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Stimulation of PC Cell-Derived Growth Factor (Epithelin/Granulin Precursor) Expression by Estradiol in Human Breast Cancer Cells

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PC cell-derived growth factor (PCDGF) is an 88 kDa glycosylated protein isolated from a highly tumorigenic mouse teratoma derived cell line which is similar to the epithelin/granulin precursor. Using Northern blot and western blot analyses, we detect the expression of PCDGF mRNA and protein in MCF-7 human breast cancer cells. We show that 17-β-estradiol stimulates PCDGF mRNA and protein expression in a time and dose-dependent manner. The stimulation of PCDGF expression by 17-β-estradiol was observed as early as 4 hours and reached a maximum at 12 hours. Maximal stimulation of PCDGF mRNA and protein expression by 17-β-estradiol was observed at a concentration of 10^{-8} M. The stimulation of PCDGF expression by 17-β-estradiol was completely inhibited by treatment with actinomycin D and with the antiestrogen 4-hydroxytamoxifen. The stimulation of PCDGF expression was also demonstrated in another human estrogen-responsive cell line T47D. The results presented here provide evidence of a novel estradiol responsive gene product in human breast cancer cell lines and give information about the hormonal control of epithelin/granulin (PCDGF) expression in these cells.

PC cell derived growth factor is an 88 kDa glycosylated protein purified from the highly tumorigenic teratoma-derived cell line PC (1). Structural analysis of PCDGF indicated that it consisted of a 68 kDa core protein and a 20 kDa carbohydrate moiety (1). Amino-acid and cDNA sequencing indicated that PCDGF cDNA was identical to the epithelin/granulin precursor (2–3). Epithelin and granulins are 6 kDa double cysteine-rich polypeptides originally purified from rat kidney (4) and from human granulocyte extracts (5) characterized by a unique highly conserved motif (6). These polypeptides have subsequently been identified throughout the entire vertebrate kingdom (6). Epithelins have been shown to act as dual growth effectors that promote or inhibit the growth of various mammalian cells in culture including keratinocytes and human epidermoid carcinoma cell line A431 (4). Interestingly, epithelin 2 can antagonize the mitogenic action of epithelin 1 (4) on keratinocyte whereas they both inhibit proliferation of human breast cancer cells (7). Cloning of cDNA for epithelins and granulins has shown that they are encoded by a 593 amino-acids common precursor called epithelin/granulin precursor containing a secretory signal peptide and seven and a half repeats of the cysteine rich 6 kDa motifs (4–5). Although it was postulated that epithelin/granulin precursor needed to be processed into biologically active 6 kDa polypeptides (4), several examples have been reported of the biological importance of the precursor. Acrogranin, a 67 kDa glycoprotein found in the acrosomal compartment of the sperm head has an amino-terminal sequence identical to the precursor (8). The 88 kDa glycoprotein PC-cell derived growth factor was shown to stimulate the proliferation of fibroblasts cells (1). Moreover, the granulin/epithelin precursor also stimulated the growth of mouse embryo fibroblasts null for the type 1 insulin-like growth factor receptor (9). In this paper, we investigated the expression of PCDGF in human breast cancer cell lines and showed that its expression is stimulated by estradiol.

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Abbreviations used: E_2: 17β-estradiol; ER: estrogen receptor; ERE: estrogen response element; FBS: fetal bovine serum; α-MEM: phenol-red free α-modified Eagle's medium; PCDGF: PC cell-derived growth factor; PFMEM: α-MEM supplemented with 5% charcoal-stripped FBS.

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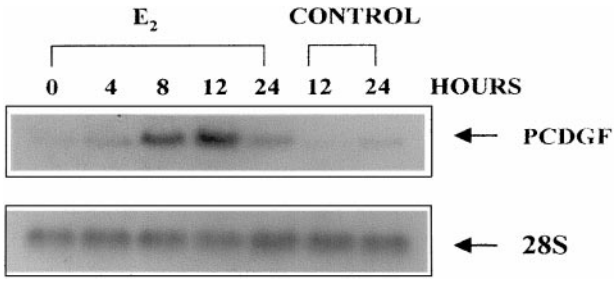

FIG. 1. Time-course of E_2 effect on PCDGF mRNA expression. MCF-7 cells were maintained in steroid-free conditions by being cultured in PFMEM medium for 24h as described in the method section. Cells were then treated with 10^{-9} M E_2. Control cells were treated with the same volume of ethanol only (0.1%). Total RNA was extracted at the indicated times. PCDGF expression was measured by Northern blot analysis. 28S ribosomal RNA expression was measured as internal control for equal RNA loading.

MATERIALS AND METHODS

Materials. 17β-estradiol (E_2), 4-OH-tamoxifen, cycloheximide, actinomycin D were obtained from Sigma. Protein A-Sepharose was from Pharmacia. Culture media and fetal bovine serum (FBS) and Trizol reagent were purchased from Life Technology. Tissue culture plasticware was supplied by Corning Incorporated.

Cell culture. Human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (ATCC) and T47D cells (ATCC) were kindly provided by Dr. Angela Brodie (University of Maryland). Both cell lines were cultivated in Dulbecco's modified Eagle's medium-Ham's F-12 medium (1:1 mixture) supplemented with 5% FBS. For all experiments, cells were cultivated as described before in 60-mm dishes. After reaching 70% confluency, cell monolayers were washed twice with phenol-red free α-modified Eagle's medium (α-MEM) and incubated for 24 hours in α-MEM supplemented with 5% charcoal-stripped FBS (PFMEM). Cells were then washed twice again with α-MEM and then incubated in PFMEM for different times in the presence and absence of various concentrations of agents to be assayed, as indicated in the figure legends.

RNA isolation and Northern blot analysis. Total RNA was extracted using Trizol reagent from duplicate 60-mm dishes. 20 μg total RNA were used to study PCDGF mRNA expression by Northern blot analysis using a human PCDGF cDNA probe carried out as described previously (10). The signals obtained by autoradiography were quantified by densitometric analysis and normalized to the level of 28S ribosomal RNA internal control.

Western blotting. MCF-7 cells were lysed in 1 ml PBS buffer containing 1% Triton X-100 with a protease inhibitor cocktail consisting of 200 μM PMSF, 1 μM leupeptin, 0.5 μM aprotinin and 1 mM EDTA from duplicate 60 mm dishes. Cells lysates and conditioned media from 4×10^6 cells were incubated overnight at 4°C with 5 μl of rabbit anti-human PCDGF polyclonal antibody. The immunocomplexes were then collected by incubation with 50 μl of protein A-Sepharose slurry for 4 hours. The Sepharose beads were washed three times with cold PBS and then boiled in 2 x reducing SDS sample buffer (2% SDS, 10% glycerol, 62 mM Tris-HCl pH 6.8, 1% β-mercaptoethanol) and loaded on a 10% SDS-polyacyamide gel. Proteins were then electrophoretically transferred to a 0.2 μm PVDF membrane (Millipore) at 100 V for 1 hour. The blot was blocked overnight at 4°C in 5% skim milk and then probed with the anti-PCDGF polyclonal antibody for 1 hour at room temperature in PBST buffer (PBS buffer containing 0.05% Tween 20) plus 1% skim milk. After washing three times, 5 min each in PBST, the blot was incubated at room temperature with goat anti-rabbit IgG conjugated to horseradish-peroxidase (KPL) for 1h in PBST containing 1% skim milk. The washing step was repeated twice. Finally, immunoreactivity on the blot was visualized by the enhanced chemiluminescence detection system (Amersham). All experiments were repeated at least three times.

RESULTS

Time-dependent stimulation of PCDGF mRNA expression by E_2. MCF-7 cells were cultivated in phenol-red free α-MEM medium supplemented with 5% charcoal-stripped FBS in the presence or absence of E_2 (10^{-9} M). Total RNA was extracted at indicated times to measure PCDGF mRNA expression by Northern blot analysis (Fig. 1). Results showed that MCF-7 cells expressed PCDGF mRNA and that PCDGF mRNA expression increased in a time-dependent fashion upon treatment with E_2. Densitometric analysis of the data indicated that the increase in PCDGF mRNA expression was 1.5-fold after 4 hours of exposure to E_2, reached a 5-fold maximum induction at 12 hours and decreased to 2-fold above basal level at 24 hours. PCDGF mRNA expression in control untreated cells remained at a low level throughout the same period.

Dose-dependent stimulation of PCDGF mRNA expression by E_2. MCF-7 cells were treated with different concentrations of E_2 (10^{-14} to 10^{-8} M) for 12h followed by RNA extraction and Northern blot analysis. As shown in Fig. 2A, treatment of MCF-7 cells with increasing concentrations of E_2 resulted in a dose-dependent increase in the level of PCDGF mRNA expression. The stimulation of PCDGF mRNA expression by E_2 was observed at concentrations as low as 10^{-12} M and was maximal with a 5-fold stimulation of PCDGF

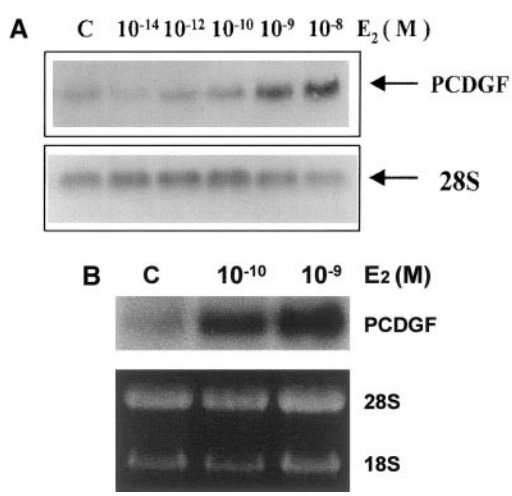

FIG. 2. Effect of increasing concentrations of E_2 on PCDGF mRNA expression. MCF-7 and T47D cells at 70% confluence were cultivated in PFMEM medium for 24h as described above. Cells were then treated with the indicated concentrations of E_2. Control cells were treated with the same volume of ethanol (0.1%). Total RNA was extracted 12h later. PCDGF mRNA expression was measured by Northern blot analysis. (A) MCF-7 cells. (B) T47D cells.

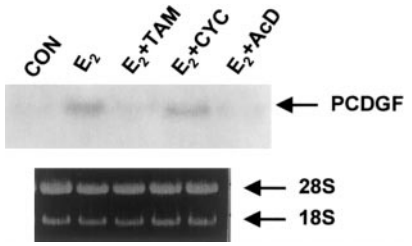

FIG. 3. Effect of 4-OH- tamoxifen and actinomycin D on the stimulation of PCDGF mRNA expression by E_2. MCF-7 cells cultivated in estrogen-depleted medium for 24h were treated for 6h with 10^{-9} M E_2 alone or in the presence of one of the following three compounds: 4-OH-tamoxifen (TAM, 1 μM), Actinomycin D (AcD, 5 μg/ml) and Cycloheximide (CYC, 10 μg/ml). Control cells (CON) not treated with E_2 but with the same volume of ethanol (0.1%) were used as negative control. Total RNA was collected to examine the level of expression of PCDGF mRNA by Northern blot analysis.

expression in cells treated at 10^{-8} M E_2, a concentration known to maximally stimulate expression of estrogen-responsive genes such as progesterone receptor in MCF-7 cells (11).

The induction of PCDGF mRNA expression was also observed in another ER-positive cell line T47D with a four-fold stimulation of PCDGF mRNA expression at 10^{-9} M E_2 (Fig. 2B).

Effect of Tamoxifen on PCDGF mRNA stimulation by E_2. Experiments were carried out to examine whether the stimulatory effect of PCDGF expression was inhibited by treatment with the antiestrogen 4-OH-Tamoxifen. We used a concentration of 1 μM of 4-OH-tamoxifen which has strong antagonist activity with no partial agonist activity (12), As shown in Fig. 3, treatment of MCF-7 cells with 4-OH-tamoxifen blocked the increase of PCDGF expression by E_2 (10^{-9} M). These data suggest that the effect of E_2 on PCDGF expression is mediated by estrogen receptor.

We then examined the effect of protein synthesis inhibitor cycloheximide (10 μg/ml) and RNA synthesis inhibitor actinomycin D (5 μg/ml) on the stimulation of PCDGF mRNA expression by short-term treatment with E_2 (10^{-9} M). As shown in Fig. 3, the stimulatory effect of E_2 on PCDGF mRNA expression was completely abolished by treatment with actinomycin D but not with cycloheximide. These data suggest that the stimulation of PCDGF expression by E2 requires *de novo* mRNA synthesis.

PCDGF protein expression is stimulated by E_2. Cell lysates and conditioned media of MCF-7 cells were collected at various times after treatment with E_2 (10^{-9} M) to examine PCDGF protein expression using immunoprecipitation and western blot analysis with anti-human PCDGF antibody as described in the materials and methods section. As shown in Fig. 4, treatment of MCF-7 cells with E_2 resulted in a time-dependent stimulation of PCDGF expression in cell lysates, reaching a maximum at 12h. E_2 treatment also caused the accumulation of PCDGF protein in conditioned medium. The cell lysates and conditioned media of control cells (0.1% ethanol only) showed a low, stable level of PCDGF protein expression throughout the same period (data not shown).

DISCUSSION

PC cell derived growth factor (PCDGF) is an 88 kDa glycoprotein corresponding to the precursor of the 6 kDa double-cysteine rich polypeptides called epithelins or granulins (1–4). We show here that human mammary carcinoma cells MCF-7 and T47 D express PCDGF mRNA and protein and that the 88 kDa precursor protein is secreted in their culture medium. Data presented here demonstrate that estrogen stimulates PCDGF expression in MCF-7 cells in a time and dose-dependent fashion. E_2 also stimulates the time-dependent production of PCDGF protein in a pattern similar to the stimulation of PCDGF mRNA expression. The stimulation of PCDGF expression by E_2 is not restricted to MCF-7 cells since it was also demonstrated in another estrogen responsive cell line T47D. PCDGF mRNA and protein were also detected in the ER-negative human breast cancer cells MDA-MB-468 and MDA-MB-453 but the level of PCDGF expression was not stimulated by estradiol treatment (Lu and Serrero, unpublished result).

The fact that 4-hydroxytamoxifen abolishes the stimulatory effect of E_2 suggests that the induction of PCDGF expression is mediated via an estrogen receptor (ER)-related pathway.

The promoter regions of mouse and human epithelin/granulin precursor have been characterized (13–14). Analysis of the sequence failed to indicate the presence of a classical estrogen responsive element (ERE) in the proximal region of the precursor promoter (6), suggesting that any steroid effect may be indirect. However, very few of the human mRNAs known to be directly regulated by estrogen in mammary epithelial cells have been shown to be induced from a canonical ERE

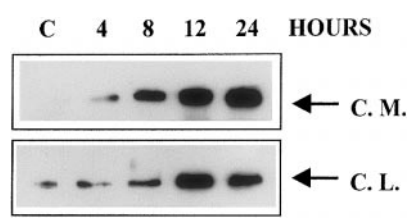

FIG. 4. PCDGF protein expression in MCF-7 cell lysates and conditioned media during incubation with E_2. MCF-7 cells were cultivated in PFMEM medium for 24h. The medium was removed and replaced with fresh PFMEM medium. Cell lysates (C.L.) and conditioned media (C.M.) were collected at indicated times after incubation with 10^{-9} M E_2 and Western blot analysis of PCDGF expression was performed as described in the materials and methods section.

(15). Most estrogen-responsive genes identified to date contain one or more imperfect EREs or multiple copies of an ERE half-site rather than a classical ERE (16–17). Such ERE half sites have been found in PCDGF promoter region (13–14). Detailed promoter studies should allow to explore this question.

The intact 88 kDa epithelin/granulin precursor, also called PCDGF, was originally purified from the conditioned medium of a teratoma derived cell line of mesenchymal origin (1). The data presented here show that cells of epithelial origin can also secrete the intact precursor. The expression of the precursor in epithelial cells as well as in fibroblasts would suggest a widespread role for PCDGF in contrast to some other growth factors that have a more restricted distribution. Receptors for the 6 kDa epithelin, the processed form of PCDGF have been characterized on human mammary epithelial carcinoma cells MDA-MB-468 by Scatchard analysis of binding studies and chemical cross-linking of ^{125}I-labeled epithelin (7). Two classes of binding sites with an apparent molecular weight of 145 kDa were reported on these cells. Cell surface binding sites for ^{125}I-PCDGF with an apparent molecular weight of 120 kDa have also been characterized by Scatchard analysis and by affinity labeling in several cell lines of mesenchymal and epithelial origins including mouse mammary epithelial cells (18). The fact that mammary epithelial cells secrete the epithelin/granulin precursor is interesting as it raises questions about the possible role of various members of the same family of polypeptide growth factors in these cells. Our preliminary results indicate that PCDGF is a mitogen for mouse and human mammary epithelial cells (Xia, Lu and Serrero, unpublished result). Experiments are performed to examine in detail the role of PCDGF in the growth of human mammary epithelial cells.

In summary, the results presented here provide novel information about the hormonal control of epithelin/granulin expression and identify a novel gene regulated by estradiol in mammary epithelial cells.

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