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Elucidating Progesterone Effects in Breast Cancer: Cross Talk With PDGF Signaling Pathway in Smooth Muscle Cell

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Abstract Several studies indicate that progesterone exerts relevant effects in breast tissue. However, the exact role of this steroid in breast cancer development and progression has not been elucidated. Here, we show that platelet-derived growth factor (PDGF)-A is one of the progesterone target genes on breast cancer MCF7 and T47D cells. A paracrine role for PDGF-A was investigated, since its receptor expression was down-regulated from breast cancer cells. Progesterone increased PDGF-A protein release as evaluated by Western blotting and ELISA. Medium from Progesterone-treated MCF7 cells resulted in phosphorylation of smooth muscle cells PDGF receptor α. This effect was not observed after treatment with PDGF inhibitor. MCF7 cells-secreted PDGF-A was able to increase smooth muscle cell viability and proliferation and decrease apoptosis, effects that were prevented by the use of a PDGF-A neutralizing antibody. Notably, cell invasion was not influenced by PDGF-A secreted by MCF7 cells. Our results elucidated for the first time the cross talk between progesterone and PDGF signaling pathway. The fact that MCF7-secreted PDGF elicited crucial roles in vascular wall smooth muscle cells, suggested a paracrine pathway for progesterone. Targeting these progesterone-induced processes may provide novel therapeutic strategies for hormone-dependent human breast cancer. J. Cell. Biochem. 100: 174–183, 2007.

Key words: angiogenesis; breast cancer; hormonal signaling; progesterone; PDGF; smooth muscle cells

Ovarian steroid hormones, estrogens and progesterone, are essential for cell growth and development of the breast. However, in contrast to estrogens, which are recognized as breast cancer growth promoters, the role of progesterone in breast cancer remains uncertain. According to the literature, progesterone plays contradictory roles in breast cancer, namely stimulating, inhibiting, or having no effect on breast cancer cell proliferation [Sutherland et al., 1988; Jeng et al., 1992; Groshong et al., 1997; Lin et al., 1999; Dabrosin, 2005a]. This controversial role of progesterone reflects our lack of knowledge concerning the progesterone-induced signaling pathways. Despite the well-established presence of progesterone receptors (PR) in breast cancer cells [Cianfrocca and Goldstein, 2004; Zheng et al., 2005], these are mainly regarded as a powerful predictive factor for adjuvant hormonal treatment [Cianfrocca and Goldstein, 2004], with no clear evidence for the role of progesterone in breast cancer progression.

Cross talk between tumor cells and adjacent neighboring cells is crucial for cancer progression. Conversely, the role of progesterone in vascular wall cells has been extensively described [Oehler et al., 2000; Dabrosin, 2005b]. Angiogenesis is a complex multistep process that comprises endothelial cell, as well as support cell proliferation, migration, and invasion [Soares et al., 2003a; Costa et al., 2004]. Several reports highlighted the effects of

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progesterone on angiogenic growth factors. However, the exact mechanism of action of progesterone remains unknown. Therefore, we sought to elucidate the signaling transduction pathways induced by progesterone in breast cancer cells, giving particular attention to the role of this hormone in tumor-induced angiogenic phenotypes.

MATERIALS AND METHODS

Chemicals

All cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Progesterone (P4) and 17β-estradiol (E2) were obtained from Sigma Aldrich (Portugal). Antibodies against progesterone receptors (PR)-A, PR-B, PDGF-A, phosphorylated-PDGFRα and βActin (Santa Cruz Biotechnology) were used for immunoblotting. Anti-PDGF neutralizing antibody and recombinant PDGF-A were purchased in Upstate (NY). Recombinant PDGF-A was used at a final concentration of 10 ng/ml in accordance with the manufacturer's instructions.

Progesterone was dissolved in 100% ethanol and added to serum-free medium of cell cultures at a final concentration range of 0.1–1000 nM. 17β-estradiol was used at a final concentration of 10^{-9} M in 100% ethanol as previously described [Soares et al., 2003b]. Ethanol was added to cell culture media at a concentration of less than 0.1%. Because progesterone receptor (PR) is an estrogen-dependent gene product, cells were maintained in 10% FBS until 70% confluence, then washed twice with PBS and immediately incubated in serum-free conditions, with no prior serum-free medium incubation. Since P4 is always accompanied by 17β-estradiol in physiological concentrations, a combination of these two steroids was used to confirm the physiological relevance.

Cell Cultures

Breast cancer cell lines MCF7, T47D, Hs578T, and SKBr-3 were obtained from the American Type Culture Collection (ATCC, Barcelona, Spain). T47D and Hs578T cells were cultured in RPMI medium. SKBr-3 cells were harvested in McCoy 5A medium, and MCF7 cells were routinely cultured in Eagle MEM medium.

Fetal aortic smooth muscle (FLTR) cells were kindly provided by Dr. James McDougall (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). FLTR cells are immortalized human fetal aortic smooth muscle (SMC) cells with no phenotypic changing after passage 30, retaining much of the phenotype of normal aortic SMC. FLTR cells were used in passages 50 through 60 [Martin et al., 2001]. Cells were cultured in high glucose Dulbeco's modified Eagle's medium (DMEM). Cell culture media, FBS and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Paisley, Scotland, UK). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. HUVECs and FLTR cell cultures at 70% confluency were starved for 16 h before treatments. Incubations were performed always in serum-free conditions and during 24 h.

Clonogenic Assay

Colony formation assay was performed as previously described [Mittal et al., 2004]. Briefly, cells were harvested at a concentration of 1×10^3 cells/ plate in six-well plates, incubated for 24 h in complete medium and then incubated with ethanol, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M P4, or combinations of P4 at each concentration with 10^{-9} M E2 for 7 days, enabling each cell to proliferate and form colonies. Cell cultures were then washed with PBS, fixed with 70% ethanol and stained with 0.04% trypan blue solution. Colonies with more than 50 cells were counted on a phase contrast microscope (Nikon, UK). Results were expressed in percentage of the colonies formed by the control cells (ethanol treated).

MTT Proliferation Assay

MCF7 and FLTR cells were cultured following standard conditions or the treatment procedures for 24 h. Cells were then washed twice with PBS and subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, an index of cell viability and cell growth. Cells were incubated with MTT solution at a final concentration of 0.5 mg/mL for 3 h and then lysed in DMSO. Optical density was measured at 540 nm. The background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean ± SD and are expressed as percentage of control, which was considered to be 100%.

cDNA Array Analyses

Total RNA obtained from MCF7 cell cultures was isolated using a TriPure method (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Arrays (Atlas Human Cancer 1.2 Arrays, Clontech) were assayed as previously described [Patriotis et al., 2001; Soares et al., 2003b]. Mean values of intensity for each spot detected from multiple arrays were generated by the computer software. Array data from vehicle-treated cells (control) and progesterone-treated cells were compared. Values rated above 2.0 were considered gene up-regulation and below 0.5 considered down-regulation, with $P < 0.0001$. Comparative differential gene expression analyses of progesterone-treated and control was performed using Gene Sight 5.0 software (Biodiscovery, USA).

Western Blotting

Proteins were isolated from MCF7 or FLTR cell lysates using Tripure (Roche Diagnostics, Basel, Switzerland). Secreted proteins were precipitated from cell medium with absolute ethanol at -20°C. Proteins were quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK) and equal amounts of protein were subjected to 8% or 15% SDS–PAGE with a 5% stacking gel. After electrophoresis proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington), using a mini-transblot electrophoretic transfer cell (Bio-Rad, laboratories, CA). Immunodetection for PDGF-A, phosphorylated PDGFRα and βActin was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences). Experiments were repeated three times with identical results. The relative intensity of each protein blotting analysis was measured using a computerized software program (Bio-Rad, Portugal) and normalized with β-Actin bands to compare the expression of proteins in different treatment groups. Experiments were repeated three times.

MCF7 Conditioned Media

MCF7 cells at 70% confluence were rinsed twice with PBS and then incubated with progesterone or ethanol in serum-free medium for 24 h at 37°C. Media were collected, centrifuged at 13,000 rpm, filtered and frozen at -80°C until usage. FLTR smooth muscle cells previously kept in serum-free medium for 16 h, were then incubated with conditioned medium removed from MCF7 cell cultures in serum-free conditions. Whenever stated, neutralizing antibody against PDGF was added to conditioned media immediately before incubation.

ELISA

MCF7 cells were incubated with progesterone or ethanol (vehicle) in serum-free conditions for 24 h. Conditioned media were then removed, centrifuged, filtered, quantified by spectrophotometry (Jenway, 6405 UV/vis, Essex, UK) and stored at -80°C. Quantification of released PDGF-AA in conditioned medium was assessed using human PDGF-AA (DAA00) Quantikine ELISA kits (R&D systems, Inc.). Optical density was measured at 490 nm in a spectrophotometer. The amount of PDGF-A in cell supernatants was expressed relative to protein content (ng/mg protein).

TUNEL Assays

Cells grown until 70% confluence onto glass coverslips were incubated with 10^{-8} M progesterone or ethanol for 24 h. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling) was performed using the in situ cell death detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a magnification 200$\times$ field. One thousand nuclei were evaluated. Three independent experiments were performed.

Invasion Assays

The invasive behavior of FLTR cells in the presence of conditioned media from MCF7 cells was determined in vitro by counting how many cells invaded through Matrigel-coated Transwell BD-Matrigel basement membrane matrix

inserts (BD-Biosciences, Belgium). Briefly, transwell inserts containing an 8 μm pore-size PET membrane coated with a uniform layer of Matrigel basement membrane were used. FLTR smooth muscle cells (1×10^4) were harvested on inserts in serum-free medium, and placed on wells containing conditioned medium from Progesterone-treated or ethanol-treated MCF7 cell cultures. Treatments were added in triplicate wells. After incubation for 24 h at 37°C and 5% CO_2, membranes were removed from inserts, stained with DAPI-methanol (Roche Diagnostics, Basel, Switzerland) for 5 min and visualized under a fluorescence microscope (Olympus, BH-2, UK). Twenty-five random fields of each membrane were counted on the microscope (×200).

Statistical Analyses

All experiments were performed in triplicate. Quantifications are expressed in mean ± standstandard deviation (SD). Samples were evaluated by the analysis of variance test. A difference between experimental groups was analyzed by Student's *t*-test, and was considered statistically significant whenever the probability value was less than 5%.

RESULTS

Presence of Progesterone Receptors in Breast Cancer Cells

To ensure that P4 was actually exerting its effects in breast cancer cells, we first confirmed the expression of progesterone receptors (PR) in four distinct breast cancer cell lines (MCF7, Hs578T, SKBr-3, and T47D) by Western blotting. Progesterone receptor (PR)-A was present in every cell lysates tested, although a higher expression was found in hormone-dependent MCF7 and T47D cells (Fig. 1). Expression of PR-B was found in every cell line except in Hs578T.

Progesterone Increased MCF7 Cell Viability in a Dose- and Time-Dependent Manner

To determine the dose-response effect of P4 on breast cancer MCF7 cells, we used the anchorage-dependent colony formation assay. Treatment of MCF7 cells with P4 at five concentrations (0.1–1000 nM) for 7 days, resulted in a significant increase in colony forming potential (Fig. 2A). The highest increase was found when cells were incubated with 1 and

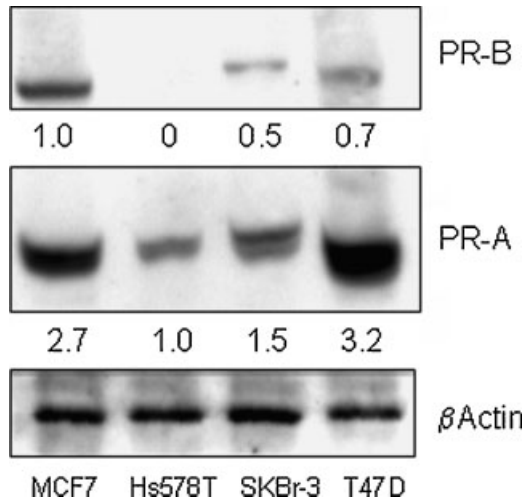

Fig. 1. Expression of PR-A and PR-B in human breast cancer cell lines. MCF7 and T47D cells expressed higher levels of the two PR isoforms. PR-B was absent from Hs578T. The relative intensity of each band after normalization to βActin is shown under each blot, expressed as mean ± SD from three independent experiments. A representative Western blotting is shown.

10 nM. Therefore, P4 was used at the concentration of 10 nM in subsequent studies. Cell proliferation was also evaluated after incubation of MCF7 cells with combinations of identical concentrations of P4 together with 10^{-9} M 17β-estradiol (E2). The presence of this steroid resulted in increased proliferation in every treated cell culture in comparison to P4-treated cells alone (Fig. 2A).

The effect of 10 nM P4 in cell viability was further investigated by MTT assay in a time course assay. Treatment with P4 resulted in a significant increase in MCF7 cells viability after 24 and 48 h ($^*P < 0.01$)(Fig. 2B). For this reason, the experiments described in the following sections were all performed at 24 h time point.

PDGF-A Is a Target Gene of P4 in MCF7 Cells

We next examined the gene expression pattern induced by P4 in MCF7 cells by cDNA arrays. Most of the genes which were increased by P4 by more than two-fold increased by progesterone encoded nuclear proteins playing a role in cell division and DNA repair (Table I). Two other up-regulated genes, platelet-derived growth factor (PDGF)-A and PDGF-associated protein, were involved in cell signaling pathways. A two-fold up-regulation of VEGF gene was also found by progesterone treatment relative to control.

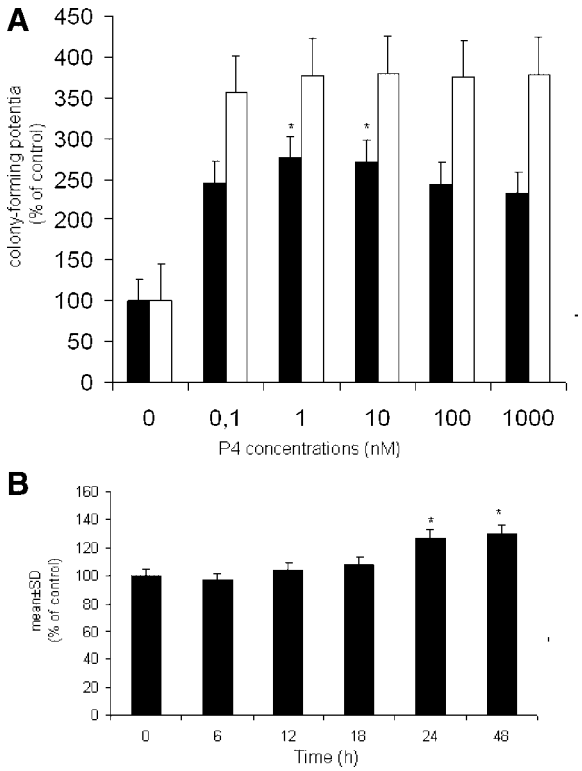

Fig. 2. In vitro treatment of human MCF7 breast cancer cells with progesterone (P4) increased proliferation potential and cell viability in a dose- and time-dependent manner. **A**: Cell proliferation assessed using the clonogenic assay in MCF7 cells. A survival percentage of 100 corresponds to the number of colonies obtained when cells were incubated with vehicle (ethanol). Treatment of cells with 1 and 10 nM P4 significantly increased colony-forming potential recorded after 7-day treatment (*$P < 0.001$ vs. control). Co-incubation of cells with P4 at the different concentrations with E2 increased proliferation rates, although no significant differences between P4 and E2 + P4 were found. Colony forming potential of cells is expressed in terms of percentage of control (0) and reported as mean ± SD. ■, P4 alone; □, distinct concentrations of P4 + 10^{-9} M E2. Experiments were repeated three times with identical results. **B**: Time course effect of P4 on viability of MCF7 cells after treatment with 10 nM P4 by MTT assay. A significant increase in cell viability was found whenever cells were incubated with P4 for 24–48 h (*$P < 0.01$ vs. control). Cell viability after treatment with P4 is expressed in terms of percentage of control cells (0), and is reported as mean ± SD.

TABLE I. Expressed Genes Up-Regulated by 10^{-8} M Progesterone in MCF7 Cells

Gene name	Progesterone/ control ratio
RAD 54	7.13
BARD	6.21
Histone H4	5.91
Aurora-associated protein kinase 1	5.45
PDGF associated protein	5.44
PDGF-A	4.77
Platelet basic protein	4.61
AP4 DNA binding protein	4.23
Cdk8	4.19
KI67	4.05
Cdk9	3.67
MTA1	3.31
G1/S specific Cyclin D1	2.62
VEGF	2.11

Intensity values are mean number of two distinct experiments performed with different RNA samples. Progesterone/control represent fold-increase values relative to vehicle-treated cells.

Progesterone-Induced PDGF-A Plays a Paracrine Role

PDGF proteins consist of a four-member family of potent mitogens and chemoattractant factors in several types of cells [Tallquist and Kazlauskas, 2004]. PDGF-A forms homodimers (PDGF-AA) or heterodimers (PDGF-AB) resulting in activation of tyrosine kinase receptor α (PDGFRα). The PDGF-AB heterodimer can also activate PDGFRβ. To evaluate whether PDGF-A was playing an autocrine role in breast cancer cells, expression of PDGF receptor was examined in the MCF7 cells. Cells incubated with either P4 or ethanol were lysed and the isolated proteins were subjected to Western blotting. Since MCF7 cells are devoid of PDGFRβ [Potapova et al., 1996] and knowing that PDGF-A polypeptide binds either homo- or heterodimers of PDGFRα [Tallquist and Kazlauskas, 2004], we evaluated by Western blotting, the activated form of PDGFR in the four breast cancer cell lines after incubation with ethanol, P4 or E2 + P4 (Fig. 3A). Following binding of PDGF-A, the PDGFRα becomes phosphorylated in its Tyr720 kinase insert domain. Activated PDGFRα was present in every cell line in very small amounts, either in the presence or in the absence of progesterone. These findings suggested that progesterone-induced PDGF-A was secreted by breast cancer cells, playing a putative role in neighboring host cells. To confirm this hypothesis, we next examined whether PDGF-A was actually present in cell culture medium after incubation with progesterone for 24 h. Proteins present in breast cancer cells conditioned media were subjected to SDS-PAGE. A 14 kDa band was clearly identified after 24 h incubation in the medium from progesterone-treated breast cancer cells, indicating that progesterone induced PDGF-A expression and secretion (Fig. 3A). Under physiological conditions P4 is always accompanied by estrogens. Therefore, we examined whether the presence of 17β-estradiol

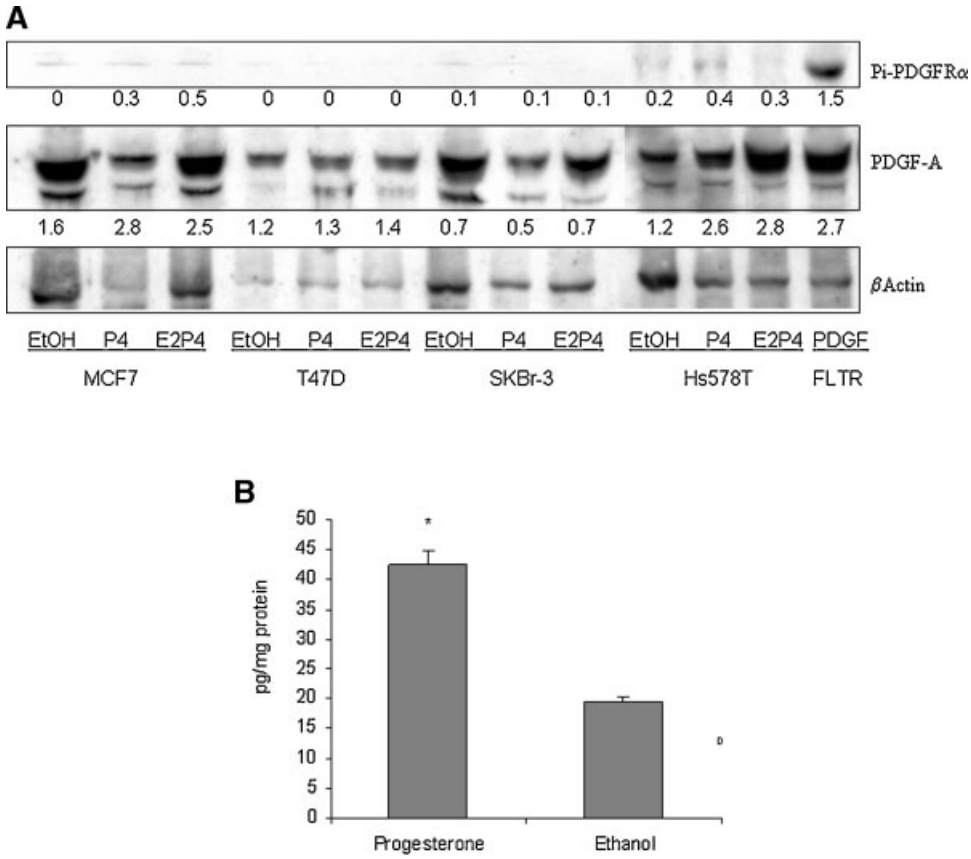

Fig. 3. Effect of P4 in the expression of phosphorylated PDGFRα in cell lysates and PDGF-A in supernatants of human breast cancer cell lines MCF7, T47D, SKBr-3, and Hs578T by Western blotting. **A**: Phosphorylated PDGFRα was found in very small amounts in every cell line independently of the treatments. PDGF-A protein was found in every cell media, although a higher expression was obtained in P4- and E2P4-treated MCF7 and T47D cells. EtOH, proteins from ethanol-treated MCF7 cell lysates/medium; P4, proteins from progesterone-treated MCF7 cell lysates/medium; E2P4, proteins from 17β-estradiol- + progesterone-treated MCF7 cell lysates/medium. Smooth muscle FLTR cells incubated with recombinant PDGF was used as a positive control (PDGF/FLTR). The relative intensity of each band after normalization for βActin is shown under each blot, expressed as mean ± SD from three independent experiments ($P < 0.01$ P4 vs. EtOH for MCF7 and for T47D cells). Representative Western blotting is shown. **B**: PDGF-AA levels in culture supernatants of MCF7 cells incubated with P4 or ethanol by ELISA. Bars represent mean ± SD of three independent experiments. (*$P = 0.02$). Statistical evaluation was performed using a one-way analysis of variance test.

(E2) interfered with PDGFRα activation or with PDGF-A up-regulation. As illustrated in Figure 3A, no significant difference in PDGFRα phosphorylation and in PDGF-A up-regulation was found between these two treatments. These findings reinforce that P4 played a relevant physiological role in inducing PDGF-A secretion independently of the presence of E2.

To quantify PDGF-A release by progesterone-induced MCF7 cells, we performed ELISA. Incubation with progesterone led to a significant increase in the release of PDGF-AA into the cell medium in comparison to vehicle treatment (P-value = 0.02) (Fig. 3B), confirming the increase in PDGF-A secretion by P4.

MCF7 Cells-Secreted PDGF-A Activates PDGF Signaling Pathway in Vascular Smooth Muscle Cells

PDGF is known to play a critical role in recruitment of pericytes to newly-formed vessels [von Tell et al., 2006]. Therefore, we evaluated whether progesterone-induced PDGF-A secretion by MCF7 cells would play a role in smooth muscle cells. Sub-confluent fetal aortic smooth muscle cells (FLTR) that retain much of the phenotype of adult smooth muscle cells were exposed to P4- or ethanol-treated MCF7 conditioned medium for 24 h. Since PDGF-A specifically binds to PDGFRα, we next examined the activation of this receptor in FLTR cell lysates.

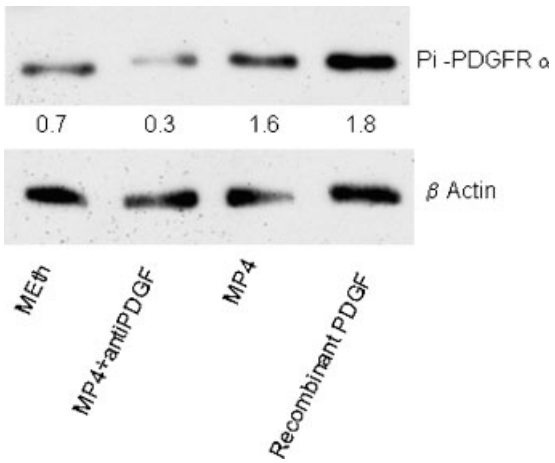

Fig. 4. P4-induced PDGF-A release by MCF7 cells activated PDGFRα signaling pathway in smooth muscle cells. FLTR cells were incubated with ethanol-treated conditioned medium (MEth), progesterone-treated conditioned medium in the presence (MP4 + antiPDGF) and in the absence (MP4) of neutralizing PDGF antibody or recombinant PDGF. Immunoblotting for phosphorylated PDGFRα (Pi- PDGFRα) showed activation of the receptor in FLTR cells incubated with progesterone-treated conditioned medium. Activation of PDGFRα is abrogated in the presence of PDGF neutralizing antibody. Recombinant PDGF was used as a positive control for PDGFRα activation. Equivalent protein loading was confirmed by probing stripped blots for βActin as shown. The relative intensity of each band after normalization for βActin is shown under each blot, expressed as mean ± SD from three independent experiments.

Activation of PDGFRα was found in cell lysates after incubation with conditioned medium from progesterone-treated cells, but not in FLTR cells incubated with ethanol-treated cells (Fig. 4). Inhibition of the PDGF pathway in FLTR cells, with the PDGF neutralizing antibody resulted in inactivation of PDGFRα (Fig. 4). Recombinant PDGF-AA was used as a positive control for PDGFRα activation. These findings indicated that progesterone-induced MCF7 secretion of the PDGF-A activated PDGF signaling pathway on smooth muscle cells through PDGFRα phosphorylation.

P4-Induced PDGF Signaling Pathway in FLTR Cells Regulates Cell Survival but not Invasion

We next investigated smooth muscle cell viability, apoptosis and invasive capacity upon paracrine PDGF signaling stimulation. Increased cell viability assessed by MTT assay, was found after incubation with P4-treated MCF7 conditioned medium (MP4) as compared with exposure to conditioned medium from vehicle-treated cells (MEth) ($P < 0.001$) (Fig. 5A). PDGF blockade with specific neutralizing antibody significantly reversed the MP4-induced increase in viability ($P < 0.01$ of MP4 + anti PDGF vs. MP4).

Consistent with the previous findings, we observed using TUNEL analysis, a significant decrease in the number of apoptotic cells in cultures exposed to MP4 alone in comparison to cells incubated with MEth (Fig. 5B). Moreover, blockade of PDGF signaling (MP4 + antiPDGF) abrogated this anti-apoptotic effect.

We then addressed the role of secreted PDGF-A in the invasive capacity of smooth muscle cell. FLTR cells (1×10^4) were cultured on inserts in serum-free conditions and distinct conditioned media were used as chemoattractant agents on a transwell assay for 24 h. No significant difference in invasive capacity of FLTR cells was observed between cells exposed to MP4, MP4 + antiPDGF, recombinant PDGF-AA or MEth (Fig. 5C). These findings indicate that PDGF-A is not involved in smooth muscle cell invasiveness capacity.

DISCUSSION

We first addressed the expression of PR-A and PR-B on four human breast cancer cell lines. As expected, the hormone-dependent cell lines, MCF7 and T47D, presented higher levels of PR-A and PR-B isoforms, whereas expression of PR in the hormone-independent Hs578T and SKBr-3 cell lines was decreased. Studies performed in knock out mice demonstrated that the PR-B isoform elicits proliferation and differentiation pathways in normal breast tissue and, in addition, PR-A acts as a PR-B function controller [Sitruk-Ware and Plu-Bureau, 2004]. Our study is consistent with those findings, since both PR isoforms were significantly expressed in MCF7 and T47D cell lines, indicating that P4 is able to activate PR-dependent signaling pathways within these two hormone-dependent cell lines. It is well established that low concentration of estrogens are able to stimulate PR expression. We also tested whether E2 affected P4-induced proliferation and PDGF gene expression. Although a slight increase in cell growth was already found after incubation with P4 alone, the presence of E2 resulted in increased proliferation. These findings are in agreement with the literature, which previously demonstrated that P4 in MCF7 cells enhanced primarily differentiation whereas

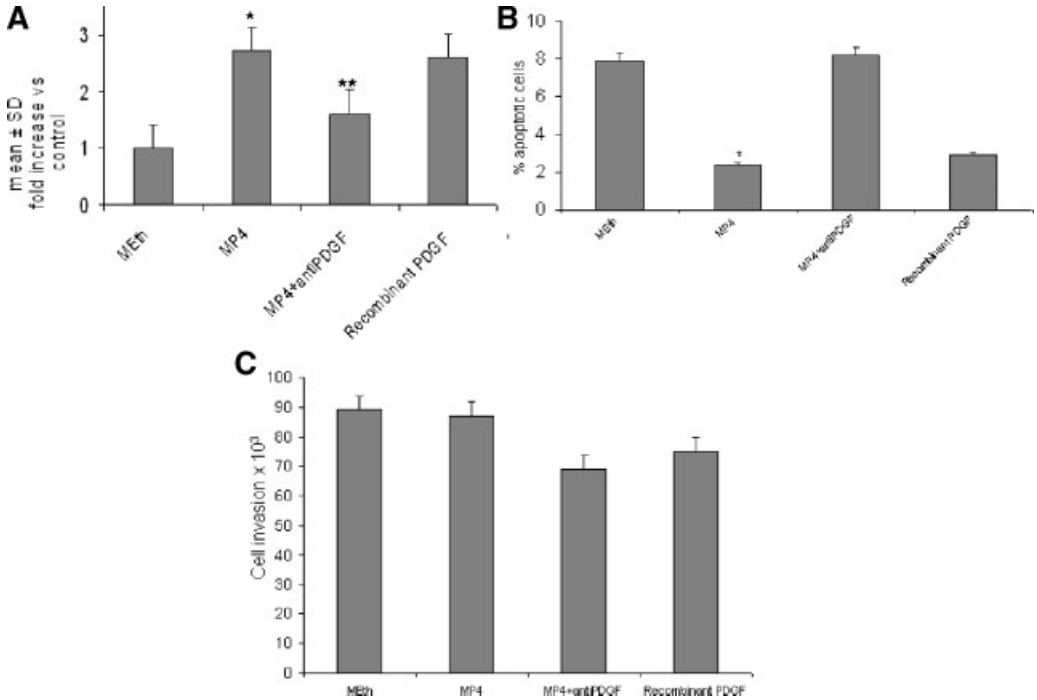

Fig. 5. Role of progesterone-induced PDGF pathway on FLTR behavior. FLTR cells were incubated with ethanol-treated conditioned medium (MEth), progesterone-treated MCF7 cell conditioned medium in the presence of PDGF neutralizing antibody (MP4 + antiPDGF) or in its absence (MP4) or recombinant PDGF protein for 24 h in serum-free conditions. **A**: MTT proliferation assay was assessed. Increased cell viability was found in cells incubated with MP4 (*$P < 0.001$ MP4 vs. MEth). A significant decrease in cell viability was observed whenever cells were incubated in the presence of antiPDGF (**$P < 0.01$ refers to differences between MP4+antiPDGF vs. MP4). Bars represent fold increase relative to cells treated with MEth, and are mean values of three experimental samples. Error bars represent standard deviations between distinct wells. Experiments were repeated three times with identical findings. **B**: Incubation of FLTR cells with MP4 led to decreased apoptosis (*$P = 0.001$ MP4 vs. MEth). Note that this effect was reversed by the presence of antiPDGF neutralizing antibody. Recombinant PDGF was used as a positive control for PDGF effects. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results. **C**: FLTR cells invasion was evaluated in transwell assays using the conditioned medium described above or recombinant PDGF-AA protein as chemoattractant. No significant difference in invasive capacity was obtained in cells incubated with the distinct treatments. Bars represent the number of invasive cells. Assays were repeated twice and performed in triplicate.

proliferation was mainly caused by E2 [Alkhalaf et al., 2002]. Nevertheless, the gene expression profile of MCF7 cells previously treated with P4 alone comprised genes involved in cell cycle and DNA repair. Accordingly, Groshong et al. [1997] proposed that treatment of breast cancer cells with progesterone first drives cells to G_1/S boundary phase and then induces cell changes that enable other growth factors to influence cancer cell behavior.

Progesterone enhances tissue remodeling of the uterus, an essential feature for embryo implantation. Stimulation of angiogenic growth factors by this hormone has been broadly described [Mirkin et al., 2003; Wu et al., 2004; Jeong et al., 2005; Liang and Hyder, 2005]. In the present study, we demonstrated for the first time that progesterone up-regulated PDGF. Furthermore, this up-regulation was independent from the presence of E2 as shown by densitometry analyses of the Western blotting assays. Estrogens did not interfere in PDGF overexpression induced by P4. The low expression of PDGFRα in MCF7 cells together with the presence of PDGF-A protein in cell culture medium prompted us to conclude that PDGF-A might play a direct or indirect role in host neighboring cells.

A possible target cell for PDGF-A is the smooth muscle cell [Edelberg et al., 2002; Machens et al., 2002; Tsutsumi et al., 2004]. PDGF has a potent mitogenic effect in cells of mesenchymal origin. In addition, the expression of the two PDGF receptors has been described in these blood vessel support cells [Taylor, 2000]. Given the fact that sprouting

vessels express PDGFRα [Dimmeler, 2005], P4-induced PDGF-A would likely influence smooth muscle cell behavior.

We first showed that conditioned medium obtained from progesterone-treated MCF7 cells (MP4) actually activated PDGFRα in smooth muscle cells. The absence of phosphorylated PDGFRα after incubation with MP4 + PDGF neutralizing antibody confirms the dependence on secreted PDGF-A. Emerging evidence shows that smooth muscle cell viability is enhanced by PDGF [Hellstrom et al., 1999; Bonello et al., 2005]. In our study, conditioned medium from progesterone-treated cells stimulated smooth muscle cells' viability/proliferation and prevented apoptosis. These results are due to the presence of PDGF-A in cell medium, since they were reversed by the PDGF inhibitor. The fact that treatment with PDGF neutralizing antibody only partially abrogated cell survival suggests that other MCF7 released factors might be involved. One possibility is VEGF, which was also found to be two-fold up-regulated in MCF7 cells (Table I), and it is known to play a role in smooth muscle cell survival [Liang and Hyder, 2005]. Noticeably, neither MP4 nor recombinant PDGF-AA induced FLTR cells invasiveness, as evaluated in double chamber invasive assay. The PDGF pathway induces survival and migration in smooth muscle cells [Wilkinson-Berka et al., 2004; Bonello et al., 2005]. However, PDGF receptors have overlapping but also distinct biological functions. According to the literature, PDGFRα is involved in proliferation of smooth muscle cells, whereas PDGFRβ induced migration of these cells [Wilkinson-Berka et al., 2004]. Our array results showed that progesterone only up-regulated PDGF-A, which specifically binds to PDGFRα. On the other hand, activation of PDGFRα inhibits PDGFRβ-induced migration [Heldin and Westermark, 1999].

In conclusion, the current study focused on the role of progesterone in smooth muscle cell activation, an essential feature in the late steps of angiogenesis. Loss of pericytes leads to vessel leakage, impairing blood flow. Thus, this cross-talk between P4 and PDGF signaling might be crucial for the steady angiogenesis that is observed in progesterone-dependent breast cancers. We are aware that secondary factors, such as the two-fold up-regulation of VEGF, might also account for this process. Additionally, P4 is probably playing relevant roles in other host cells, including endothelium and fibroblasts. Tumor-secreted PDGF-A is known to result in VEGF-producing fibroblast recruitment [Dong et al., 2004], reinforcing the complex interaction between tumor cells and distinct host cells through growth factors. According to our in vitro study, the use of hormonal therapy against progesterone would target not only progesterone-dependent cancer cells, but also block this paracrine role exerted by the hormone, also preventing tumor vessel stability. In vivo studies are now required in order to determine whether progesterone-blocking therapy is important in decreasing tumor angiogenesis. Taking advantage of the Novartis inhibitor, Imatinib, which inhibits PDGF tyrosine kinase activity, further studies are underway to address the mechanisms of this cross-talk between progesterone and PDGF signalling pathway both in in vitro and in vivo models.

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