PC cell-derived growth factor (PCDGF/GP88, progranulin) stimulates migration, invasiveness and VEGF expression in breast cancer cells

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Metastasis is a multi-step process involved in the progression of breast cancer to a disease with poor prognosis. Growth factor and/or growth factor receptor over-expression have been reported to play an important role in this process. The 88 kDa glycoprotein PC cell-derived growth factor (PCDGF/GP88), also known as progranulin, has been shown to play a major role in breast tumorigenesis by stimulating proliferation, mediating survival and conferring resistance to tamoxifen. In the present paper, the metastatic potential of PCDGF/GP88 was examined in breast cancer. Using MCF-7 cells, we showed that PCDGF/GP88 over-expression stimulated anchorage-independent cell growth and accelerated cell migration through matrigel. Similar results were obtained with MCF-7 cells treated exogenously with PCDGF/GP88. Furthermore, gelatin zymograph and immunoblot revealed that matrix metalloprotease-9 was up-regulated by PCDGF/GP88. PCDGF/GP88 stimulated VEGF expression in MCF-7 cells. These results suggest that PCDGF/GP88 could act to promote metastasis and angiogenesis in human breast cancer cells in addition to stimulating their proliferation and survival.

Introduction

The 88 kDa glycoprotein PC cell-derived growth factor (PCDGF/GP88) is an autocrine growth factor, first isolated from the highly tumorigenic mouse teratoma PC cells (1). PCDGF/GP88, also known as progranulin, is the largest member of the granulin/epithelin family of cystein-rich polypeptide growth modulators (2, 3). It has been reported that PCDGF/GP88 stimulated the proliferation and survival of several cell types of mesenchymal and epithelial origin by stimulating proliferation, mediating survival and conferring resistance to tamoxifen. In the present paper, the metastatic potential of PCDGF/GP88 was examined in breast cancer. Using MCF-7 cells, we showed that PCDGF/GP88 over-expression stimulated anchorage-independent cell growth and accelerated cell migration through matrigel. Similar results were obtained with MCF-7 cells treated exogenously with PCDGF/GP88. Furthermore, gelatin zymograph and immunoblot revealed that matrix metalloprotease-9 was up-regulated by PCDGF/GP88. PCDGF/GP88 stimulated VEGF expression in MCF-7 cells. These results suggest that PCDGF/GP88 could act to promote metastasis and angiogenesis in human breast cancer cells in addition to stimulating their proliferation and survival.

Abbreviations: MMP-9, matrix metalloprotease-9; PCDGF, PC cell-derived growth factor.
Cells were maintained in DMEM/F12 supplemented with 5% FBS in the presence of 400 μg/ml G418.

**RT-PCR**

MCF-7 EV cells were cultivated in phenol red-free DMEM/F12 supplemented with 1% charcoal-stripped serum and 1 mM 17-β estradiol (E2), PCDGF/GP88 (400 ng/ml) or vehicle only. O4 cells were cultivated in phenol red-free DMEM/F12 with 1% charcoal-stripped serum. For the experiments with tamoxifen, 1 mM tamoxifen was added to the culture medium. Medium was changed at day 3. RNA samples were collected at day 3 using Trizol. Five micrograms of total RNA were reverse transcribed into single strand cDNA by Superscript II (Gibco), using 250 ng of random hexamer (Gibco) as primer. The RT reaction was carried out for 1 h at 42°C in 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, DTT 0.01 M and dNTP (each 0.5 mM). A total of 30-35 PCR cycles depending on the gene amplified was performed, followed by electrophoresis on 1% agarose gel. The specific primer pairs are: for glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer 5′-TGAAGGTCGGAATCCACCAAGATTGGT-3′; reverse primer 5′ CATGGGGCCTGAGGCTCACCAC 3′; for VEGF: forward primer 5′ ATGAACTTTCTGCTGTCTTGGGT 3′, reverse primer 5′ TCACCGCTCCGGCTTTGTCAG 3′.

**Matrigel migration assay**

PCDG/GP88 over-expressing cells (O4) and MCF-7 EV were seeded at a density of 1 × 10⁵ cells onto matrigel coated inserts (100 μM/cm²; 8 μM pore size) in 24-well plates (Beckton Dickinson Labware, Bedford, MA). The cells were incubated in phenol red-free DMEM/F12 medium supplemented with 1% charcoal-stripped serum with or without 400 ng/ml of PCDGF/GP88. After a 12-h incubation, the cells on the inserts were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS and stained with hematoxylin qs (Vector Laboratories, Burlingame, CA). The cells on the upper side of the inserts were removed with a cotton swab. Fixed cells that migrated to the lower side of the filters were counted with a microscope.

**Soft agar assay**

Anchorage-independent cell growth was determined by soft agar colony forming assay. 10 000 MCF-7 or O4 cells were inoculated in 1 ml of 0.33% agar in tissue culture medium containing 10% FBS in 35 mm plates over a bottom layer of 1.5 ml of 0.6% agar in DMEM/F12 supplemented with 10% FBS. The cells were allowed to grow for 21 days with re-feeding with 200 μl of DMEM/F12, every 3 days. Colonies were stained with 0.005% crystal violet for an hour. Experiments were carried out in quadruplicate.

**Zymograph assay and western blot for MMP-9**

Five million cells were plated in DMEM/F12 medium with 5% FBS. After 24 h incubation and subsequent washing, the medium was replaced with serum-free medium supplemented with vehicle only or 400 ng/ml of PCDGF/GP88. After a 72-h incubation, the medium was replaced with serum-free medium supplemented with vehicle only or 400 ng/ml of PCDGF/GP88. Medium was collected 24 h later and concentrated with Centriprep YM-10 (Millipore, Bedford, MA). The amount of concentrated medium used in the assay was normalized to the cell number. Samples were diluted in 50 mM Tris-HCl pH 7.4 without reduction and separated in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min and incubated for 16 h at 37°C in 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM CaCl₂. Gels were stained with Coomasie brilliant blue R-250 and destained in 40% methanol, 10% acetic acid until clear bands appeared.

For western blot analysis of MMP-9 expression, conditioned media were collected and concentrated as described above. Samples were mixed with Laemmli buffer in reducing conditions and separated in a 7.5% SDS-PAGE. Proteins were transferred to PVDF membrane for western blot analysis of MMP-9 expression with 1 μg/ml anti-human MMP-9 mouse monoclonal antibody from Oncogene Research (Boston, MA), followed by incubation with HRP-conjugated goat anti-mouse-IgG. Immunoreactive proteins were detected by enhanced chemiluminescence kit (Pierce, Rockford, IL).

**Statistical analysis**

All experiments were conducted in triplicates or quadruplicates and repeated at least three times. Data were analyzed by Student’s t-test for mean comparison and statistical significance (P < 0.05). The values are reported as mean ± SEM.

**Results**

**PCDG/GP88 expression stimulates anchorage-independent growth in vitro**

The ability of PCDGF/GP88 to stimulate anchorage-independent growth of MCF-7 cells was first examined. For this purpose, we compared the ability of MCF-7 cells over-expressing PCDGF/GP88 (O4 cells) and control cells (MCF-7 EV) to form colonies in soft agar. As shown in Figure 1, there was a 4.5-fold increase in the number of colonies formed by O4 cells when compared with MCF-7 EV cells (P < 0.05).

**Effect of PCDGF/GP88 on cell invasion through matrigel**

To assess the effect of PCDGF/GP88 on extracellular matrix invasion, we examined the ability of PCDGF/GP88 to stimulate cell migration through matrigel. As shown in Figure 2, the number of cells migrating through matrigel was significantly higher in MCF-7 EV cells treated with PCDGF/GP88 when compared with untreated cells (2.9-fold increase, P < 0.05).

Similarly, increased cell migration was also observed in PCDGF/GP88 over-expressing MCF-7 cells (O4 cells) when compared with control MCF-7 EV cells (3.2-fold increase,
The data suggest that PCDGF/GP88 induces cell migration through extracellular matrix.

**PCDGF/GP88 promotes MMP-9 secretion by MCF-7 cells**

We investigated the effect of PCDGF/GP88 on MMP-9 expression using two assays. A zymograph assay, using gelatin as substrate, was used to determine whether PCDGF/GP88 stimulated the secretion of gelatinase from MCF-7 cells. As shown in Figure 3 (upper panel), untreated MCF-7 EV cells displayed a minimal gelatinase activity. Treatment of the cells with 400 ng/ml of PCDGF/GP88 for 24 h prior to collecting the medium resulted in the presence of a strong gelatinolytic activity migrating at a molecular weight corresponding to MMP-9. Increased gelatinolytic activity was also observed in PCDGF/GP88 expressing MCF-7 cells (O4 cells).

The stimulation of MMP-9 secretion by PCDGF/GP88 was examined by immunoblot analysis using anti-MMP-9 specific antibody. As shown in Figure 3 (lower panel), the level of MMP-9 protein secreted in the culture medium was greater in MCF-7 EV treated with PCDGF/GP88 and O4 cells than in control untreated MCF-7 EV cells (11.4- and 9.8-fold, respectively).

**PCDGF/GP88 promotes VEGF expression in MCF-7 cells**

VEGF is the major angiogenic factor involved in tumor neovascularization. The ability of PCDGF/GP88 to stimulate VEGF expression in MCF-7 cells was investigated. Reverse transcription PCR was used to examine VEGF expression in MCF-7 cells. The primer set selected for VEGF (see Materials and methods section) permitted the detection of the six VEGF transcripts: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206 (22). Only three transcripts VEGF121, VEGF165 and VEGF189 were detectable in MCF-7 cells. As shown in Figure 4, treatment of MCF-7 cells for 5 days with PCDGF/GP88 induced a 2.4-fold increase in the expression of VEGF transcripts. This stimulation was comparable with the one observed with a 5 day treatment with estradiol (3.2-fold), a known stimulator of VEGF expression in MCF-7 cells (23,24).

Interestingly tamoxifen, which inhibited the effect of estradiol on VEGF, was unable to prevent the stimulation of VEGF observed with PCDGF/GP88 in MCF-7 EV cells (Figure 5).

Increased VEGF expression was also observed in PCDGF/GP88 over-expressing cells when compared with MCF-7 EV cells. As shown in Figure 6, tamoxifen treatment of the PCDGF/GP88 over-expressing cells (O4 cells) further stimulated VEGF expression in PCDGF/GP88 over-expressing cells.

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**Fig. 3.** Stimulation of MMP-9 expression by PCDGF/GP88. Gelatinolytic activity of MCF-7 EV, MCF-7 EV + PCDGF/GP88 (P) and O4 cells was determined as described in the Materials and methods section (upper panel). MMP-9 expression in the cells was determined by western blot using anti-MMP9 antibody (lower panel).

**Fig. 4.** Effect of PCDGF/GP88 and estradiol on VEGF expression in MCF-7 cells. MCF-7 EV cells were cultivated in phenol red-free medium supplemented with 1% charcoal-stripped serum supplemented with vehicle only, 400 ng/ml PCDGF/GP88 or 1 nM estradiol (E2). VEGF expression was determined by RT-PCR as described in the Materials and methods section. GAPDH expression was measured as internal standard. Upper panel: ethidium bromide staining of amplified products after agarose gel electrophoresis. Lower panel: determination of relative intensity of VEGF transcripts normalized to GAPDH expression. The data were expressed as fold stimulation above control corresponding to the level of expression of transcripts in MCF-7 EV cells treated with vehicle only.

**Fig. 5.** Effect of PCDGF/GP88 treatment in VEGF expression. MCF-7 EV cells were cultivated as described in the Materials and methods section. 400 ng/ml PCDGF/GP88 (P) 1 μM Tamoxifen (T) or vehicle only was added into the culture and incubated for 5 days. Estradiol (E2) was used as a positive control. RNA samples were isolated and subjected to RT-PCR for VEGF expression determination. Upper panel shows PCR product of VEGF. Lower panel shows the relative density of VEGF mRNA expression. Band intensity was normalized to GAPDH and compared with the level of expression in MCF-7 EV cells treated with vehicle only (control).
Neovascularization or angiogenesis is a crucial process required for solid tumor progression beyond a certain size. We have shown previously that PCDGF/GP88 over-expression stimulates VEGF expression in MCF-7 cells in culture. The data would suggest that the increased VEGF expression observed in the tumors was due to a direct stimulation of VEGF expression in the tumor cells by PCDGF/GP88 rather than to an indirect effect on stromal cells found in the tumor environment. Several growth factors including EGF, PDGF, TNF-α, TGF-β stimulate VEGF expression in several cells (36,37). Interestingly, He et al. showed that PCDGF/GP88 induced human microvascular endothelial cell proliferation and promote tube-like structure, suggesting the role in normal angiogenesis (26). Our results showing that PCDGF/GP88 induced VEGF expression would provide a pathway by which PCDGF/GP88 stimulated vascularization is mediated.

Several reports have demonstrated an association between VEGF and MMP-9 expression in several cancer types. VEGF enhanced expression of MMP-9 through Flt-1 in vascular smooth muscle cells (38) whereas Bergers et al. found that MMP-9 acted as an angiogenic switch by causing greater accessibility of VEGF to its receptors (39). In addition, Kurizaki et al. found that pro-MMP-9 expression correlated with the higher intratumoral microvessel density and thymidine phosphorylase expression (14). Taken together, the fact that PCDGF/GP88 stimulates both MMP-9 secretion and VEGF would suggest that PCDGF/GP88 participates in facilitating this correlation.
between matrix metalloproteases expression and tumor angiogenesis in human breast cancer cells.

Our previous in vivo study showed that PCDGF/GP88 over-expressing MCF-7 cells are more tumorigenic in nude mice xenograft model than MCF-7 cells. Moreover, treatment of the PCDGF/GP88 over-expressing cells with tamoxifen further stimulated tumor growth instead of inhibiting it as observed for MCF-7 cells (10). It is suggested that the stimulation of VEGF expression by PCDGF/GP88 and its potentiation by tamoxifen would support the increased tumor growth observed in vivo. Over-expression of VEGF in MCF-7 cells has been shown to promote cell proliferation and provide estrogen-independent tumor growth in vitro and in vivo, suggesting an autocrine/paracrine-activation loop by VEGF (40). It is possible that the potentiation of tumor growth in vivo, observed with tamoxifen and PCDGF/GP88, may be mediated by their stimulatory effect on VEGF expression. We show here that the stimulation of VEGF expression by tamoxifen is also observed in vitro in PCDGF/GP88 over-expressing cells as well as in MCF-7 cells treated with estradiol and PCDGF/GP88. The mechanism underlying this effect is at present unknown. We have shown previously that PCDGF/GP88 up-regulated Bcl-2 expression in MCF-7 and prevented its down regulation by tamoxifen (10). A relationship between bcl-2 and VEGF expression has recently been reported in human melanoma via stimulation of VEGF mRNA stability and promoter-activation (41). The existence of a similar positive association between bcl-2 and VEGF in MCF-7 cells could explain the VEGF stimulation observed with the combination of tamoxifen and PCDGF/GP88 treatment in MCF-7 cells treated with estradiol. Interestingly, estrogen and tamoxifen have been also shown to stimulate VEGF expression in a variety of cell systems including cultured vascular muscle cells (42), uterus (43,44) and endometrial cells (45). In MCF-7 cells, tamoxifen has been occasionally reported as stimulating VEGF mRNA expression in vitro (46). In vivo, tamoxifen treatment has been associated with higher plasma VEGF level in pre- and postmenopausal women (47).

We have shown previously that PCDGF/GP88 over-expression in MCF-7 cells mediated tumorigenicity, tamoxifen resistance and estrogen-independent growth. The data presented here would propose a potential role of PCDGF/GP88 in mediating breast tumor metastasis and angiogenesis via promoting MMP-9 and VEGF expression, implying that PCDGF/GP88 plays a significant role in many aspects of breast cancer.

Acknowledgements

The authors wish to thank Huifang Dai for preparing PCDGF/GP88 used in these experiments. This work was supported by grants R01 CA 85367 from the National Institutes of Health, DAMD 17-01-1-0580 and DAMD 17-01-1-0551 from the Department of Defense Breast Cancer Research program and 9857- AFF and BCTR2000-356 from the Susan G. Komen Breast Cancer Foundation. Wisit Tangkeangsirisin is the recipient of a postdoctoral scholarship from the Royal Thai Government.

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Received January 23, 2004; revised April 8, 2004; accepted April 9, 2004