c-*myc*, and actin protein expression as described in Materials and Methods.

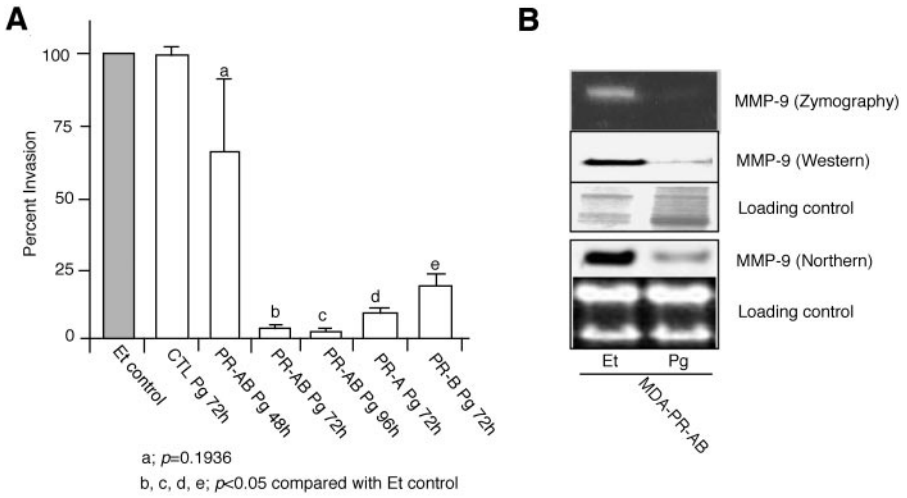

Fig. 4. Effect of Pg on cell invasion and MMP. *A,* effect on invasiveness. The indicated cells were treated with Pg for 48, 72, or 96 hours and then assayed in Matrigel invasion as described in Materials and Methods. Bars represent the mean percentage in six independent assays with SE. The percentage of all of the cells treated with ethanol was defined as 100%. *B,* analysis of Pg-treated MDA-PR-AB cells for MMP-9 protein and activity and MMP-9 mRNA. Cells were treated with Pg, and zymography, Western blot analysis, and Northern blot analysis were carried out as described in Materials and Methods.

Effect of Ectopic Expression of Id-1 in PR-Transfected MDA-MB231 Cells. To determine the importance of Id-1 in the mediation of Pg effects in MDA-MB231 cells, Id-1 was constitutively expressed into MDA-PR-AB cells. Ectopic *Id-1* gene was introduced using the retroviral infection method, and transgene expression was confirmed by Northern blot analysis (Fig. 5*A*). On treatment with Pg, the morphologic change previously observed in MDA-PR-AB cells still could be observed in MDA-PR-AB-Id-1 cells (Fig. 5*B*). Western blot analysis revealed that p21 also still was up-regulated in MDA-PR-AB-Id-1 cells. However, ectopic expression of Id-1 abolished the Pg effect on Id-1 expression and on c-*myc* down-regulation (Fig. 5*C*). Most importantly, MDA-PR-AB-Id-1 cells were able to maintain a high level of proliferation in the presence of Pg (to ~80% of ethanol-treated control cells; Fig. 5*D*). Like parental MDA-MB231 cells, MDA-PR-AB-Id-1 cells treated with Pg also could invade Matrigel (Fig. 5*E*), even though they exhibited the same morphologic change as the MDA-PR-AB-CTL cells, which did not invade the matrix. Interestingly, Pg did not suppress MMP-9 activity in MDA-PR-AB cells, which ectopically expressed Id-1. Conversely to MDA-PR-AB cells (Fig. 4*B*), MDA-PR-AB-Id-1 cells still showed strong MMP-9 expression and gelatinase activity on Pg treatment (Fig. 5*F*).

DISCUSSION

The status of the female sex steroid hormone receptors ER and PR is considered a strong prognostic factor and sometimes is used as a clinical marker in routine treatment of patients with breast cancer (3, 4). However, there is considerable debate on the effect of estrogen and Pg on the malignant phenotype of breast cancer. Many studies have shown that there is substantial cross-talk between signal transduction pathways and steroid hormone receptors, in addition to the conventional hormone-dependent regulation. Pg can activate the mitogen-activated protein kinase pathway, and this activation was reported to depend on the existence of ER (28). Ligand-occupied PR can suppress estradiol-stimulated ER activity (29). Moreover, the expression level of PR can be modulated by estradiol (30). Thus, PR and ER interact extensively in the modulation of biological responses. The T47D cell line has a high level of PR and ER (31) and therefore is widely used as a model system to study sex steroid hormone effects on human breast cancer cells. A poorly differentiated breast cancer cell line, MDA-MB231, is known to possess no PR and only small amounts of endogenous ER-b (32) and therefore makes this cell line a good model of hormone-independent aggressive breast cancer.

PR exists as two isoforms, the M_r 120,000 PR-B and the NH_2-terminally truncated M_r 94,000 PR-A, that display different regulatory functions in the target cells and therefore are considered to be two distinct transcription factors (33). In normal breast tissues, PR-A and PR-B are coexpressed within the same cells in comparable amounts, but in breast cancer cells, expression of one or the other may predominate (34). Recent studies suggest that the relative expression level of the two PR isoforms may prove to be of prognostic value not only in breast cancer patients (35) but also in patients with endometrial cancers, which also are affected by sex steroid hormones during development and tumorigenesis (36). In the present study, we found that MDA-MB231–transfected cells expressing only one form of PR were able to down-regulate proliferation, invasion, and Id-1 expression on Pg treatment.

Id-1 repression is an early event in response to Pg treatment and could be detected <1 hour after treatment of T47D (13) and MDA-PR-AB cells (data not shown). We believe that this effect results from direct repression of the Id-1 promoter, and we currently are investigating this in our laboratory. Most importantly, we found that Pg-induced growth inhibition was correlated with Id-1 mRNA reduction. We previously reported that in T47D cells, an antisense oligonucleotide that reduced Id-1 protein levels reduced the ability of estrogen to stimulate cell proliferation, whereas constitutive Id-1 expression rendered cells refractory to growth inhibition by Pg (13). This accumulating evidence led us to conclude that Id-1 could act as an important mediator of the effects of Pg on cell proliferation.

Id-2, another Id family protein, could be detected at high levels in nonaggressive T47D cells; however, there was no expression of Id-2 in PR-transfected MDA-MB231 cells on Pg treatment. We previously have shown that Id-2 is highly expressed in differentiated mammary epithelial cells *in vivo* and in culture, that Id-2 antisense transcripts blocked mammary epithelial cell differentiation (15), and that Id-2 is expressed at low levels in undifferentiated breast cancer cells. Another Id family gene, *Id-3*, also was regulated by Pg in PR-transfected cells. Id-1 and Id-3 are coexpressed temporally and spatially during murine neurogenesis and angiogenesis (37). However, most of the aggressive breast cancer cell lines possess specifically high levels of Id-1 expression that was still maintained in serum-free conditions (13). We recently have described the mechanisms involved in the loss of proper physiologic regulation of Id-1 expression in metastatic breast cancer cells (14), and the loss of regulation is considered to link with aggressive phenotype. Thus, we speculate that Id-1 plays a more important role than Id-3 in MDA-MB231 cells.

Pg-induced growth inhibition was accompanied by p21 up-regulation,

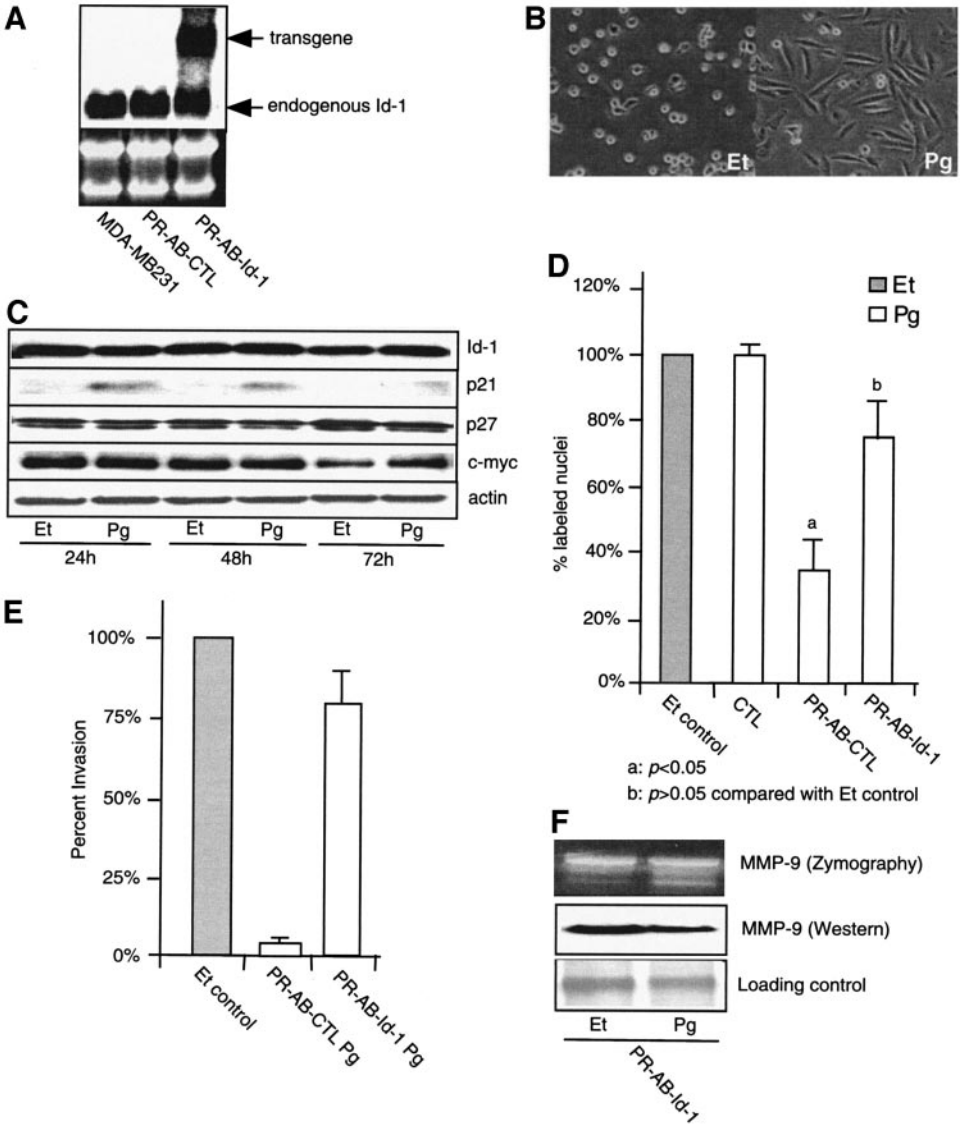

Fig. 5. Effects of ectopic Id-1 expression in MDA-PR-AB cells. *A,* confirmation of the expression of human Id-1 transgene. RNA was extracted from parental MDA-MB231, MDA-PR-AB-CTL, and MDA-PR-AB-Id-1 cells and analyzed by Northern blot analysis. *B,* MDA-PR-AB-Id-1 cells exhibited the same morphologic change as seen in MDA-PR-AB cells after Pg treatment. MDA-PR-AB-Id-1 cells were cultured in 10% serum and photographed after treatment with either ethanol (*Et*) or Pg for 24 hours. *C,* effect of Pg on cell growth/cell cycle-related genes in MDA-PR-AB-Id-1 cells. Cells were cultured for the indicated times in the absence or presence of Pg and analyzed for Id-1, p21, p27, c-*myc*, and actin protein expression as described in Materials and Methods. *D,* effect of Pg on levels of labeled nuclei in MDA-PR-AB-Id-1 cells. Cells were cultured in 1% serum with either ethanol (Et) or Pg for 48 hours. [^{3}H]Thymidine was included in the last 16 hours. The mean thymidine incorporation of labeled nuclei of all of the cells treated with ethanol was defined as 100%. Other values are given relative to this level. MDA-PR-CTL cells were MDA-MB231 cells transfected with an empty vector. *E,* Matrigel invasion analysis of MDA-PR-AB-CTL and MDA-PR-AB-Id-1 cells on Pg treatment. The indicated cells were treated with Pg for 72 hours and then assayed in Matrigel invasion as described in Materials and Methods. Bars represent the mean percentage in six independent assays with SE. The percentage of all of the cells treated with ethanol was defined as 100%. *F,* zymographic analysis of MMP-9 gelatinase secreted by MDA-PR-AB-Id-1 cells on Pg treatment. Cells were treated with Pg, and zymography and Western blot analysis were carried out as described in Materials and Methods.

which generally plays a critical role in regulating cell growth inhibition, and by c-*myc* repression. Because of some previous reports indicating a relationship between Pg and human telomerase reverse transcriptase expression (38), other proliferative markers, such as telomerase activity and presence of its catalytic subunit human telomerase reverse transcriptase, also were investigated (data not shown). Although a slight reduction of telomerase activity was detected in T47D cells on Pg treatment, we could not detect any significant change in MDA-PR-AB cells. Therefore, telomerase activity may depend on the cell type and the steroid hormone receptor content, including ER.

Pg-treated PR-transfected MDA-MB231 cells showed drastic morphologic change, being more flattened and spread out than the parental MDA-MB231 cells or ethanol-treated PR-transfected MDA-MB231 cells. This is consistent with previous reports in which PR expression was reactivated in aggressive breast cancer cells (39, 40). The morphologic changes may partly explain the decrease in cell migration and invasion observed in Pg-treated PR-transfected MDA-MB231 cells. Moreover, we observed a difference in morphology between cell populations with PR-A and PR-B. Cells with only PR-A exhibited a more flattened, stellate, and multipolar shape. This change found in PR-A–expressing cells was similar to that induced by RU486, an anti-Pg agent (41). It has been suggested that PR-B is a major activator of gene transcription, and PR-A is a repressor of PR-B activity (42). However, more recent studies using cells (43) and mice (44) suggest that PR-A and PR-B can activate gene transcription. The difference in morphologic appearance suggests that PR-A acts differently than PR-B and similarly to the status of PR occupied with Pg antagonist. This change was masked in the cells that had both receptors, suggesting that PR-B might be dominant. These findings led us to speculate that there must be a distinct pathway through PR-A or PR-B in this morphologic change. However, the effects of Pg on proliferation were similar in PR-A– and PR-B–transfected cells.

In addition to proliferative inhibition, reduction of anchorage-independent growth, and morphologic change, Pg reduced the invasive capacity of the PR-transfected MDA-MB231 cells. Lin *et al.* (45) reported that Pg, besides its effects on the reduction of cell proliferation, also could increase cell migration (46). However, they concluded that Pg still could inhibit the invasive property because of the down-regulation of urokinase plasminogen activator and up-regulation of tissue-type plasminogen activator. In our experiments, we pretreated the cells before conducting the invasion assays. The cells were treated for different periods before seeding. After 72-hour pretreatment with Pg, the invasive capacity of PR-transfected MDA-

MB231 cells was markedly inhibited in cell populations with PR-A and/or PR-B. Therefore, continuous treatment for a few days was needed to induce the inhibitory effect of Pg on invasiveness. Conversely, this inhibitory effect was abolished in MDA-PR-AB cells, which ectopically expressed Id-1.

MMP-9, one of the MMP family proteins, is important for the degradation of basement membrane and extracellular matrix during cancer invasion. In response to Pg treatment, MMP-9 was drastically down-regulated in MDA-PR-AB cells, which could explain why the cells showed reduced invasive capacity. Interestingly, a rapid reduction of MMP-9 expression occurred at the mRNA level, suggesting that a direct effect of PR on MMP-9 promoter might occur. However, Id-1 transfection could overcome the Pg effect on MMP-9 and on the invasive capacity determined by Matrigel invasion assay. We speculate that ectopic Id-1 expression induced the production of MMP-9 protein, which promoted invasion into Matrigel, even though the cells were treated with Pg.

In conclusion, we have shown that Pg inhibits metastatic breast cancer cell proliferation and invasion after PR transfection and that Id-1 is an important mediator of the Pg response. Moreover, reintroduction of only one form of Pg receptor, either PR-A or PR-B, could significantly modify the aggressive phenotype of metastatic breast cancer cells treated with Pg. Using antisense strategies, we recently determined that a small reduction of Id-1 protein expression could reduce breast cancer cell metastasis *in vivo* (26). Here, we show that Pg treatment can almost completely abolish Id-1 expression, thus suggesting the possibility that PR signaling could efficiently reverse breast cancer cell metastasis. This finding represents an important basis for future clinical applications.

ACKNOWLEDGMENTS

We thank Professor Pierre Chambon for generously providing the vectors hPR1 and hPR2 containing human PR cDNAs and Dr. Andrew P. Smith for editing.

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Reduction of Human Metastatic Breast Cancer Cell Aggressiveness on Introduction of Either Form A or B of the Progesterone Receptor and Then Treatment with Progestins

Tomoki Sumida, Yoko Itahana, Hiroyuki Hamakawa, et al.

Cancer Res 2004;64:7886-7892.