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G. Sellers

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Second Progress Report for US Army Grant Proposal DAMD 17-96-1-6072
Epithelin/granulin precursor expression in human breast carcinoma
Ginette Serrero, Ph.D. Principal Investigator.

Epithelin/granulin precursor is an 88 kDa glycoprotein originally purified as an autocrine growth factor from the highly tumorigenic mouse teratoma-derived cell line PC. For this reason this growth factor is also defined and known as PC cell derived growth factor (PCDGF). PCDGF belongs to a novel family of double cysteine rich polypeptides that include the 6 kDa epithelins and granulins originally purified from kidney extracts (epithelins) or granulocyte extracts (granulins). Experiments in the mouse cell lines indicated that PCDGF was overexpressed in the highly tumorigenic cells and that was positively correlated to the tumorigenic properties of the cells.

RATIONALE AND SPECIFIC AIMS

The purpose of the grant proposal DAMD 17-96-1-6072 was to design experiments in order to establish a solid foundation for the role of PCDGF in mammary epithelial cell biology and the involvement of its autocrine expression in the progression of the tumorigenicity in human breast carcinomas.

The specific aims originally proposed were:

1- Comparison of PCDGF expression in normal human mammary epithelial cell strains and in ER+ and ER- breast carcinoma cell lines.
2- Biological activity of PCDGF and its processed form epithelin 1 in normal mammary epithelial cells and mammary carcinoma cells.
3- Effect of inhibition of PCDGF expression (antisense approach) or action (neutralizing antibody or competitive inhibitor approaches) on the growth of malignant breast carcinoma in vitro and in vivo.

SECOND PROGRESS REPORT

The first report indicated that progress had been accomplished in the first and third specific aims of the original grant application. In the past few months, since the submission of the first report additional progress has been made in these specific aims and also in specific aim 2 which focuses on examining the effect of PCDGF and its processed forms in normal mammary epithelial cells and in mammary carcinoma cell lines. Thus, we are now in a good position to accomplish all the goals set in the original application. Moreover, this investigation has opened a new light in understanding estrogen action in mammary epithelial cells as described below.
The specific aims, rationale and methods used are the sames as the ones proposed in the original application and therefore have not been reiterated in the progress report. Only the progress accomplished since submission of the first progress report is described here.

Specific aim 1: Estradiol stimulates the expression of PCDGF in human breast carcinoma cell lines MCF7 and T47D.

The studies had been initiated and described in the first progress report. These studies have been finalized and a manuscript has been submitted for publication: "R. Lu and G. Serrero (1998)

Expression of PC cell-derived growth factor (epithelin/granulin precursor) in human breast cancer cells and its regulation by estrogen" submitted to publication.
These data were described in the previous progress report and thus were not described again here. However a copy of the manuscript is included with this report.

In summary the data presented in this paper summarized here show that PCDGF mRNA and protein is expressed in estrogen-dependent human breast carcinoma cell lines MCF-7 and T47D. Treatment of human breast carcinoma cell lines MCF-7 and T47D with estradiol stimulated PCDGF mRNA and protein expression in a dose (5-fold stimulation above control with ^{10}-9M E2) and time-dependent manner (maximal stimulation at 12 hours after addition of E2). This stimulation is blocked by treatment of the cells with anti-estrogen tamoxifen (1 μM). Moreover, it is also inhibited by actinomycin D suggesting that estradiol effect on PCDGF expression is at the transcriptional level.

The fact that PCDGF acts as an autocrine growth factor on the teratoma cells from which it was originally isolated (Zhou et al, 1993), is required for tumor growth (Zhang and Serrero, accepted to publication see Appendix) and that PCDGF expression is stimulated by estradiol in human breast cancer cells, has prompted us to formulate the hypothesis that PCDGF, expressed by human breast cancer cells, mediates the mitogenic activity of estrogen in the cells.
For these studies, we used the MCF-7 cells which are well characterized and widely studied. These studies are very important for the point of view of breast cancer biology since 70% of the diagnosed breast cancer starts as being estrogen receptor (ER) positive and estrogen-responsive but eventually become ER negative. Identifying new estrogen-responsive genes and new targets mediating mitogenicity is very important as possible new targets for breast cancer therapy and diagnosis.
Several types of experiments are currently carried out in our laboratory to examine this hypothesis.

A- To determine the effect of PCDGF on the growth of MCF-7 cells maintained in the absence of estradiol and examine whether PCDGF can effectively replace estradiol to stimulate the proliferation of the cells.
B- To isolate MCF-7 cells overexpressing PCDGF by transfection of expression vector containing PCDGF cDNA in the sense orientation and examine if these cells show a reduced or a loss of estradiol requirement for their growth in vitro and in vivo.
C- To isolate MCF-7 cells in which PCDGF expression has been inhibited by transfection of antisense PCDGF cDNA and examine if growth of cells is inhibited and unresponsive to the growth stimulatory activity of estradiol.

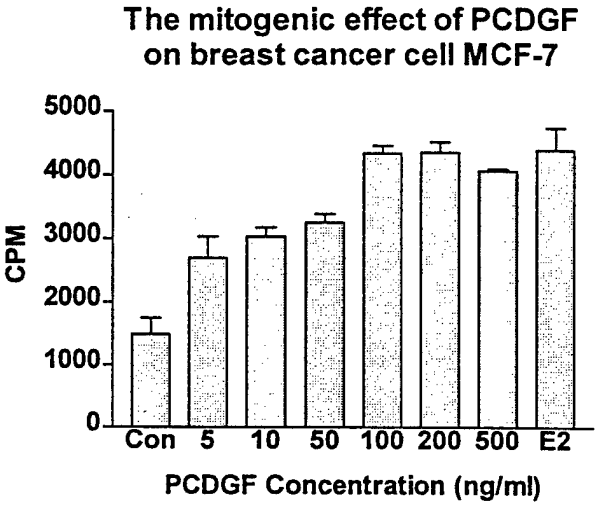
These experiments were not originally proposed in the initial application but the impact they have in breast cancer biology and the progress we have made in developing all the tools and cell lines necessary to carry out this investigation have prompted us to initiate them under this present application and to propose them in details in an academic award submitted in July 1998 to be activated in 1999 if approved and funded.

1) Effect of PCDGF on the growth of MCF-7 cells in the absence of estradiol.

The experiments described below have bee dependent on availability of biologically active PCDGF. Rather than relying exclusively on PCDGF purified from PC cell conditioned medium,

we have expressed and purified recombinant purified PCDGF and showed that it is biologically active (You and Serrero, unpublished results). Experiments were carried out to examine the effect of PCDGF on the growth of MCF-7 cells maintained in the absence of estradiol in phenol red free alpha MEM medium supplemented with charcoal-extracted fetal bovine serum (FBS). these conditions, the cells do not grow and PCDGF expression is very low. However when PCDGF was added to the culture medium a dose dependent increase of thymidine incorporation onto DNA was observed (figure1).

Fig. 1:



MCF-7 cells were cultivated in 24 well dishes in 1 ml of phenol red-free alpha MEM medium supplemented with 10% charcoal extracted FBS. After two days, cells were treated in the absence or presence of increasing concentrations of human PCDGF for 48 hours followed by addition of ^{3}H-thymidine for 6 hours to measure thymidine incorporation into DNA.

The data of figure 1 show that PCDGF stimulate thymidine incorporation in MCF7 cells maintained in the absence of estrogen. A 4-fold maximal stimulation was observed in the presence of 100 ng/ml of PCDGF. This fold-stimulation of DNA synthesis is similar to the one observed by adding estradiol to the culture medium in lieu of PCDGF (E2 10^{-9} M).

These data suggest that PCDGF stimulates DNA synthesis of MCF-7 cells in estrogen–depleted medium similarly to estradiol.

2) Isolation of MCF-7 cells overexpressing PCDGF.

We then focused on isolating MCF-7 cells overexpressing PCDGF by transfection of pcDNA3 expression vector containing human PCDGF cDNA in the sense orientation to determine whether increased PCDGF constitutive expression in MCF-7 cells results in a reduced or loss of estrogen requirement for growth.

We have isolated several clones that overexpress PCDGF as seen by northern blot and western blot analyses. As control, MCF-7 cells were transfected with empty vector pcDNA3.
One of these clones called O4 was particularly studied. Growth properties of MCF-7 control cells and overexpressing (O4) were examined in estrogen depleted medium (fig. 2).
Results show that O4 cells can proliferate in the absence of estrogen in contrast to MCF-7 cells comparative to the ones of MCF-7 control cells cultivated in the presence of estradiol. Both empty vector control cells and MCF-7 cells show an 80% inhibition of growth when cultivated in the absence of estradiol.

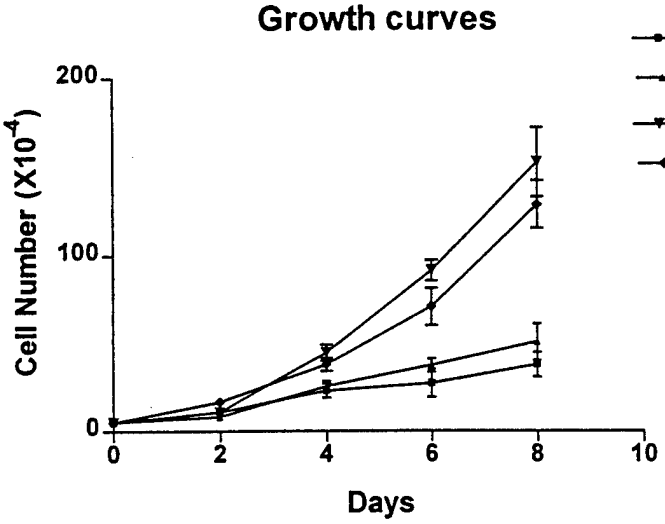


Fig. 2: *Growth curve of MCF-7, con1 (empty vector control) and 04 (PCDGF cDNA trans-fected) cells cultivated in estrogen-depleted medium. Cell number was determined every two days. As a positive control, MCF-7 cells cultivated in the presence of estradiol (MCF-7 +E2 10^{-9} M) was also measured.*

Comparison of the responsiveness of the MCF-7 control cells and the overexpressing O4 and O7 cells to estradiol for stimulation of growth (fig.3) indicated that the cells which overexpressed PCDGF had a reduced response to estradiol (1.5 fold) compared to the MCF-7 cells (4-fold).

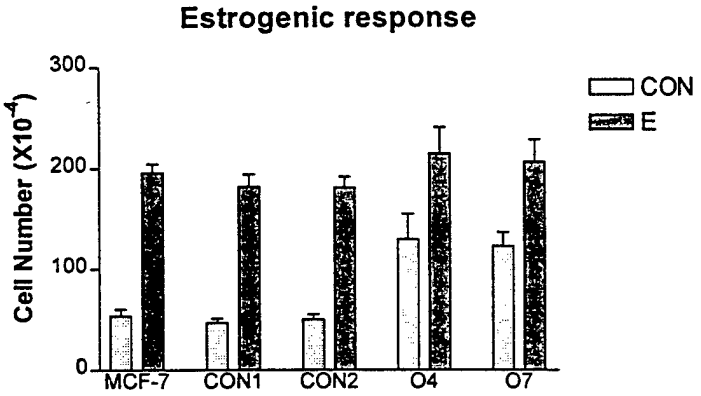


Fig 3: *Effect of estradiol on the growth of MCF-7 cells and overexpressing O4 and O7 cells. Cells were cultivated in phenol red-free medium supplemented with charcoal extracted fetal bovine serum in the absence (con) or in the presence of estradiol (E 10^{-9}M). Cells were counted at day 5.*

The results of figure 3 suggest that overexpression of PCDGF results in an increase capacity of the cells to proliferate in the absence of estradiol and a reduced responsiveness to estradiol added exogenously. Experiments were carried out to determine that the reduced estrogen response in PCDGF overexpressing cells was not due to a reduced estrogen receptor number or a reduced estrogen responsiveness. For this purpose, experiments were carried out to measure the activation by estradiol of an estrogen receptor response element luciferase reporter gene construct in control and overexpressing cell lines (Fig 4).

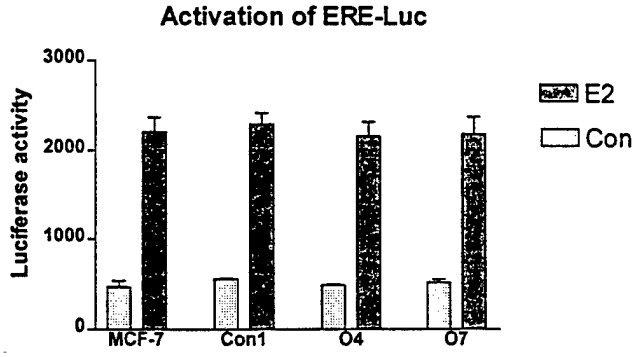


Fig 4: *Comparison of estrogen response element luciferase reporter gene (ERE-Luc) activity in MCF-7 cells and in PCDGF overexpressing cells. ERE-Luc plasmid DNA was transfected in MCF-7 or O4, O7 cells cultivated in the absence (Con) or in the presence of estradiol (E 2 10^{-9}M). Extracts were prepared to measure luciferase activity using a luminometer.*

Fig 4 showed that O4 and O7 (PCDGF overexpressing) cells had maintained the same ability to activate ERE-Luc reporter gene construct in response to estradiol. Additional experiments are carried out to measure estrogen receptors binding and estrogen receptor expression in both types of cells to confirm the data obtained by measuring ERE-LUC activity. The results of Figs. 3 and 4 indicated that the increase of PCDGF production is correlated to the increased capacity of the cells to proliferate in the absence of estradiol and to the decreased capacity of estrogen to stimulate cell proliferation. These in vitro studies are presently completed and prepared for submission of a manuscript.

These results are very important because they strongly suggest that the novel growth factor PCDGF mediate the mitogenic activity of estradiol in human breast cancer cells.

At present time, experiments are proposed to examine in more detail the growth properties of the PCDGF overexpressing human breast carcinoma cells in particular their ability grow in vitro and in vivo in the absence of estradiol. One important question to examine is whether overexpression of PCDGF in human breast cancer cells results in (and is sufficient) to confer escape from estrogen requirement for growth and would be one mechanism by which highly tumorigenic cells lose estrogen dependency and resist to anti-hormone therapy.
We are proposing to initiate these experiments during the 6-months of no-cost extension on the grant-funding period and hopefully during the course of academic award that has been submitted in July 1998 should this award be granted.

In addition, we have developed a neutralizing antibody to the human PCDGF. We are proposing to examine the capacity of this antibody to inhibit the growth of human breast carcinoma in the presence of estradiol. According to our hypothesis that estradiol mediates its mitogenic activity on human breast cancer cells by stimulating PCDGF expression and production in the cells, then neutralizing antibody to PCDGF added extracellularly should block the mitogenic activity of estrogen. This would confirm by a different approach the evidence that we already have gathered with experiments described in the present report in Figs 1-4.

Specific aim 2: Biological activity of PCDGF and processed forms.

As mentioned above, we have succeeded in expressing human PCDGF in recombinant form that is biologically active. We have expressing 6 kDa processed epithelin forms in mammalian expression system stably transfected into CHO cells. We have used this 6 kDa form as antigen and developed a polyclonal antibody to epithelin. Thus during the past months since the submission of the first progress report we have developed adequate tools to allow us to examine the presence and action of processed as well as precursor form in human breast carcinoma cells.

Concerning the effect of PCDGF on cell proliferation, two types of experiments are carried out:
The first type of experiments is focused on the characterization of PCDGF receptors. Recombinant PCDGF has been iodinated and characterization of its cell surface binding sites has been achieved by Scatchard analysis and by affinity labeling on a variety of cell lines. There is presence of two classes of affinity of cell surface receptors with an apparent molecular weight deduced from SDS-PAGE of 120 kDa. These studies have been published in 1998 in an article

provided in the progress report (**Xia, X and Serrero, G 1998, Biochem. Biophys. Res. Commun. 245, 539-543**) and therefore are not described in detail here.
The second type currently initiated is to examine the signal transduction pathway by which PCDGF mediates its growth proliferating activity on mammary epithelial cells. These studies are done with MCF-7 cells but also with a mouse mammary epithelial cells line C57MG cells since they do not produce PCDGF and therefore, there is no need to be concerned with the effect of endogenously produced factor.

Specific aim 3: Effect of inhibition of PCDGF expression or action (neutralizing antibody) or competitive inhibitor approaches on the growth of malignant breast carcinoma in vitro and in vivo.
These studies were well on the way at the time of submission of the first year progress report.
In particular we had developed MDA-MB-468 cells where PCDGF expression had been inhibited by antisense PCDGF cDNA transfection. Since submission of the progress report, in vivo studies have been initiated to examine the tumorigenic properties of antisense Cells when compared to empty vector transfected control cells.
Experiments carried out by subcutaneous injection of cells (2 x 105 cells/mouse) into athymic nude mice.

Table 1
In vivo growth of human ER negative MDA-MB-468 cells transfected antisense PCDGF cDNA in nude mice.

Cells	Mice with tumors	Tumor weight (g)
468 control cells	7/7	0.3 ± 0.06
AS3 cells	2/7	0.05 ± 0.01

Results of these experiments show that cells where PCDGF expression has been inhibited by antisense cDNA transfection show a 75% reduction in tumor growth.

These results emphasize the importance of PCDGF expression in human breast cancer growth.

FUTURE PERSPECTIVES

The data show that we have been successful in accomplishing or initiating all of the specific aims proposed. The results bring important information about the novel growth factor PCDGF and its importance as a possible mediator of estrogen mitogenic signal in human breast cancer cells. At present time, experiments are initiated with the University of Maryland Cancer Center to examine the expression of PCDGF in various stages of human breast tumors and determine when is PCDGF overexpressed in these tumors.

PUBLICATIONS SUPPORTED BY DAMD 17-96-1-6072

Zhang, H. and Serrero, G. (1998) Inhibition of tumorigenicity of the teratoma-derived PC cell line by transfection of with antisense cDNA for PC cell-derived growth factor.
Proc. Natl. Acad. Sci. USA. accepted for publication

Xia, X and Serrero, G. (1998) Identification of cell surface binding sites for PC cell-derived growth factor, PCDGF, (epithelin/granulin precursor) on epithelial cells and fibroblasts. Biochem. Biophys. Res. Commun. 245, 539-543.

Lu, R and Serrero, G. (1998) Expression of PC cell-derived growth factor (epithelin/granulin precursor) in the human breast carcinoma cell line MCF-7 and its regulation by estrogen. Submitted to publication.

Lu, R and Serrero, G (1998) Resveratrol, a natural product derived from grapes exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. Submitted to publication.

These 4 articles (reprints or manuscripts) are provided with the progress report.

Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF).

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Manuscript has 23 pages, 8 figures and one table.

Abstract:

The PC cell line is a highly tumorigenic insulin-independent teratoma-derived cell line isolated from the non-tumorigenic insulin-dependent 1246 cell line. Studies of the PC cell growth properties have led to the purification of an 88 kDa secreted glycoprotein called PC cell-derived growth factor (PCDGF) shown to stimulate the growth of PC cells as well as 3T3 fibroblasts. Sequencing of PCDGF cDNA demonstrated its identity to the precursor of a family of 6 kDa double cysteine-rich polypeptides called epithelins or granulins (epithelin/granulin precursor). Since PCDGF was isolated from highly tumorigenic cells, its level of expression was examined in PC cells as well as in non-tumorigenic and moderately tumorigenic cells from which PC cells were derived. Northern blot and western blot analyses indicate that the levels of PCDGF mRNA and protein were very low in the non-tumorigenic cells and increased in tumorigenic cell lines in a positive correlation with their tumorigenic properties. Experiments were performed to determine whether the autocrine production of PCDGF was involved in the tumorigenicity of PC cells. For this purpose, we examined the in vivo growth properties in syngeneic C3H mice of PC cells where PCDGF expression had been inhibited by transfection of antisense PCDGF cDNA. The results show that inhibition of PCDGF expression resulted in a dramatic inhibition of tumorigenicity of the transfected cells when compared to empty vector control cells. These data demonstrate for the first time the importance in tumor formation of overexpression of the novel growth factor PCDGF.

RESULTS

Comparison of PCDGF mRNA expression in 1246, 1246-3A and PC cells.

The cell lines 1246 (non-tumorigenic), 1246-3A (moderately tumorigenic) and PC (highly tumorigenic) were cultivated in DME/F12 medium supplemented with 2% fetal bovine serum (FBS). When the cells were 80% confluent, total RNA was extracted and PCDGF mRNA expression was measured by northern blot analysis using a radiolabeled PCDGF cDNA probe. RPL32 mRNA expression was measured as an internal control for equal RNA loading. As shown in Figure 1, the level of expression of PCDGF was very low in the non-tumorigenic 1246 cells and in the moderately tumorigenic 1246-3A cells. In contrast, PCDGF mRNA expression increased by at least 20-fold in the highly tumorigenic PC cells. The size of the PCDGF mRNA transcript expressed by the cells was 2.2 kb.

PCDGF protein expression in the three cell lines.

Next we examined the expression of PCDGF protein in the three cell lines. Since PCDGF is a secreted protein (8), we examined its level not only in cell lysates but also in the culture medium of the three cell lines by a combination of immunoprecipitation and western blot analysis using an anti-PCDGF antibody. As shown in Figure 2, the level of expression of PCDGF in cell lysates and conditioned media of the highly tumorigenic PC cells was much higher than in 1246 and 1246-3A cells, similar to the differences observed for PCDGF mRNA expression (Figure 1). PCDGF protein expression was very low in the 1246-3A cell lysate and culture medium and undetectable in 1246 cells. The data presented in Figures 1 and 2 suggest that the level of PCDGF expression in the three cell lines correlated with the degree of tumorigenicity of the cells. Based on these results, it was hypothesized that PCDGF overexpression in PC cells was

directly associated with their tumorigenic properties. To examine this hypothesis, experiments were carried out to isolate PC cells in which PCDGF expression had been inhibited by stable transfection with PCDGF antisense cDNA and to compare the tumorigenic properties of these antisense transfected cells with empty vector transfected PC cells.

Isolation of antisense PCDGF transfected PC cells.

PC cells were co-transfected with the pAS-PCDGF plasmid (Figure 3) and with the pRSVneo plasmid as described under Material and Methods. Control PC cells were co-transfected with empty pCMV4 and pRSVneo plasmids. In both cases, transfected cells were selected for growth in medium containing G418. Colonies were assayed for the presence of the transfected plasmids by PCR analysis of genomic DNA (Figure 4). Antisense pAS-PCDGF and empty vector control transfected cells were further tested to determine the level of PCDGF protein expression by western blot analysis (Figure 5). All antisense clones tested exhibited a significantly reduced level of PCDGF expression both in cell lysates and in conditioned media when compared to empty vector controls (P6 and P14) and to wild type PC cells. Three antisense clones (AS-II1, AS-II15 and AS-II18) and two empty vector control clones (P6 and P14) were chosen and further studied. Inhibition of PCDGF protein expression was observed in the three antisense transfected clones to varying degrees (Figure 5). The level of PCDGF protein secreted in the conditioned media of empty vector control cells (P14 and P6) was similar to that secreted by untransfected PC cells.

INTRODUCTION

The C3H mouse teratoma-derived cell line 1246 is an adipogenic cell line requiring insulin to proliferate and differentiate in defined medium (1, 2). Insulin-independent variant cell lines were isolated from 1246 cells maintained in insulin-free medium. One cell line called 1246-3A was analyzed in detail and shown to have lost the ability to differentiate and to have become moderately tumorigenic when 10^6 cells were injected into syngeneic host C3H mice (3). It was shown that the 1246-3A cells synthesized and secreted several factors that affected their proliferation and differentiation and the factors were biochemically characterized and identified (4-6). In order to establish a cell culture model of increased tumorigenicity, an *in vitro-in vivo* shuttle technique (7) was applied to isolate from the 1246-3A cells a highly tumorigenic cell line called PC (5). These highly tumorigenic PC cells gave rise to tumors when 10^4 cells were subcutaneously injected into syngeneic C3H mice (5). These three cell lines ranging from differentiating and non-tumorigenic cells to differentiation-deficient, highly tumorigenic cells represented a unique model system to analyze the cellular and biochemical changes associated with the acquisition of tumorigenic properties. Comparative studies of the growth properties of the cell lines indicated that PC cells had lost the ability to respond to the growth factors required by 1246 and 1246-3A cells and instead became dependent on their own conditioned medium for proliferation (8). The factor responsible for this growth stimulation called PC cell-derived growth factor (PCDGF) was purified to homogeneity and sequenced (9). PCDGF was an 88 kDa glycoprotein consisting of a 68 kDa core polypeptide and a 20 kDa carbohydrate moiety (8), shown by amino acid and cDNA sequencing to be identical to the epithelin/granulin precursor (9, 10). Epithelin and granulins are 6 kDa double cysteine-rich polypeptides originally purified from rat kidney (11) and from human granulocyte extracts (12). Although no function was attributed

to granulins during their purification and characterization, epithelins were shown to be dual growth effectors for epithelial cells (10-11). Cloning and sequencing of epithelins and granulins cDNAs showed that the 6 kDa polypeptides were encoded by a common precursor cDNA (9-10). This epithelin/granulin precursor corresponded to a 63 kDa polypeptide contained 7 ½ repeats of the 6 kDa epithelin or granulin polypeptides and several putative glycosylation sites (9-10). As PC was a highly tumorigenic cell line that synthesized and secreted PCDGF, it was important to investigate the possible role of PCDGF on the growth of the teratoma cells. For this purpose, two experimental approaches were taken. One was to determine whether PCDGF was preferentially expressed in the highly tumorigenic cells. This was done by comparing the levels of expression of PCDGF mRNA and protein in the tumorigenic PC cell derivatives with the ones found in the non-tumorigenic 1246 and moderately tumorigenic 1246-3A parent cells. The second approach was to examine the role of PCDGF expression on the tumorigenic properties of the PC cells by preventing the factor from being synthesized and examining the resulting changes in the growth properties of the cells. This was achieved by transfecting PC cells with an antisense PCDGF cDNA construct that blocked the growth factor expression. This approach has been widely used to examine the effect of inhibiting the expression of growth factors or their receptors on the growth properties of producer cells (13-19). Experiments presented here examined the in vitro and in vivo growth properties of PC cells in which PCDGF expression had been inhibited by transfection with antisense PCDGF cDNA.

MATERIALS AND METHODS

Cell culture

Stock cultures of PC and 1246-3A cells were maintained in defined media as described previously (6). 1246 stock cells were cultivated in DME/F12 nutrient medium (1:1 mixture) (Gibco/BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS) (Gibco). For comparative studies, the three cell lines were cultivated in DME/F12 medium supplemented with 2 % FBS.

Northern blot analysis of PCDGF mRNA expression

Total cellular RNA was isolated by RNAzol method (Cinnabiotex, Friendswood TX). Fifteen micrograms of total RNA were separated by electrophoresis on a denaturing 1.2% agarose gel containing 0.22M formaldehyde in 1X MOPS (20). RNA samples were blotted on to nitrocellulose membrane (MSI Inc., Westboro, MA) by overnight capillary transfer in 10X SSC, and then hybridized at 42^0C overnight in hybridization solution (50% formamide, 5X SSPE, 1 % SDS, 5X Denhardt's solution, 1 μg/ml poly-A and 100 μg/ml denatured salmon sperm DNA) with approximately 10^6 cpm/ml of randomly primed ^{32}P-labeled mouse PCDGF cDNA probe to measure PCDGF mRNA expression. Filters were washed and exposed to X-ray film (Kodak X-Omat AR) for autoradiography. Ribosomal protein L32 mRNA (RPL32) was detected as an internal standard for normalizing RNA loading (21).

Immunoprecipitation and western blot analysis of PCDGF protein

Since PCDGF is a secreted protein, its expression was measured in cell lysates and conditioned media collected in the presence of a protease inhibitor cocktail of 200 μM phenyl methyl

sulfonyl fluoride (PMSF), 1μM leupeptin, 0.5μM aprotinin, and 1 mM ethylene diamine tetra acetic acid (EDTA) (all obtained from Sigma, St. Louis, MO). Cells were lysed in phosphate buffer saline (PBS) containing 1% Triton X-100 followed by sonication and centrifugation. For comparative studies of PCDGF expression, the samples used for immunoprecipitation and western blot analysis were normalized to equivalent cell number (see figure legends), determined by counting cells from duplicate sets of dishes. Immunoprecipitation of PCDGF was carried out by incubating samples for 4 hours with 5μg of affinity-purified anti-PCDGF IgG conjugated to agarose beads followed by centrifugation at 10,000 x g for 10 minutes. Immune complexes were resuspended in Laemmli sample buffer (22), boiled for 5 minutes and separated by electrophoresis on a 10% polyacrylamide gel in the presence of SDS. Proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% non-fat milk overnight at 4^0C and then incubated for 1 hr at room temperature with 10 ng/ml of anti-PCDGF IgG conjugated to horseradish peroxidase, in the presence of 1% BSA. Immunoreactivity was visualized by the enhanced chemiluminescence (ECL) detection system (Amersham).

Construction and transfection of antisense PCDGF cDNA expression vector in PC cells

PC cells were transfected with a PCDGF antisense cDNA fragment cloned into the expression vector pCMV4 (23) as described below. A 228 bp PCDGF cDNA fragment that included the start codon region was obtained by digesting full length PCDGF cDNA with SmaI and XbaI enzymes. This fragment was cloned in the antisense orientation into XbaI and SmaI site of $pCMV_4$ and is referred hereafter as pAS-PCDGF. Transfection of PC cells with pAS-PCDGF by the calcium phosphate method (20) was performed when the cells reached 80% confluence in

DME medium supplemented with 10 % FBS. A calcium phosphate precipitate added to the cells contained 20 μg of pAS-PCDGF plasmid, 2 μg of pRSVneo plasmid carrying the neomycin resistant gene as a selectable marker and 20 μg of pSK plasmid as carrier. After seven hours, the cells were shocked with 10% DMSO for 2-3 min., washed twice and fed with DME/F12 medium supplemented with 10% FBS. One day after transfection, cells were subcultured at 1/3 ratio and cultivated in serum-supplemented medium in the presence 400 μg/ml of Geneticin (G-418 Sulphate, Gibco-BRL). This medium was first changed 2 days later and every 3-4 days thereafter. After 10-14 days, colonies of G418 resistant cells were picked with cloning rings and cultivated in DME/F12 medium supplemented with 10% FBS and 400 μg/ml G418. Control PC cells were transfected with pCMV4 and pRSVneo plasmid DNAs and isolated as described above. Presence of the transfected DNA (pAS-PCDGF or pCMV4) was determined by PCR analysis of genomic DNA isolated from G418 selected transfectants. For empty vector control cells, PCR analysis was performed using the primer pair of SP647: 5'-CCTACTTGGCAGTACATCTACGTA-3' and

AP912: 5'-CTGACGGTTCACTAAACGAGCTC-3' corresponding to CMV promoter region.

The sense primer SP647 (described above) and antisense primer

SP7 5'-CGAGAATTCAGGCAGACCATGTGGGTC-3' located in the start codon region of PCDGF cDNA were used to test for the presence of antisense PCDGF cDNA in the genomic DNA of antisense transfectants. The transfectants were lysed in buffer A (100mM KCl, 10mM Tris-HCl [pH 8.3], 0.45% Tween 20 and 0.45% NP40) and 120 μg/ml proteinase K (Boehringer Mannheim). Then, they were incubated at 60^0C for 1 hr, followed by boiling for 15 min. 50 -100 ng DNA of each clone was used as template for PCR reactions. DNA from non-transfected cells was used as negative control. Plasmid DNA was used as a positive control. PCR was performed

in a 20 µl reaction mixture containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 0.5 units Taq DNA polymerase (Promega), 20 ng of each primer, and 50ng of genomic DNA template. The reaction tubes were heated to 95 ^{0}C for 3 minutes, and then subjected to 40 cycles of 95 $^\circ$ C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes with a 10 minute 72^{0}C extension in a thermocycler (MJ Research, Inc., Watertown, MA). 10 µl of PCR product was analyzed on a 1% agarose gel and stained with ethidium bromide.

Measurement of cell proliferation

Proliferation of pAS-PCDGF and control transfected PC cells was examined. Cells were plated in 12 well plates (Corning) at a density of 3 x 10^4 cells/well in 2 ml of either 2F defined medium consisting of DME/F-12 medium supplemented with 2 µg/ml human fibronectin (UBI, Lake Placid, NY) and 10 µg/ml human transferrin (Sigma) or in DME-F12 medium supplemented with 2% FBS. On day 5, cells were washed with PBS and enumerated with a Coulter Counter after trypsinization of cells from duplicate wells.

Tumorigenicity study

Six week-old female C3H mice from Taconic Farms (Germantown, NY) were injected subcutaneously with 10^6 pAS-PCDGF or control transfected PC cells. The appearance and size of tumors were examined daily. The mice were sacrificed 40 days after injection to measure tumor weight.

All experiments described here were repeated at least twice.

In vitro growth properties of antisense and control transfected cells.

The morphology of antisense and control transfected cells was examined. Phase contrast micrographs indicated that the pAS-PCDGF transfected cells did not spread as well as the control cells and they maintained a rounded morphology when compared to control transfected PC cells (Figure 6).

Growth of both types of transfected cells was measured in serum-supplemented medium and in serum-free defined medium (Figure 7). pAS-PCDGF transfected cells cultivated either in defined medium (2F medium, bottom panel) or in serum-containing medium (top panel) had a reduced proliferative capacity when compared to control transfected cells and untransfected PC cells cultivated in similar culture conditions. After 5 days in culture, the number of pAS-PCDGF transfected cells was reduced by 90% in defined medium and by 50 % in serum-supplemented medium compared to control cells cultivated in the same conditions. Moreover, after 5 days, the number of cells in defined medium was 10 % of those in serum-containing medium. In contrast, empty vector control cells had the same proliferation capacity as non-transfected PC cells, and they displayed only a 50 % reduction in cell number after 5 days in defined medium when compared to serum-containing medium.

Tumorigenic properties of antisense and control PC cells.

The tumorigenic properties of the three pAS-PCDGF transfected clones and two empty vector control clones were examined by subcutaneous injection of 10^6 cells/mouse into 6 week-old syngeneic C3H female mice. Tumor formation was followed by daily monitoring of the mice, and tumor growth was determined by measuring the dimensions of the tumors. Figure 8 shows mice injected with control cells and pAS-PCDGF transfected cells 40 days after injection. The

mice were sacrificed and the tumors were collected and weighed. As shown in Table 1, all of the mice injected with either one of the control transfected PC cell lines (P14 and P6) developed tumors rapidly. Tumors were visible as early as 15 days after injection for both the control transfectants and non-transfected PC cells. These results indicated that the growth properties of the control cells had not been significantly affected by the transfection and selection processes. In contrast, all three clones that had been transfected with pAS-PCDGF showed a marked inhibition of tumor growth. For AS-II1 and AS-II18, inhibition was complete since after 40 days, none of the mice injected with either of these clones had tumors. For AS-II15, 2 of the 5 mice injected developed tumors, but the inhibition of tumor growth was still significant as AS-II15 tumors were 5-10% of the weight of the control transfected PC cell tumors. Even 60 days after injection in C3H mice, AS-II1 and AS-II18 did not form palpable tumors (data not shown).

DISCUSSION.

PCDGF (PC cell-derived growth factor) is a growth factor belonging to a family of double cysteine-rich polypeptides that includes the 6 kDa cysteine-rich polypeptides epithelins and granulins (8-10). PCDGF is secreted as an 88 kDa glycoprotein by the highly tumorigenic PC cell line. PC cells are insulin-independent teratoma cells isolated for their high tumorigenic properties in syngeneic C3H mice (3). Histological analysis of the tumors generated by subcutaneous injection of PC cells in C3H mice indicate that PC cells form malignant fibrous histiocytomas (Zhang and Serrero, unpublished data). Sequencing of full length PCDGF cDNA isolated from PC cell cDNA library indicated that PCDGF was identical to mouse epithelin/granulin precursor (10-11) and that PCDGF expressed by the highly tumorigenic cells did not contain any mutations (Zhang and Serrero, unpublished data). Time course studies of PCDGF synthesis and secretion in PC cells have shown that PCDGF is secreted as early as 2 hours after synthesis (data not shown). This observation and the fact PC cells were growth stimulated by PCDGF (8) and presented PCDGF cell surface binding sites (24) indicated that secreted PCDGF was an autocrine growth factor for the highly tumorigenic cells. Based on these results, it was hypothesized that PCDGF expression may be increased in tumorigenic cells when compared to their normal counterparts. Increased expression of several growth factors in many different tumor cells such IGF-I in glioblastoma (14), and transforming growth factor-α in human breast cancer cells (25) haa been observed. The model system consisting of non-tumorigenic 1246 cells (1), moderately tumorigenic 1246-3A (2) and highly tumorigenic PC cells (5) provided an experimental system to investigate directly the role of PCDGF (epithelin/granulin precursor) in tumorigenic cell lines derived from the same non-tumorigenic

and hormone-responsive parent cell line. The analysis of PCDGF mRNA and protein levels in the three cell lines indicated that PCDGF expression was undetectable in the non-tumorigenic 1246 cells and significantly increased in the highly tumorigenic cells in correlation with the degree of tumorigenicity of the teratoma cells.

To determine whether increased PCDGF expression in PC cells contributed to their high tumorigenic properties, we used the antisense cDNA transfection approach to block PCDGF expression in PC cells and examine their growth characteristics. Antagonizing mRNA with the help of "hybridization competitor" in inhibiting protein synthesis was first introduced by Zamecnik, et al (26) and Plesner, et al (27). Recent years have seen considerable progress in studying the role of antisense RNA as an inhibitor of oncogenic protein production (13). The development of stable transfected clones with antisense cDNA is advantageous in that it allows a continuous supply of antisense RNA to disrupt protein synthesis, and it is well suited for in vivo tumorigenic assays. Our results demonstrate that decreasing PCDGF protein synthesis and secretion by expression of antisense PCDGF mRNA in the highly tumorigenic PC cells reduced their growth ability in vitro and inhibited their tumorigenicity in vivo. Phase contrast microscopic analysis showed that the antisense transfected cells with inhibited PCDGF expression had a decreased ability to spread on the tissue culture substrate. Many cells were rounded and floated in the culture medium. In contrast, empty vector control transfectants expressing normal PCDGF levels were well spread and grew as well as untransfected PC cells. Localization of PCDGF in PC cells by immunofluorescence with anti-PCDGF antibody revealed PCDGF staining at sites of cell-cell contact (Zhang and Serrero, unpublished data). This evidence suggests that PCDGF may affect cell growth by a complex mechanism including autocrine

stimulation of PC cell growth and contribution in the regulation of adhesion and communication between cells.

In vivo studies indicated that inhibiting PCDGF expression in the teratoma cells resulted in an inhibition of tumor growth. Comparison of tumorigenicity (Table 1) and western blot analysis showing the level of residual PCDGF expression in the pAS-PCDGF transfected cells (Figure 6) suggests that the degree of inhibition of tumorigenicity correlates with the degree of inhibition of PCDGF expression in the cells. The 2 cell lines (AS-II1 and AS-II18) with the lowest level of PCDGF production and secretion were not tumorigenic since none of the injected mice developed tumors even after 60 days while tumors appeared in 15 days in mice injected with control cells. In contrast, AS-II15 cells, in which inhibition of PCDGF expression by antisense PCDGF cDNA transfection was not as efficient, maintained some degree of tumorigenicity (although a reduced one) since 2/5 mice developed smaller tumors which first appeared after 36 days. Teratocarcinoma stem cells are known to originate from a breakdown in the normal regulatory processes controlling proliferation and differentiation rather than from mutations in genes responsible for the normal control of cell behavior (28). The abnormal behavior of such embryonic stem cells can be reversed if they are placed in a normal environment. Little is known about the molecular mechanism of the malignant transformation of teratoma. The fact that malignant teratoma arise from relatively undifferentiated cells implies the existence of a relation between tumorigenesis and differentiation (29). The three cell lines 1246, 1246-3A and PC with increasing tumorigenic properties were derived from a C3H mouse teratoma and provide a good model system to study the molecular mechanisms involved in the loss of differentiation properties and the acquisition of tumorigenic properties. The studies with this model system described here demonstrate for the first time that the epithelin/granulin precursor also known as

PC cell-derived growth factor (PCDGF), is an essential autocrine modulator resulting in the autonomous growth of undifferentiated highly tumorigenic PC cells in vitro and in vivo. The data presented here also demonstrate that overexpression of epithelin/granulin precursor, PCDGF, may play an important function in teratocarcinogenesis and in tumorigenesis.

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Figure legends

Figure 1. Comparison of PCDGF mRNA expression in 1246, 1246-3A, and PC cells.

Cells were cultured in DME/F12 medium supplemented with 2% FBS until 80% confluent. Medium was changed 24 hours before RNA was collected. Fifteen micrograms of total RNA were analyzed by northern blot with ^{32}P-labeled PCDGF cDNA probe to measure PCDGF mRNA expression. Ribosomal protein RPL32 mRNA expression was used as internal standard for RNA loading.

Figure 2: PCDGF protein expression in 1246, 1246-3A, and PC cells.

Cells were cultivated as described in the legend of Figure 1. Cell lysates and conditioned media were collected and normalized by cell number. Samples corresponding to 18 x10^5 cells (cell lysates) and 3 x 10^5 cells (conditioned media) were used to measure PCDGF expression by immunoprecipitation and western blot using an anti-PCDGF antibody as described under Material and Methods.

Figure 3. Construction of antisense PCDGF cDNA

A 228bp PCDGF cDNA fragment (- 45 to +183bp) including the start codon region was ligated in the antisense orientation into the XbaI - SmaI site of pCMV$_4$ expression vector. Immediate early promoter region (CMV, stippled block), a DNA copy of a segment of the alfalfa mosaic virus 4 RNA that contains a translational enhancer (A), transcription termination and polyadenylation signals from the human growth hormone gene (hGH, gray block) and the SV40

origin of DNA replication and early region enhancer sequences ($SV40_{ori}$, white block) are shown. Locations of primers used for PCR analysis of transfectants are indicated.

Figure 4. PCR analysis of antisense and control transfected clones

PCR analysis of DNA from the selected clones was performed with primer pairs described in Material and Methods. **A:** SP647 (sense primer) and the AP912 (antisense primer) both located in the CMV promoter (figure 3) were used to amplify a 266 bp fragment from cells transfected with the CMV promoter expression vector (antisense and empty vector transfected cells). **B:** Sense primer SP647 (described above) and antisense primer SP7 located in the start codon region of PCDGF cDNA (Figure 3) were used to amplify a 551bp DNA fragment in the pAS-PCDGF transfectants only. No amplified band was obtained with DNA from control transfected cells. pAS-PCDGF plasmid DNA and genomic DNA from non-transfected PC cells were used as positive and negative control template for PCR, respectively.

Figure 5. PCDGF protein expression in antisense and control transfected clones.

PC cells, antisense and control transfectants were cultivated in DME/F12 medium supplemented with 2%FBS. The medium replaced with fresh medium 24 hours before the samples were collected. Cell lysates and conditioned media were normalized by cell number of $18x10^5$ and $3x10^5$ cells respectively were immunoprecipitated with anti-PCDGF IgG and analyzed by western blot as described in Material and Methods.

Figure 6. Morphology of antisense and control transfectants in monolayer culture.

Phase contrast micrographs of antisense and control transfectant cells. (Original magnification, 100x).

Figure 7. In vitro growth properties of antisense and control transfected clones

PC cells, antisense cDNA transfected cells and empty vector control transfected cells were plated either in DME/F-12 medium supplemented with 2μg/ml human fibronectin and 10 μg/ml human transferrin (2F medium, bottom panel) or with 2% FBS added 12hrs after plating (top panel). At day five, cells from duplicate wells were trypsinized and counted with a Coulter Counter. The experiment was repeated twice. Each bar represents the mean number of cells (± S.D.) calculated from both experiments.

Figure 8. Comparison of mice injected with antisense and control transfected cells.

C3H female mice were photographed 40 days after subcutaneous injection of 10^6 cells antisense (top panel) or control PC transfectants (bottom panel).

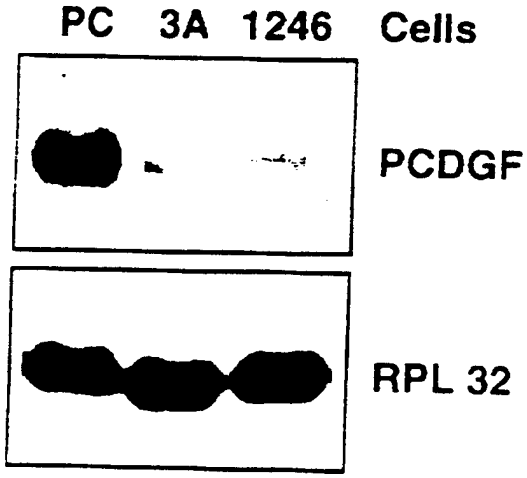
Table 1

In vivo tumorigenicity of PC cells transfected with antisense PCDGF cDNA and with empty vector.

Cells injected	Day of appearance ± SD	Mice with tumors	Weight (g) ± SD
AS-II1	–	0/5	–
AS-II18	–	0/5	–
AS-II15	36 ± 14	2/5	0.35 ± 0.05
P14	15 ± 3	5/5	5.4 ± 2.0
P6	15 ± 9	5/5	3.6 ± 1.9
PC	15 ± 4	5/5	6.4 ± 2.6

C3H female mice were injected subcutaneously with 10^6 cells PCDGF antisense cDNA transfectants (AS-II1, AS-II18, and AS-II15), control transfectants (P14, P6) or non-transfected PC cells. Average day of tumor appearance, number of mice with tumors at 40 days and tumor weight in g ± SD are provided.

Figure 1



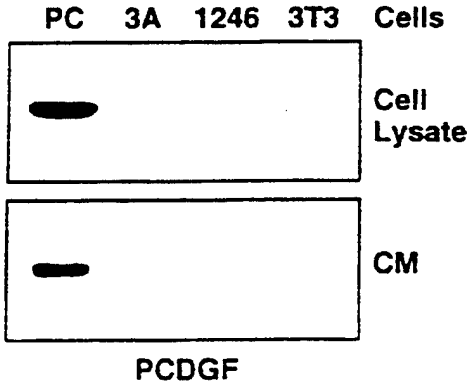
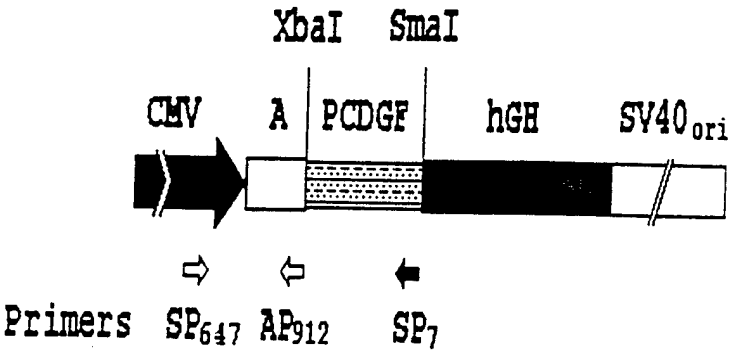

PC
3A
1246
3T3
Cells
Cell Lysate
CM
PCDGF

Figure 3



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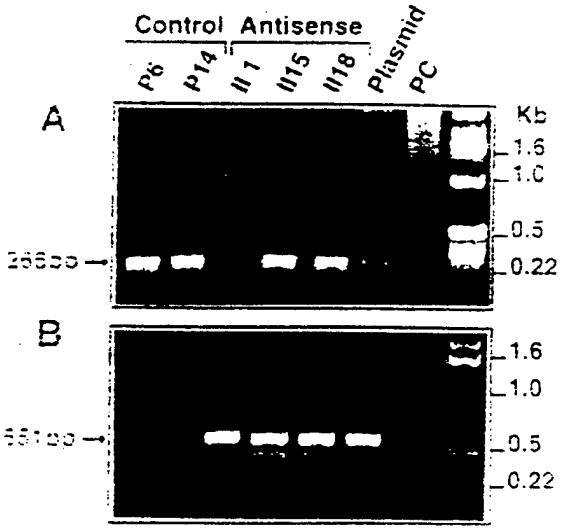
Control
Antisense
P6
P14
II 1
II15
II18
Plasmid
PC
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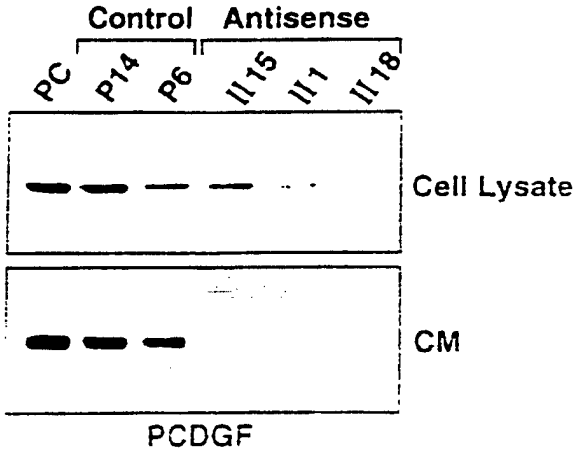
B
1.6
1.0
0.5
0.22


Control
Antisense
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P14
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Il1
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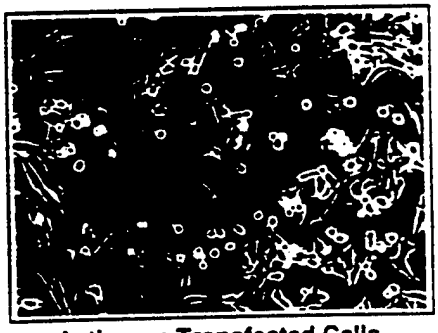
Cell Lysate
CM
PCDGF

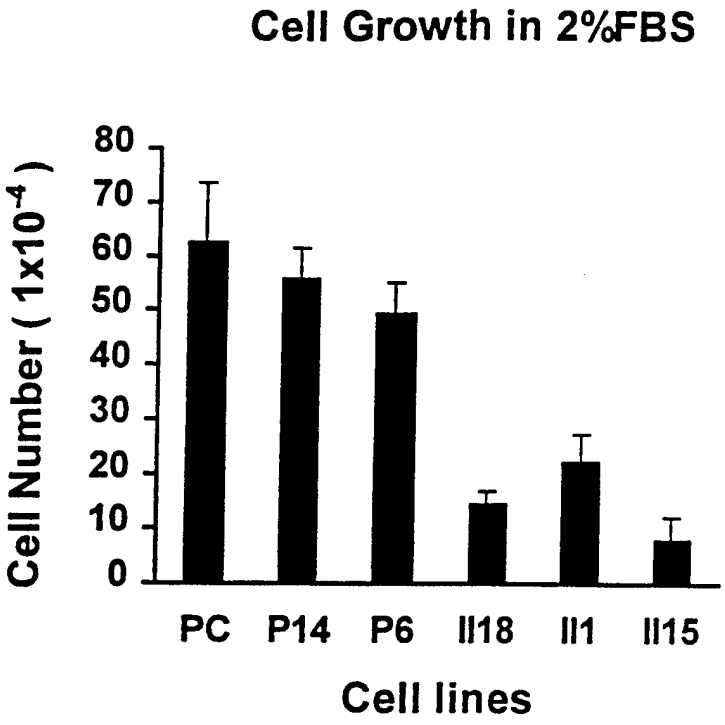
Figure 6

Control Transfected Cells



Antisense Transfected Cells

Figure F.


Cell Growth in 2%FBS
Cell Number (1x10-4)
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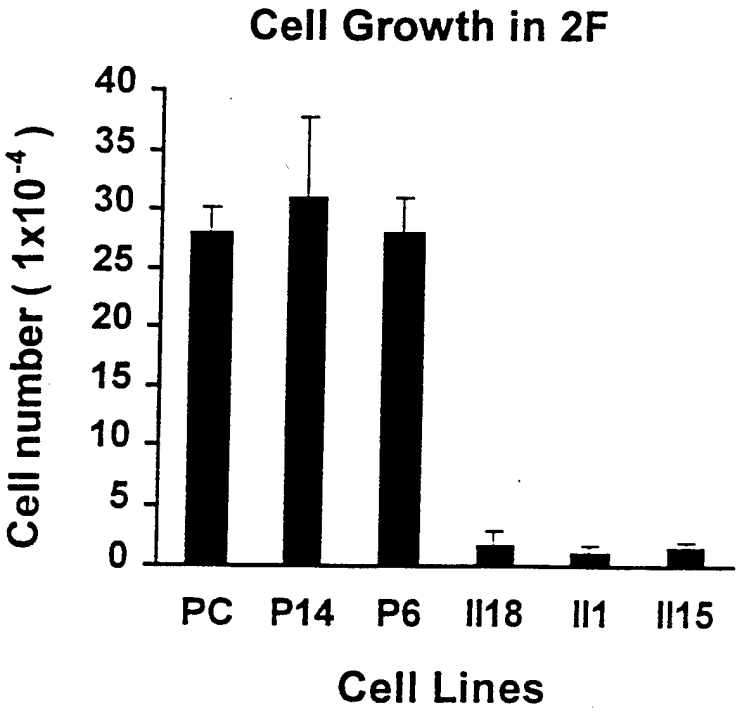
70
60
50
40
30
20
10
0
PC
P14
P6
Il18
Il1
Il15
Cell lines


Cell Growth in 2F
Cell number (1x10-4)
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35
30
25
20
15
10
5
0
PC
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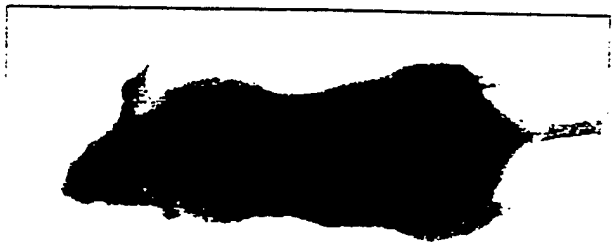
Cell Lines



Antisense PCDGF Construct

Empty VectorControl

Title: Expression of PC cell-derived growth factor (epithelin / granulin precursor) in the human breast carcinoma cell line MCF-7 and its regulation by estrogen

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Running Title: Regulation of PC-cell derived growth factor (PCDGF) by estrogen

Key Words: Epithelin / granulin precursor. Estrogen. MCF-7. breast carcinoma. PC cell-derived growth factor (PCDGF).

Abstract

We report here the identification of a novel estrogen-responsive gene in human breast cancer cell MCF-7. PC cell-derived growth factor (PCDGF) is an 88 KD protein originally isolated from a highly tumorigenic mouse teratoma derived cell line which is similar to epithelin / granulin precursor. Using Northern blot and western blot analyses, we demonstrate here the expression of PCDGF mRNA and protein in MCF-7 human breast cancer cell. We show that 17-β-estradiol stimulates PCDGF mRNA expression in a time and dose-dependent manner. 17-β-estradiol also stimulates the level of expression of PCDGF protein in cell lysate as well as its accumulation in the conditioned medium of MCF-7 cells. Time-course study indicates that the stimulation of PCDGF expression by 17-β-estradiol is observed as early as 4 hours and reaches a maximum at 12 hours. Maximal stimulation of PCDGF mRNA and protein expression by 17-β-estradiol is observed at a concentration of 10^{-8} M. The stimulation of PCDGF expression by 17-β-estradiol is completely inhibited by treatment of the cells with actinomycin D and the antiestrogen 4-hydroxytamoxifen. The stimulation of PCDGF expression is also demonstrated in another estrogen-responsive human breast cancer cell line T47D. The results presented here provide evidence of a novel estradiol responsive gene product in human breast cancer cell lines, hence a potential new marker for therapeutic intervention.

Introduction

Breast cancer is a significant worldwide public health problem. Estrogen is known to be the main stimulator for breast cancer cell growth *in vivo* and *in vitro* (1-3). Binding of estrogen to estrogen receptor (ER) and subsequent interaction of ER with estrogen response elements (EREs) are believed to mediate the transactivation of estrogen-responsive genes (4,5). Identification of estrogen responsive genes in human breast cancer cells is very important since they can contribute to understanding the molecular mechanism of estrogen action; can provide novel targets for breast cancer treatment; and can be used clinically as markers of estrogen responsiveness. Endocrine therapy has been widely used for breast cancer treatment. However, only ER positive breast cancer patients can benefit from hormonal therapy. Measuring estrogen-responsive genes expression can have predictive value to follow the responsiveness of breast cancer patients to endocrine therapy.

Our laboratory has been investigating the cellular and molecular changes associated with the loss of differentiation ability and acquisition of tumorigenicity in cells. Using a mouse teratoma model system of increasing tumorigenic properties, it was demonstrated that the highly tumorigenic cells called PC required for proliferation, the presence of an autocrine growth factor called PC cell derived growth factor (PCDGF) which was purified and structurally characterized (6). Amino acid and nucleotide sequencing demonstrated that PCDGF was identical to the epithelin / granulin precursor encoding a family of double cysteine-rich 6 KD polypeptides that promote or inhibit the growth of various cells in culture including human breast cancer cells (7,8). In the present paper, we investigated the expression of PCDGF in the human breast cancer cell line MCF-7 and showed that the

expression is regulated by estradiol.

Materials and Methods

Materials. 17β-estradiol (E_2), 4-OH-tamoxifen, cycloheximide, actinomycin D were obtained from Sigma. Protein A-Sepharose was bought 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 were kindly provided by Dr. Angella Brodie. Both cells were cultivated in Dulbecco's modified Eagle's medium- Ham's F-12 medium (1:1 mixture) supplemented with 5% fetal bovine serum. For all the 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. Northern blot analysis was carried out as described previously (9). 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 protease inhibitors from duplicate 60 mm dishes. Cell lysates and conditioned media were incubated overnight at 4°C with 5 µl of a rabbit anti-human PCDGF polyclonal antibody on a rotating shaker . 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 2x 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 to PCDGF 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 twice.

Results

1. 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 various times for measurement of PCDGF mRNA level (Fig. 1). Northern blot analysis showed that MCF-7 cells expressed PCDGF mRNA and that PCDGF mRNA expression increased upon treatment with E_2 in a time-dependent fashion. Densitometric analysis of the data indicated that a 1.5-fold increase in PCDGF mRNA expression was observed after 4 hours of exposure to E_2. A 5-fold maximum induction was achieved after 12 hours which decreased to 2-fold above basal level at 24 hours. PCDGF mRNA expression in control untreated cells remained at a low steady state level throughout the same period.

2. Dose-dependent stimulation of PCDGF mRNA expression by E_2

MCF-7 cells were cultivated with different concentrations of E_2 (10^{-14} to 10^{-8} M) for 12h. As shown in Fig. 2, 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 could be observed at concentrations as low as 10^{-12} M and was maximal at 10^{-8} M. Densitometric analysis of the autoradiography data showed a 5-fold maximum stimulation of PCDGF expression in cells treated with 10^{-8} M E_2, a concentration that is also known to maximally stimulate the expression of other estrogen-responsive genes in MCF-7 cells, such as progesterone receptor (10). The induction of PCDGF mRNA expression was also observed in another ER-positive breast cancer cell line T47D. For which a four-fold stimulation of PCDGF mRNA expression was observed at 10^{-9} M E_2 (data

not shown).

3. Effect of Tamoxifen on PCDGF mRNA stimulation by E_2

Experiments were then 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, a concentration at which 4-OH-tamoxifen has strong antagonist activity with no partial agonist activity (11), As shown in Fig. 3, 4-OH-tamoxifen blocked the increase of PCDGF expression by E_2 (10^{-9} M).

We then examined the effect of protein synthesis inhibitor (cycloheximide) and RNA synthesis inhibitor (actinomycin D) 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 abolished by treatment with actinomycin D, but not by cycloheximide. These data suggest that the effect of E_2 on PCDGF expression is mediated by estrogen receptor and requires *de novo* mRNA synthesis. *De novo* protein synthesis is not involved in PCDGF mRNA induction by E_2.

4. 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 KD glycoprotein found to be the precursor of the 6 KD double-cysteine rich polypeptides epithelin / granulin. We show here that PCDGF synthesized by the human mammary breast cancer cell line MCF-7. Data also show that the cells secrete the growth factor in their culture media. Data presented here demonstrate for the first time that estrogen stimulates PCDGF expression in human breast carcinoma cells in a time and dose dependent fashion. E_2 also stimulates the time-dependent production of PCDGF protein in a pattern that is similar to the stimulation of PCDGF mRNA expression. The promoter regions of mouse and human epithelin / granulin precursor (PCDGF) have been characterized (12,13). Analysis failed to indicated the presence of classical estrogen responsive element (ERE) in PCDGF promoter. In fact, only a few of the human mRNA regulated by estrogen in breast cells have been shown to be induced from a canonical ERE (14). 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 (15,16). There are several ERE half sites found in PCDGF promoter region (12, 13). 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 stimulation of PCDGF expression by E_2 is also demonstrated in another estrogen responsive human breast cancer cell line T47D (data not shown), suggesting it is not restricted to MCF-7 cells.

One intriguing feature about PCDGF is its ubiquitous expression in cells of epithelial lineage as well as fibroblast suggesting it may play a basic role in the maintenance of normal cell growth in contrast to previously described growth factors that have a more

restricted distribution. Receptors for the 6 Kd epithelin, processed form of PCDGF have been characterized on MDA-MB-468 breast cancer cells (17). Scatchard analysis and chemical cross-linking of ^{125}I-labeled epithelin binding to MDA-MB-468 cells indicated the presence of two classes of 145 KD binding sites. 120 KD cell surface binding sites for ^{125}I-PCDGF have been characterized by Scatchard analysis and by affinity labeling in several cell lines of mesenchymal and epithelial origins (18).

One of the major problems in breast cancer treatment occurs when breast cancer cells become estrogen-independent for growth and hence resistant to conventional antiestrogen therapy (19). The mitogenic effect of estrogen is believed to be mediated by a group of polypeptide growth factors, such as Tranforming Growth Factor α (TGF-α), and Insulin like Growth Factor I, and II (IGF-I, IGF-II) (20,21). One potential mechanism for the transition of breast cancer cells from hormone dependence to independence is the influence of autocrine and paracrine growth factors pathways (19). Identification of the growth factor that mediates the mitogenic effect of estrogen and directly targeting it for breast cancer treatment may offer an alternative approach to solve this dilemma. Based on the fact that PCDGF act as a growth modulator (6,22) and the finding here that E_2 stimulates the expression of PCDGF in MCF-7 breast cancer cells, it is tempting to speculate that PCDGF could be a novel autocrine growth factor mediating the mitogenic response of estrogen in mammary epithelial cells. Experiments are currently underway in our laboratory to examine this hypothesis.

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Legend

Fig 1. Time-course of E_2 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.

Fig 2. Dose-response of E_2 effect on PCDGF mRNA expression

MCF-7 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

Fig 3. Stimulation of PCDGF mRNA expression by E_2 is inhibited by treatment with 4-OH- tamoxifen, actinomycin D.

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 (1 μM), Actinomycin D (5 μg/ml) and Cycloheximide (10 μg/ml). Control cells received the same volume of ethanol (0.1%). Total RNA was collected to examine the level of expression of PCDGF mRNA by Northern blot analysis.

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.

Fig. 1

Fig. 1

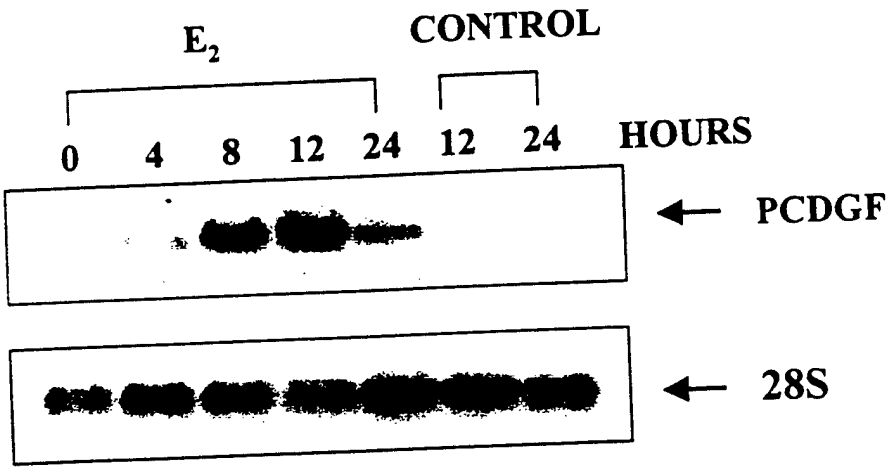


Fig. 2

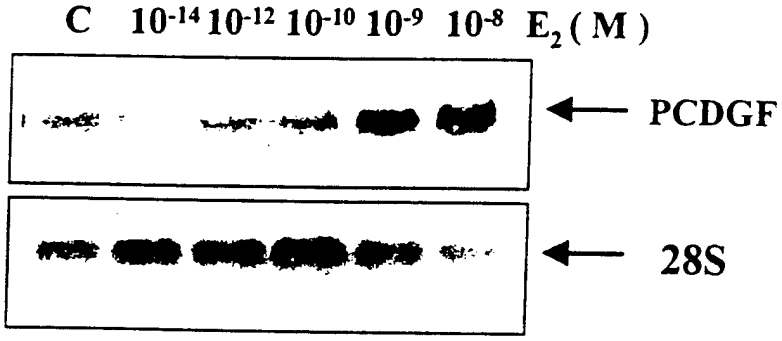
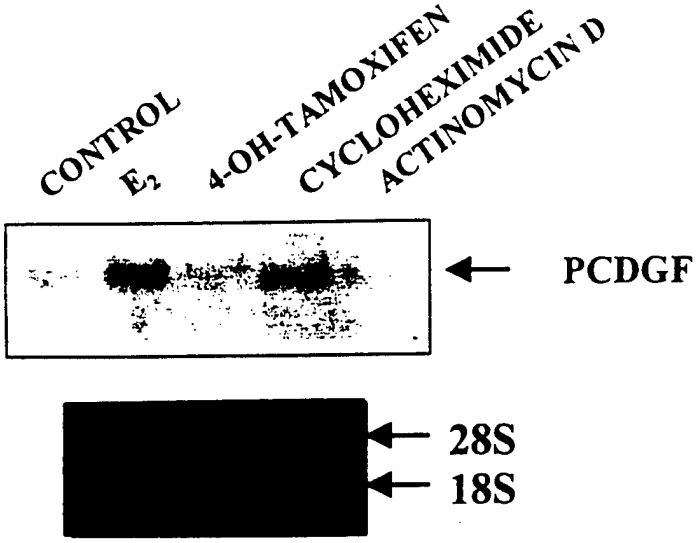


Fig. 3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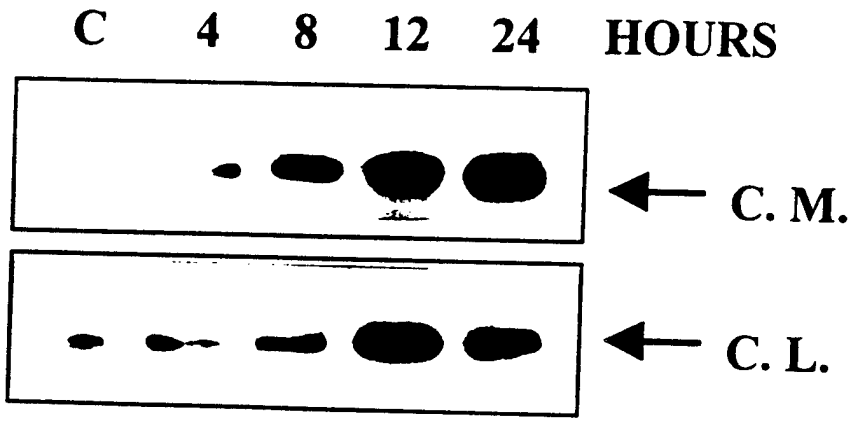
CYCLOHEXIMIDE
ACTINOMYCIN D
PCDGF
28S
18S

Fig. 4



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Identification of Cell Surface Binding Sites for PC-Cell-Derived Growth Factor, PCDGF, (Epithelin/Granulin Precursor) on Epithelial Cells and Fibroblasts[1]

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PC cell derived growth factor (PCDGF) is an 88-kDa glycoprotein purified from the culture medium of the highly tumorigenic mouse teratoma-derived cell line PC. PCDGF was shown to stimulate the proliferation of 3T3 fibroblasts and PC cells. Amino acid sequencing of PCDGF indicated its identity to the precursor for the 6-kDa polypeptides epithelins and granulins. In this paper, we investigated the binding of PCDGF to the mink lung epithelial cell line CCL64. Scatchard analysis indicates that ^{125}I-PCDGF binding to CCL64 cells is curvilinear, corresponding to the existence of two classes of binding sites: high affinity binding sites (560±170 sites/cell) with a K_{d1} of 43±15 pM and low affinity binding sites (16,350±5900 sites/cell) with a K_{d2} of 3.9±1.9 nM. ^{125}I-PCDGF was chemically crosslinked to cell surface receptors on CCL64 cells with disuccinimidyl suberate. A major crosslinked band of about 190 kDa with radiolabeled PCDGF was detected after SDS-PAGE, suggesting the presence of PCDGF binding sites with molecular weight of about 120 kDa. ^{125}I-PCDGF crosslinking studies indicate the presence of PCDGF binding sites with a molecular weight similar to those of binding sites on CCL64 cells on the surface of two other PCDGF-responsive cell lines, 3T3 fibroblasts and PC cells. These data suggest that the receptors for PCDGF are widely distributed on cells of distinct embryonic origin.

The C3H mouse teratoma-derived cell line 1246 is an adipogenic cell line which requires insulin to proliferate and differentiate in defined medium (1, 2). Insulin-independent variant cell lines have been isolated from 1246 cells maintained in the absence of insulin. One of them called 1246-3A cell line was particularly studied (2). These cells are unable to differentiate and become tumorigenic when 10^6 cells are injected into syngeneic hosts C3H mice. By an *in vitro-in vivo* shuttle technique, a highly tumorigenic cell line called PC was isolated. PC cells give rise to tumors even when 10^4 cells are injected into C3H mice (3). Comparison of the growth properties of 1246, 1246-3A and PC cells indicated that PC cells had lost binding and response to several growth factors to which the 1246 and 1246-3A cells responded (3-5). In contrast, PC cells became dependent for their growth at low cell density on the presence of their own conditioned medium. A growth-promoting factor called PC-cell derived growth factor (PCDGF) present in the PC conditioned medium was purified to homogeneity as an 88-kDa glycoprotein (6). Deglycosylation of the protein indicated that PCDGF has a 68-kDa protein core and a 20-kDa carbohydrate moiety (6). N-terminal sequencing and partial sequencing of internal peptide fragments demonstrated that PCDGF contained regions of sequence identity to that deduced from the granulin or epithelin precursor cDNAs (6-8). Definite identification of PCDGF to epithelin/granulin precursor was demonstrated upon cloning full length PCDGF cDNA from a PC cell cDNA library.[3] Granulins and epithelins are 6-kDa cysteine-rich polypeptides originally purified from granulocytes (9) and kidney extracts (10). 5 granulins were purified with no known function (9). Epithelins 1 and 2, the two 6-kDa polypeptides purified from rat kidney extracts, were shown to inhibit the growth of A431 cells and the

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Abbreviations used: BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; EGF, epidermal growth factor; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCDGF, PC cell-derived growth factor.

[3] H. Zhang and G. Serrero, manuscript submitted for publication.

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ration of the human breast carcinoma cell line MB-468 (10, 11). Only in mouse keratinocytes pithelin 1 and 2 shown to have distinct biological ies since epithelin 1 stimulated the growth of nocytes whereas epithelin 2 inhibited the action helin 1 (10). cDNAs encoding epithelins and gra- were independently isolated showing that the s encoding the 6-kDa granulins and epithelins identical. (7, 8). In both cases, the cDNA encoded kDa protein containing a 17-amino acid signal le which is absent from the amino acid sequence reted PCDGF (6) and seven and a half predicted al sequence repeats of about 50 amino acids cor- nding to the epithelins/granulins sequences. Pres- of several glycosylation sites was also predicted the deduced amino acid sequences (7, 8). It was sed that epithelins and granulins were synthe- as an immature precursor that was processed into a biologically active polypeptides (8). A 68-kDa in called acrogranin similar to the precursor of elins/granulins had been isolated from the acroso- ompartment of the guinea pig (12). The function of polypeptide remains to be elucidated. In addition, elial cell specific transforming growth factor -e), a 20-kDa polypeptide with a N-terminal se- ce identical to human granulin A, was purified bovine kidney (13). It is not clear at this time her TGF-e is the partially processing product of elins/granulins precursor or is encoded by another . The study with PC cells provided the first evi- e that the unprocessed precursor had biological ity and was acting as a growth modulator (6).

owth factors regulate cell proliferation and differ- ation by binding to specific cell surface receptors h, once activated, will mediate a cascade of second sengers pathways leading to biological cellular re- ses. Therefore, it is very important to characterize interaction of newly characterized growth modula- to cell surface binding sites in cells where they t biological response. Since PCDGF is a novel wth factor, experiments were carried out to investi- e the properties of its binding to cells.

has been shown that epithelin/granulin precursor NA is expressed by many tissues and cells in culture Thus we first screened several cell lines to find one t did not express significant level of PCDGF so that ding studies would not be hampered by possible oc- ancy of cell surface binding sites by secreted DGF. We found that the mink lung epithelial cell e CCL64 expressed extremely low level of PCDGF NA and PCDGF inhibited the proliferation of L64 in a dose-dependent manner.[4] Thus initial ^{125}I-DGF binding studies were performed using CCL64 ls. In this paper, we report results about the biochemical analysis and identification of PCDGF cell surface binding sites for these cells and also for other cell lines.

MATERIALS AND METHODS

Expression of PCDGF in insect cells and purification of PCDGF. PCDGF cDNA was ligated into the *Eco*RI-*Sma*I sites of Baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA). Plasmid of pVL1392-PCDGF was used to cotransfect insect cells (SF9) with Baculovirus DNA (BaculoGold, PharMingen, San Diego, CA) and Baculovirus-PCDGF was selected according to the method of Summers (14). After infection of Baculovirus-PCDGF the insect cells (SF9 or High5) were cultured in Grace's medium for 48 hours at 27°C. The conditioned medium was collected and rPCDGF was purified by immunoaffinity chromatography on an anti-PCDGF antibody sepharose column. Details of the purification procedures are reported elsewhere.[4]

Iodination of PCDGF. Affinity purified PCDGF was iodinated by the chloramine T method at 4°C. Briefly, 1 μg of PCDGF was incubated for 2 min with $Na^{125}I$ (100 μCi) that had been preincubated for 90 seconds with 2 μg chloramine T (Sigma, St. Louis, MO). The reaction was quenched with the addition of 100 μl saturated tyrosine, 10 μl of a solution of 1% BSA and 2 μg sodium metabisulfite. After addition of 100 μl PBS, the iodinated protein was separated from free $Na^{125}I$ by gel filtration on a Sephadex-G50 column (Pharmacia, Uppsala, Sweden) that had been preblocked with PBS containing 1% BSA then extensively washed with PBS. The labeled proteins were eluted with PBS and fractions monitored for radioactivity. Amount of incorporated radioactivity was estimated by TCA precipitation. Specific activity of ^{125}I-PCDGF was typically 30-50 μCi/μg. Labeling conditions to reach higher specific activity always led to loss of biological activities for the radiolabeled protein.

^{125}I-PCDGF binding to CCL64 cells. The binding assays were performed using cells in suspension. Mink lung epithelial cell line, Mv1Lu (CCL64) (15) was obtained from the American Type Culture Collection (Rockville, MD). CCL64 cells were cultivated as monolayers in DME medium supplemented with 10% fetal bovine serum (FBS) until they reached confluency. At that time, cells were washed with PBS and detached by brief incubation with a solution of 0.25 mg/ml of trypsin and 1 mM EDTA. The cells were harvested by centrifugation, extensively washed with culture medium containing 1% BSA and proteinase inhibitors and counted with a hemocytometer. For binding assays, 10^6 cells were resuspended in 500 μl of binding buffer consisting of DME medium supplemented with 1% BSA in 1.5 ml Eppendorf tubes. Equilibrium binding was achieved by incubating CCL64 cells for 2 h at 22°C with 10^5 cpm of ^{125}I-PCDGF and increasing concentrations of unlabeled PCDGF from 0 to 100 ng/ml. At the end of the incubation period, binding was stopped by centrifugation followed by 3 successive washings at 4°C with ice-cold binding buffer followed by centrifugation. Cell pellets were counted with a LKB gamma counter. Scatchard analysis of binding data was done by using the LIGAND computer program. Values described in the result section correspond to the average of three separate experiments each performed with duplicate determinations per experimental point.

Crosslinking studies of PCDGF to various cell lines. 3T3 fibroblasts were cultivated in DMEM-F12 medium supplemented with 10% CS and PC cells were cultivated in defined medium as described previously (6). PC cells were detached by brief incubation with PBS containing 1 mM EDTA. 3T3 fibroblasts were detached by brief exposure to trypsin solution. In both cases, the cells were washed extensively with DMEM-F12 medium supplemented with 1% BSA prior to doing the binding assay. For crosslinking studies, 5×10^5 cells were resuspended in 250 μl of binding buffer in Eppendorf tubes. ^{125}I-PCDGF was added in 50 μl of binding buffer with or without

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100-fold excess unlabeled competing ligand. Binding was performed as described in the previous paragraph. At the end of the incubation period. the cells were washed twice with 0.2% BSA-DME and once with PBS before crosslinking was carried out. The cells were resuspended in 200 μl PBS containing 1 mM disuccinimidyl suberate (DSS. Pierce. Rockville. IL) which had been prepared at a stock concentration of 100 mM in DMSO just prior to being used. Incubation was carried out at room temperature for 20 min. After crosslinking, 10 μl of 1 M Tris-HCl buffer (pH 7.4) was added to quench the reaction. The cells were centrifuged, washed and extracted with 25 μl extraction buffer (PBS containing 1% Triton X-100, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C for 1 h. Samples were centrifuged for 5 min at 13,000 x g and 25 μl of supernatant from each sample was mixed with 4 μl of 20% SDS and 15 μl 3× Laemmli's sample buffer (16) containing β-mercaptoethanol and boiled for 5 min. Electrophoresis of samples was carried out on 7% SDS polyacrylamide slab gel according to Laemmli (16) using a mini-gel apparatus (Bio-Rad, Richmond, CA). The dried gels were exposed to X-ray films for autoradiography at −70°C.

RESULTS

Binding and Scatchard Analysis of ^{125}I-PCDGF to CCL64 Cells

Binding of ^{125}I-PCDGF to CCL64 cells was examined. PCDGF was iodinated with chloramine T as described in the method section. Preliminary experiments established that ^{125}I-PCDGF bound to CCL64 cells in a time-dependent manner, with maximal specific binding obtained at 22°C for 2 h (data not shown). To measure ^{125}I-PCDGF binding, CCL64 cells were incubated with labeled tracer in the presence of increasing amounts of unlabeled PCDGF. 2.8% of added ^{125}I-PCDGF bound to the cells in the absence of unlabeled competitor. A concentration-dependent inhibition of iodinated PCDGF binding was observed in the presence of unlabeled PCDGF. A 90% inhibition of total binding was observed at the highest concentration of unlabeled PCDGF tested (300 ng/ml).

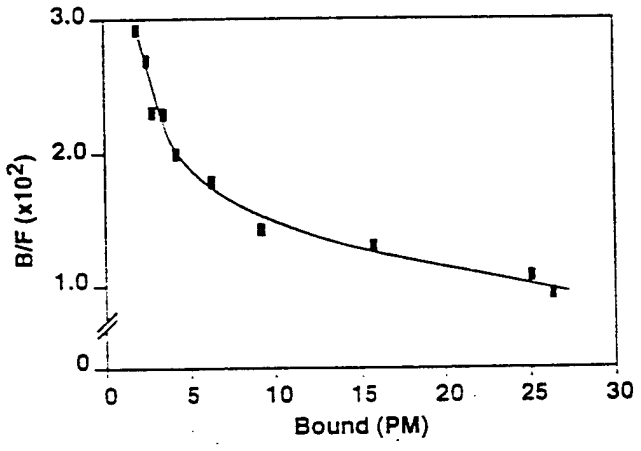

FIG. 1. Scatchard analysis of ^{125}I-PCDGF binding to CCL64 cells. CCL64 cells were incubated fir 2 h at 22°C with ^{125}I-PCDGF (10^5 cpm/tube) in the presence of increasing concentrations of unlabeled PCDGF (0-100 ng). The cells were washed three times and cell-associated radioactivity was measured in a gamma counter. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled PCDGF. Scatchard analysis of PCDGF binding data was done using LIGAND computer program. Scatchard representation of one of three experiments is shown here.

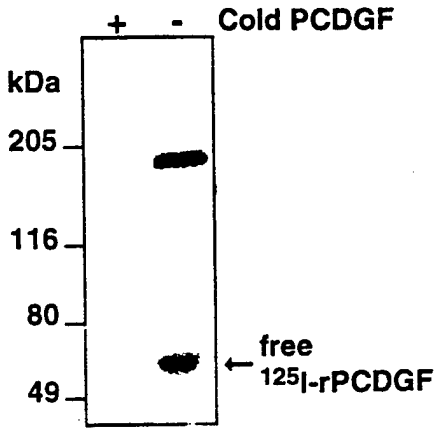

FIG. 2. Affinity labeling of ^{125}I-PCDGF binding sites on CCL64 cells. ^{125}I-PCDGF was incubated with 5 × 10^5 CCL64 cells in the presence or absence of 100-fold excess of unlabeled PCDGF for 2 h at 22°C and was crosslinked to its receptor with 1 mM DSS (final concentration) for 20 min at 4°C. The crosslinking reaction was quenched by the addition of Tris-HCl buffer (pH 7.4), and the cells were extracted by 1% Triton X-100. The supernatant was mixed with 3 × SDS-PAGE sample buffer containing β-mercaptoethanol and electrophoresed on a 7% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to the X-ray film.

Equilibrium binding data were analyzed by the LIGAND computer program (17). Scatchard representation from one typical experiment is shown in Fig. 1. The data indicated that binding of ^{125}I-PCDGF to CCL64 cells was curvilinear corresponding to the presence of two classes of cell surface receptors for PCDGF. Based on three independent experiments, binding parameters calculated were K_{d1} = 43 ± 15 pM with 560 ± 170 sites/cell for the high affinity sites, and K_{d2} = 3.9 ± 1.9 nM with 16350 ± 5900 sites/cell for the low affinity sites.

Crosslinking of ^{125}I-PCDGF to CCL64 Cells

^{125}I-PCDGF bound to CCL64 cells was crosslinked by the addition of the chemical crosslinker DSS. Crosslinked material was separated by electrophoresis on 7% polyacrylamide gels followed by analysis by autoradiography. As shown in Fig. 2, autoradiographic analysis revealed the presence of one major crosslinked band with an apparent molecular weight of 190 kDa. The intensity of the radiolabeled crosslinked band was significantly decreased in the lanes where binding was carried out in the presence of excess cold PCDGF, showing that unlabeled PCDGF competed with ^{125}I-PCDGF. Crosslinked band could not be detected if experiment was performed in the absence of crosslinker DSS and

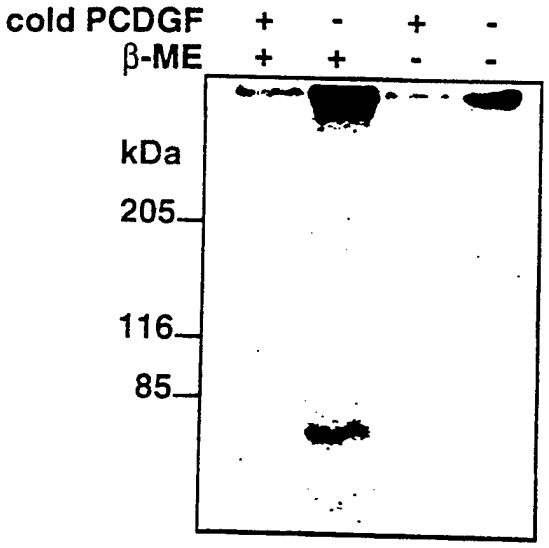

FIG. 3. Comparison of affinity labeling of ^{125}I-PCDGF to CCL64 cells in reducing and non-reducing conditions. Binding and crosslinking of ^{125}I-PCDGF to CCL64 cells were done as described previously. The extracts were mixed with SDS-PAGE sample buffer in the presence or absence of 10 mM β-mercaptoethanol (β-ME) and separated on a 7% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to the X-ray film. In non-reducing condition the migration of PCDGF in SDS-PAGE was changed due to the high cysteine content of the protein.

when using cells which were not responsive to PCDGF (data not shown). As shown in Fig. 3, the molecular weight of the crosslinked band was not significantly changed whether samples were treated or not with β-mercaptoethanol prior to performing the electrophoresis. These data would suggest that if the receptors are multimeric, they are not linked by disulphide bridges.

PCDGF Receptors Are Ubiquitously Expressed in Cells of Different Embryonic Origin

We have shown previously that PCDGF is a growth stimulator for mouse 3T3 fibroblasts and for the highly tumorigenic teratoma-derived PC cell line from which PCDGF was originally purified (6). Binding and crosslinking experiments of ^{125}I-PCDGF to both cell types were then performed to determine the size of crosslinked PCDGF receptors on both types of cells. SDS-PAGE of the crosslinked products indicated the presence of a major wide labeled band which migrated with an apparent molecular weight similar to the ones in CCL64 cells. The formation of crosslinked bands was prevented by addition of unlabeled PCDGF, showing the binding of ^{125}I-PCDGF to these cells was specific (Fig. 4). Because PC cells were cultivated in defined medium in the absence of serum, these results indicate that the crosslinked band does not correspond to a possible interaction between PCDGF and a binding protein present in serum. These data suggest that PCDGF cell surface receptors are expressed in many different types of cells responsive to PCDGF and the molecular weight of these receptors on these different cells is similar.

DISCUSSION

As a member of a new growth factor family, PCDGF may play an important role in cellular proliferation and differentiation. The characterization of receptors which mediate the effects of growth factors such as PCDGF is of importance due to the possible involvement of these proteins in physiological and/or pathological processes. The data presented here investigate for the first time the binding of PCDGF (epithelin/granulin precursor) to cell surface receptors. Our experiments show that PCDGF can be iodinated and that ^{125}I-PCDGF specifically binds to several cell types. Scatchard analysis of the equilibrium binding data with the epithelial cell line CCL64 indicates the existence of two classes of high and low affinity PCDGF receptors. Crosslinking experiments reveals the presence of one major class of binding sites for PCDGF on CCL64 with the molecular weights of about 120 kDa. Furthermore, it is shown that binding sites for PCDGF are present on several PCDGF-responsive cell lines in addition to CCL64 cells.

The existence of two classes of high and low affinity receptors for PCDGF by Scatchard analysis suggests either possible interactions between identical binding sites, or multiple independent sites. However, it is now

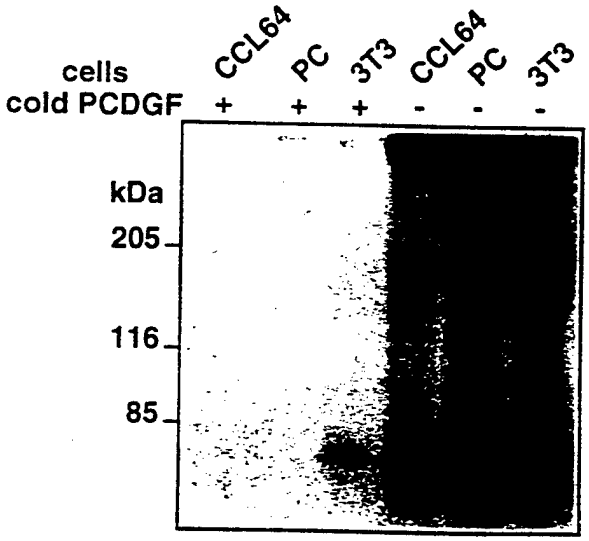

FIG. 4. Presence of PCDGF binding sites on the cell surface of several cell lines. 3T3 fibroblasts, PC cells and CCL64 cells were prepared as described under Materials and Methods. Cell suspensions were incubated for 2 h at 22°C with ^{125}I-PCDGF in the presence or absence of unlabeled PCDGF. After incubation, the cells were treated with DSS for 20 min at 4°C and extracted in 1% Triton X-100. The extracts were analyzed by SDS-PAGE and autoradiography performed as mentioned before.

well documented for other growth factors that the formation of high affinity sites may be the result of receptor subunit interaction, such as homodimerization of low affinity receptors as it has been shown with EGF receptor (18), heterodimerization of α and β chains as shown for interleukin-2 receptor (19), or heterodimerization of a low affinity receptor with an affinity converter subunit as recently reported between the receptors for interleukin-6, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor and the gp130 protein (20, 21). Crosslinking experiments of ^{125}I-PCDGF binding to cell surface binding sites revealed the presence of a single band of 190-kDa after SDS-PAGE and autoradiography. This does not necessarily imply that only a single binding species is present.

Like many other cytokines and growth factors (20, 22, 23), PCDGF may be multifunctional and exhibit pleiotropic biological effects on cells. To date, the only physiological role known to PCDGF or epithelins is related to cell proliferation. Unlike epithelins, PCDGF affects proliferation both on epithelial and non-epithelial cells (6). Dependent on the cell types, PCDGF either stimulates or inhibits the cellular proliferation. The effective concentration of PCDGF for inhibition or stimulation on the cellular proliferation was similar.[5] An interesting question remained to be answered is whether the different biological effects of PCDGF are mediated by the different classes of binding sites independently or by the diversified downstream signal pathways in cells.

PCDGF is the precursor of epithelins/granulins. Up to now there is only one report about the characterization of receptor for epithelins (11). In addition, the binding sites on epithelial cells for TGF-e, a protein with the N-terminal sequence identical to the human granulin A, is reported (24). Scatchard analysis revealed there were two different binding sites with different affinity for epithelins and results from crosslinking showed there was a single band of ligand-receptor complex with about 145 kDa on the surface of MDA-MB-468. Furthermore, it is observed that epithelin 1, 2 and 3 seemed to share same receptor because they all specifically competed with iodinated epithelin 1 for the binding to the membrane. Despite the evidence that showed that PCDGF receptor is different from epithelins receptors, we cannot totally exclude the possibility that PCDGF and epithelins share same receptors or some classes of the receptors. Molecular cloning and expression of PCDGF receptors should enable us to study the exact nature of the interactions between PCDGF and epithelins/granulins receptors. Even if PCDGF receptors are different from epithelins receptors, cross-talk and transmodulation between these receptors remain potentially important regulatory events to consider, as has been shown to occur between EGF receptor (25) and platelet-derived growth factor receptors (26).

[5] X. Xia and G. Serrero, unpublished observation.

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Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells

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Running title: Antiestrogenic activity of resveratrol

No. of figures: 5

Abstract

Resveratrol is a natural phytoalexin compound found in grape and other food products. In this paper, the effect of resveratrol on the growth of cultured human breast cancer cells was examined. Results show that resveratrol inhibits the proliferation of estrogen receptor positive MCF-7 cells in a dose dependent fashion. Detailed studies with MCF-7 cells demonstrate that resveratrol antagonizes the mitogenic effect of E_2 in a dose-dependent fashion at both the cellular (cell proliferation) and the molecular (gene activation) levels. At 5 μM, resveratrol abolished the growth stimulatory effect mediated by concentrations of E_2 up to 10^{-9} M. The antiestrogenic effect of resveratrol could be observed at concentrations as low as 1 μM. The antiestrogenic effect of resveratrol has also been demonstrated at the molecular level. Resveratrol in a dose-dependent fashion antagonized the stimulation by E_2 of progesterone receptor gene expression in MCF-7 cells. Moreover expression of Transforming Growth factor α and Insulin like Growth Factor I receptor mRNA were inhibited while Transforming Growth Factor β2 mRNA was significantly elevated in MCF-7 cells after 6 days incubation with resveratrol (10 μM). In summary, our results show that resveratrol, a partial ER agonist itself, acts as an ER antagonist in the presence of estrogen leading to inhibition of human breast cancer cells, suggesting its possible role as a chemopreventive agent.

Introduction

Resveratrol (3,5,4'- trihydroxystilbene) is a bioflavonoid found in many plants, including grapes, and mullberries. In the plant world, resveratrol, regarded as antibiotic, plays an important role in host defense mechanism against infection and injury (Dercks and Creasy, 1989). Red wine is believed to be the main source of resveratrol in human diet. Recent studies have associated resveratrol with the cardioprotective effect observed among people with moderate wine consumption. For example, it has been reported that resveratrol has protective effects against oxidation of lipoproteins (Frankel et al., 1993), an important step in atherogenesis; it also inhibits platelet aggregation and alters eicosanoids synthesis (Soleas et al., 1997). Morerover, resveratrol has been found to possess chemopreventive activity: it is an inhibitor of ribonucleotide reductase itself (Fontecave et al., 1998) and can inhibit cellular event associated with cell proliferation, tumor initiation, promotion and progression (Jang et al., 1997; Mgbonyebi et al., 1998).

Bioflavonoids are major constituents of plants and vegetables (Harborne, 1994). Some of them have been categorized as phytoestrogens because these environmentally derived compounds bind and activate the estrogen receptor (ER) (McLachlan, 1985). It also has been shown that these phytochemicals are weak estrogens and less active than endogenous estrogens (McLachlan, 1985). Recent studies have suggested that some of the flavonoids have antiestrogenic effect through preventing more potent endogenous estrogen binding to ER, a mechanism similar to tamoxifen (Makela et al., 1995; Markaverich et al., 1995;

Collins et al., 1997). For example, phytochemicals, such as enterolactone, narigenin and phloretin have all been shown to possess mixed agonist / antagonist activity of ER (Mousavi and Aldercreutz, 1992; Miksicek, 1993; Ruh et al., 1995). Indeed, the chemopreventive effect of bioflavonoids has been used to explain the low incidence of breast cancer and prostate cancer among vegetarians and Orientals who normally have higher blood level of phytoestrogens (Setchell and Adlercreutz, 1988; Adlercreutz et al., 1992; Makela et al., 1995). Recently, Gehm et al, reported that resveratrol can bind and activate ER and stimulate the growth of ER positive breast cancer cells (Gehm et al., 1997). However, effect of resveratrol on the ER positive breast cancer cell growth in the presence of E_2 have not been thoroughly studied. In the present paper, we examined the effect of resveratrol either alone or in combination with estrogen on the growth of breast cancer cell MCF-7 both at cellular and molecular levels.

Experimental procedures

Cell culture

MCF-7 cells obtained from the American Type Culture Collection (ATCC) were maintained in Dulbecco' Modified Eagle's medium (DME)-Ham's F12 medium (1:1 mixture; Gibco) supplemented with 5% fetal bovine serum (FBS).

Cell proliferation studies using cells cultivatied in DME-F12 medium supplemented with 5% FBS.

To determine the effect of resveratrol on cell proliferation, cells were plated at 10^5 cells per well in 6-well plates in the same medium mentioned above in the presence or absence of increasing concentrations of resveratrol (Sigma). The cell number was measured every two days till day 6 after trypsinizing the cells.

Cell proliferation studies using cells cultivated in estrogen-depleted medium.

To examine the effect of resveratrol and 17-β- estradiol (E_2) (Sigma) on cell proliferation, MCF-7 cells were plated in 24-well plates at $5x10^4$ cells per well in phenol-red free α-modified Eagle's medium (α-MEM)(Life Technologies, Inc) supplemented with 5% charcoal-stripped FBS (PFMEM) for 24h. The medium was then removed and replaced with fresh PFMEM medium in the presence or absence of various concentrations of resveratrol and E_2 as indicated in the figures. Cells were counted at day 6.

RNA isolation and RT-PCR

For the study of Progesterone receptor (PR) mRNA expression, MCF-7 human breast cancer cells ($3X10^5$ cells / well) were plated in 6-well plates in DME-F12 medium plus 5% FBS. Two days later, the cells were washed and cultivated in

estrogen free PFMEM medium for 24h. MCF-7 cells were incubated with different concentrations of E_2, resveratrol and 4-OH-Tamoxifen (Sigma) for 24h since stimulation of PR mRNA expression reaches a maximum after 24h (May et al., 1989).

For the study of the expression of autocrine growth regulators: Transforming Growth factor α (TGF-α), Transforming Growth Factor-β1,-β2,-β3 (TGF-β1,β2,β3) and Insulin like Growth Factor I (IGF-I) and Insulin like Growth Factor II (IGF-II) or growth factor receptors: IGF-I receptor (IGF-IR), TGF-β receptors and Epidermal growth factor receptor (EGFR), MCF-7 cells were seeded at 10^5 cells / well in 6-well plate in DME-F12 medium plus 5% FBS in the presence or absence of different concentrations of resveratrol for six days.

Total RNAs were isolated using TRIZOL reagents (Gibco) according to the manufacture's protocol. Specific mRNAs expression was examined by semiquantitative RT-PCR based on the comparison with an internal control, human β-actin mRNA expression. The RT-PCR reaction in the exponentially amplifying cycle allows semiquantitative comparison of the mRNA expression (Ide et al., 1997). Briefly, 5 μg each of total RNAs from MCF-7 cells was reverse-transcribed by random primer and Super-script II reverse transcriptase (Life Technologies, Inc). The resulting cDNA was subjected to PCR with the primers described below. The amplification reactions were performed with an initial incubation step at 94°C for 3 min followed by 25 cycles each (30 cycles for TGF-β2, TGF-α) at 94 °C for 1 min, 60 °C for 45s, 72 °C for 2 min. These cycles were followed by a final incubation step at 72 °C for 7 min. The samples were

subjected to electrophoresis in 1.2% agarose gel and stained with ethidium bromide. The identity of PCR products was confirmed by restriction enzyme digestion. The gels were blotted onto nylon membrane (BIO-RAD) in 10X SSC, and southern blot was conducted as described before (Eisinger and Serrero, 1993; Quinn et al., 1996) using corresponding sense primer for each PCR product as a probe. The probes were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Life technology,Inc). The RT-PCR analysis was repeated at least three times for each of the two independent experiments. The primers used for amplification of PR and IGF-I receptor, were synthesized according to published sequences (Quinn et al., 1996; Hobisch et al., 1997). The primers for TGF-α, TGF-β2, β-actin are obtained from CLONTECH. The sizes of amplified PCR fragments were: 297bp for TGF-α, 755bp for IGF-IR, 838bp for β-actin, 415bp for TGF-β2, and 742bp for PR, respectively.

All experiments described in this paper were repeated at least three times. Values are expressed as means $\pm$SD

Results

Effect of resveratrol on the proliferation of MCF-7 cells cultivated in regular medium (DME/F12 supplemented with 5% FBS).

MCF-7 cells were plated in DME-F12 medium supplemented with 5% FBS. The effect of resveratrol on the proliferation of MCF-7 cells was determined at different concentrations. As shown in Fig. 1, resveratrol inhibited the growth of MCF-7 cells in a dose-dependent fashion. At 10 μM, resveratrol had a dramatic inhibition of MCF-7 cell growth after six days (about 80-90% inhibition) while at 1 μM, only a 10% inhibition was observed at day 6. At 100 μM, resveratrol completely inhibited the growth of MCF-7 cells. MCF-7 cells from which resveratrol (10 μM) had been removed after three days incubation failed to regain any growth advantage over the cells continuously maintained in the presence of resveratrol (data not shown). These data would suggest that the Inhibitory effect of resveratrol on MCF-7 cell growth is irreversible.

In order to examine whether the inhibitory effect of resveratrol was due to the presence of estradiol in the complete culture medium, we then examined the effect of resveratrol on MCF-7 cells cultivated in the absence or presence of estradiol in estrogen-depleted medium.

Effect of resveratrol on the proliferation of MCF-7 cells cultivated in estrogen-depleted medium.

MCF-7 cells were seeded in estrogen-depleted PFMEM medium for 24h, as described in the methods section. Resveratrol was added at the indicated concentrations and cells were counted at day 6. Fig. 2 shows that resveratrol

alone had a weak mitogenic effect on MCF-7 cells cultivated in estrogen-depleted medium. Resveratrol as low as 100 nM, stimulated the growth of MCF-7 cells. The mitogenic effect of resveratrol reached a maximum at 1 μM. However, resveratrol was not as potent a growth stimulator as E_2 since the maximal stimulation of MCF-7 cell growth with 1 μM of resveratrol was two fold over the control when compared to a four fold stimulation observed with 10^{-9} M E_2. In contrast, a 10 μM concentration of resveratrol completely inhibited the growth of MCF-7 cells cultivated in PFMEM medium.

Resveratrol antagonizes E_2-mediated stimulation of MCF-7 cell proliferation

Since E_2 is the major mitogen in MCF-7 cell growth, we next examined the effect of resveratrol on E_2 mediated MCF-7 cell proliferation. MCF-7 cells were seeded in PFMEM medium for 24h. Then resveratrol and E_2 were added at the indicated concentrations and cells were counted at day 6. First we examined the effect of 5 μM of resveratrol on the proliferative effect mediated by different concentrations of E_2 on MCF-7 cells. Fig. 3A shows that resveratrol (5 μM) dramatically inhibited the proliferative effect of E_2 at all concentrations tested (from 10^{-12} to 10^{-9} M). When added in the presence of E_2 up to 10^{-10} M, growth inhibition was complete since the cell numbers after 6 days were similar to the ones measured for control cells maintained in estrogen-depleted medium only.

In the next experiment, E_2 concentration (10^{-9} M) was maintained constant and resveratrol concentrations varied in order to examine the antiestrogenic potential of different concentrations of resveratrol. As shown in Fig. 3B, starting from as low as 1 μM, resveratrol antagonized the effect of E_2 in a dose-dependent

manner. A 50% inhibition of E_2 effect was observed at a resveratrol concentration of 5 μM. Maximal growth inhibition was achieved in the presence of 10 μM of resveratrol.

In summary, we show that resveratrol, itself a weak mitogen, antagonizes the mitogenic effect of E_2 in a dose-dependent fashion.

Resveratrol antagonizes the PR expression stimulated by E_2

In order to confirm the antiestrogenic action of resveratrol, we examined whether resveratrol antagonizes the effect of E_2 on the expression of an E_2 inducible gene product such as progesterone receptor (PR) mRNA expression. PR is the most responsive of all the estrogen regulated RNAs studied so far (May et al., 1989). Using RT-PCR technique, PR mRNA expression was examined in MCF-7 cells treated or not with resveratrol and E_2. As shown in Fig. 5, resveratrol alone stimulated PR expression, reaching a maximum of 10 fold at 10 μM whereas stimulation by E_2 (10^{-9} M) alone was 22 fold. Together with E_2 (10^{-9} M), resveratrol inhibited PR expression in a dose dependent manner similar to the antiestrogen 4-OH-tamoxifen. At 10 μM, resveratrol inhibited PR expression by 50% and by 75% at 30 μM. The fact that the maximal stimulation obtained by resveratrol treatment was only about half of the maximum stimulation observed with E_2 only is in agreement with the observed effect of resveratrol on MCF-7 proliferation in the absence of E_2 (Fig. 2), indicating that resveratrol can act as a weak estrogen.

Effect of resveratrol on the mRNA expressions of autocrine growth factors and their receptors

Proliferation study had shown that long term incubation with resveratrol dramatically inhibited the growth of MCF-7 cells (80%-90% inhibition after 6 days at 10 μM of resveratrol), Experiments were then performed to investigate the expression of growth factors and growth factor receptors known to be important in the growth control of breast cancer cells after 6-day resveratrol treatment. Using RT-PCR, the mRNA expression for TGF-α, TGF-βs, IGF-I, IGF-II as well as their receptors were examined at 5 μM and 10 μM concentrations of resveratrol respectively. The most noticeable changes were the significant inhibition of TGF-α and IGF-IR mRNA expressions as well as a dramatic elevation of TGF-β2 mRNA expression where cells were treated with resveratrol. As shown in Fig. 6, resveratrol inhibited the mRNA expression of TGF-α and IGF-IR mRNA in a dose dependent fashion. 10 μM resveratrol inhibited the expressions of TGF-α by 87% and IGF-IR by 90%, respectively. There was a slight increase of IGF-II mRNA expression while IGF-I mRNA was not detected by RT-PCR (data not shown). No changes were observed in the mRNA expressions of IGF-II receptor and EGF receptor after resveratrol treatment (data not shown). TGF-β2 has been regarded as a negative growth regulator for breast cancer cells and a marker of antiestrogenic action *in vitro* and in *vivo* (Butta et al., 1992; Kopp et al., 1995; Kopp et al., 1995; Knabbe et al., 1995; MacCallum et al., 1996; Muller et al., 1998). A 6-fold increase of TGF-β2 mRNA expression was observed at 5 μM concentration of resveratrol. At 10 μM, resveratrol significantly

elevated TGF-β2 mRNA expression up to 15-fold above the control. In contrast to TGF-β2, no change in the expression of TGF-β1, TGF-β3 and TGF-β receptors mRNAs were observed in these conditions (data not shown). Finally, resveratrol (10 μM) had no effect on the mRNA expression of ER (data not shown).

Discussion

The results presented in this paper show that resveratrol inhibits the growth of ER-positive MCF-7 human breast cancer cells. We further demonstrate that resveratrol, itself a partial ER agonist, antagonizes the mitogenic effect of E_2 in a dose dependent fashion at both cellular level (cell proliferation) and molecular level (gene activation). At 5 μM, resveratrol significantly inhibited the proliferation mediated by all concentrations of E_2 tested (10^{-12} M to 10^{-9} M). The antiestrogenic effect of resveratrol could be observed when added at concentration as low as 1 μM. At the molecular level, resveratrol antagonized PR mRNA expression stimulated by E_2 in a dose-dependent manner. Resveratrol alone activated PR mRNA expression, but when combined with E_2 (10^{-9} M) it inhibited PR mRNA expression in a dose dependent manner.

Since autocrine growth factors have been shown to play an important role in the growth regulation of breast cancer cells (Yee et al., 1988; May and Westley, 1995; Johns and Clemmons, 1995), we decided to examine the expression of several autocrine growth factors and their receptors after resveratrol treatment. The most obvious changes observed were a significant inhibition of TGF-α mRNA expression (87% inhibition at 10 μM of resveratrol) and IGF-IR mRNA expression (90% inhibition at 10 μM of resveratrol). The results are in agreement with the conclusion that resveratrol has antiestrogenic effect since these two genes are known to be estrogen-responsive. TGF-α has a mitogenic effect on human breast cancer cells in culture. Its expression is induced by E_2 and suppressed by antiestrogen (Lippman et al., 1988; Dickson, 1990). IGF-IR is also

a target of estrogen regulation. Research showed IGF-IR mRNA expression increased 6 fold following E_2 stimulation, and its induction is important for E_2 mediated proliferative effects (Stewart et al., 1990). Since the mitogenic effect of E_2 requires the presence of TGF-α, IGF-IR, the inhibition of their expression by resveratrol might be detrimental to cell growth.

Our results also show for the first time that resveratrol dramatically stimulated TGF-β2 mRNA expression (15 fold) in MCF-7 cells while no changes in TGF-β1, TGF-β3 were observed. This stimulatory effect on TGF-β2 expression was also observed with cells cultivated in estrogen depleted medium (data not shown). TGF-βs have been shown to be negative autocrine regulators that inhibit the growth of most breast cancer cell lines (Knabbe et al., 1987; Wang et al., 1995; Wang et al., 1996). TGF-β family includes three isoforms, TGF-β1, TGF-β2, TGF-β3. Growth stimulation of estrogen dependent breast cancer cells with E_2 is associated with down-regulation of TGF-β2, TGF-β3 mRNAs (Jeng et al., 1993); growth inhibition of the cell lines by the antiestrogen (tamoxifen) is associated with elevated TGF-β2 mRNA expression. In fact, part of the growth inhibitory effect of antiestrogen is thought to be mediated through the induction of TGF-βs (Knabbe et al., 1987; Zugmaier et al., 1989; Arteaga et al., 1990; Arrick et al., 1990; Jeng et al., 1993). It is believed that in MCF-7 cells, the antiestrogen first induce the secretion of TGF-β1 via a nontranscriptional pathway; TGF-β1 itself induces TGF-β2 by a direct transcriptional mechanism (Knabbe et al., 1995). This may explain why we did not observe any significant changes in TGF-β1 mRNA expression in the presence of resveratrol. There are still some

controversies regarding the role of TGF-βs in the growth inhibition mediated by antiestrogen. Recently, Koli et al (Koli et al., 1997) reported that blockade of TGF-β signaling using dominant negative TGF-β2 receptor in combination with neutralization assay failed to prevent antiestrogen-mediated growth inhibition of breast cancer cell MCF-7. Regardless of the conflicting reports in the interpretation of the importance of TGF-β in the growth of breast cancer cells, TGF-β2 has been widely accepted as a marker of antiestrogen action *in vitro* and *in vivo* (Knabbe et al., 1995; Kopp et al., 1995; MacCallum et al., 1996; Muller et al., 1998). Thus the results presented here that resveratrol stimulates TGF-β2 mRNA expression confirm the conclusion of the antiestrogenic effects of resveratrol as well as provide a possible mechanism for its growth inhibition.

The competitive binding of radiolabeled estradiol by resveratrol has been examined in a clone of MCF-7 cells (Gehm et al., 1997). The data showed that resveratrol is a weak ligand for ER. The IC_{50} for resveratrol to inhibit 0.1 nM ^{125}I-estradiol binding to ER is about 10 μM. In this study, it was shown that resveratrol, acting as a pure ER agonist, stimulated the growth of a clone of MCF-7 cells in the absence of estrogen. However, the effect of resveratrol on cell growth was not examined in the presence of E_2. In our studies, we show here that resveratrol, like tamoxifen, is a partial agonist of ER at low concentrations and whereas in the presence of E_2 will antagonize the E_2 effect at higher concentrations resulting in growth inhibition of breast cancer cells. The most likely mechanism for the antiestrogenic effect of resveratrol could be due to the direct competition of resveratrol with E_2 for binding to ER as was suggested for

some phytochemicals (Adlercreutz et al., 1992; Miksicek, 1993; Ruh et al., 1995). But other mechanism might also be involved, such as prevention of ER binding to ERE or of ER-mediated transactivation. Finally, data also indicate that antiestrogenic effect is not the sole possible mechanism mediating resveratrol growth inhibition of breast cancer cells because resveratrol is also capable of inhibiting the growth of the ER-negative MDA-MB-468 cells, even though less effectively than for MCF-7 cells (Lu and Serrero unpublished result). Resveratrol has been found to be a potent inhibitor of ribonucleotide reductase and cyclooxygenase-2 (Fontecave et al., 1998; Subbaramaiah et al., 1998), and can also inhibit NADH:ubiquinone oxidoreductase (Fang and Casida, 1998) and DNA polymerase (Sun et al., 1998). The inhibition by resveratrol of these key enzymes involved in cell metabolism may explain some of antiproliferative effect of resveratrol.

There is no pharmacokinetics information available about resveratrol metabolism in human body. But it is believed that a couple of glasses of wine could supply the body with two-digit micromolar concentration of resveratrol, the concentration at which most pharmacological effects of resveratrol are found (Jang et al., 1997). The finding that resveratrol possesses antiestrogenic ability and also inhibit the growth of ER negative breast cancer cells raises an interesting question about its potential role as chemopreventive agent. Even though no "French Paradox" has been found in term of cancer prevention, reports in the literature indicate an inverse relationship between breast cancer and wine consumption and not simply with alcohol consumption (Renaud and

deLorgeral., 1993; Gronbaek et al., 1995). Based on this report and our data presented here, resveratrol is definitely an interesting compound worthy of further investigation for its chemopreventive potential.

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Figure legends

Fig. 1. Effect of resveratrol on the proliferation of MCF-7 cells cultivated in regular medium. MCF-7 cells were plated as described in the materials and methods section in DME-F12 medium plus 5% FBS either in the absence (0.1% ethanol only) or in the presence of indicated concentrations of resveratrol. Cell number was determined every other day until day 6. Values are means±SD of triplicate determinations.

Fig. 2. The effect of resveratrol on the proliferation of MCF-7 cells cultivated in estrogen-depleted medium. MCF-7 cells were plated in phenol-red free α-MEM plus 5% charcoal-stripped FBS (PFMEM) for 24h as described in the materials and methods section. The medium was then removed and replaced with the same PFMEM medium in presence of various concentrations of resveratrol or 10^{-9} M E_2 only (E) as indicated in the figure. Control cells (C) received 0.1% ethanol only. The cell number was measured at day 6. Values are means±SD of triplicate determinations.

Fig. 3. Resveratrol antagonizes the E_2 mediated proliferation in MCF-7 cells. The culture conditions of MCF-7 cells were similar to the ones used in the legend of Fig. 2 with indicated concentrations of E_2 and resveratrol. Cells were counted at day 6. Values are means±SD of triplicate determinations. **A,** effect of 5 μM resveratrol on the MCF-7 cell proliferation mediated by different concentrations of E_2. **B,** effect of different concentrations of resveratrol on the MCF-7 cells

proliferation mediated by 10^{-9} M E_2. The control cells received either 0.1% ethanol only (C) or 10^{-9} M E_2 only (E).

Fig. 4. The effect of resveratrol on the mRNA expression of PR gene stimulated by E_2. MCF-7 cells were plated in DME-F12 medium supplemented with 5% FBS. Two days later, cells were starved in PFMEM medium for 24h. Cells were treated either with 0.1% ethanol as control (C); 10^{-9} M E_2 only (E); increasing concentration of resveratrol alone or in the presence of 10^{-9} M E_2; or indicated concentrations of 4-OH-Tamoxifen in the presence of10^{-9} M E_2. Total RNA was isolated 24h later. PR mRNA expression was examined using RT-PCR as described in the methods section. The PR signals were scanned and normalized to β-actin internal control and the results were expressed as fold induction in comparison to control. Values are means±SD of three independent experiments

Fig. 5. Effect of resveratrol on the mRNA expressions of TGF-α, IGF-IR and TGF-β2. MCF-7 cells were plated in DME-F12 medium plus 5% FBS in the absence (0.1% ethanol only as control) or presence of indicated concentrations of resveratrol. Total RNAs were isolated at day 6 and examined for the expressions of TGF-α (A), IGF-IR (B) and TGF-β2 (C) mRNA by RT-PCR. The signals were normalized to β-actin internal control and the results were expressed as fold induction in comparison to control. Values are means±SD of three independent experiments.

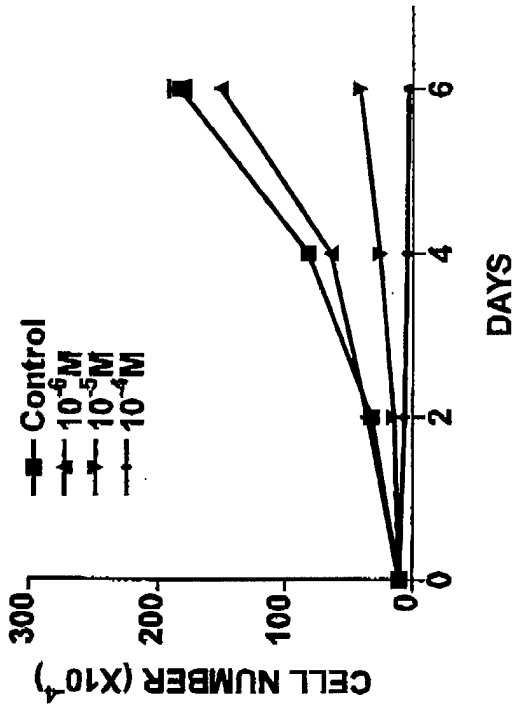
CELL NUMBER (X10⁴)
300
200
100
0
0
2
4
6
DAYS
Control
10⁻⁶M
10⁻⁵M
10⁻⁴M

Fig. 1

Fig. 1

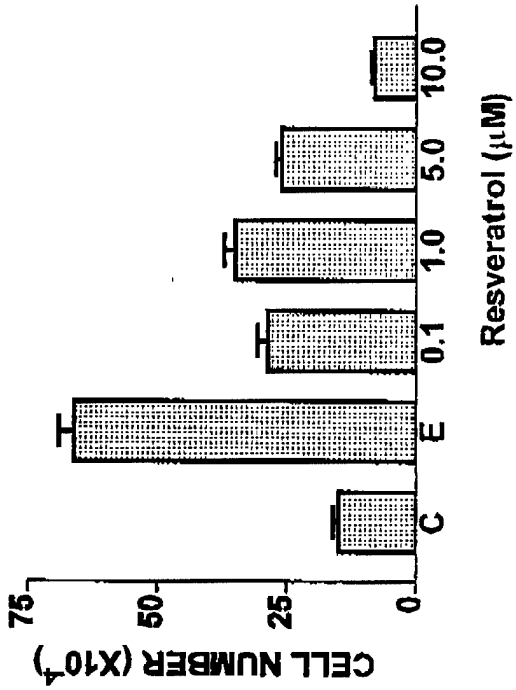

Fig. 2

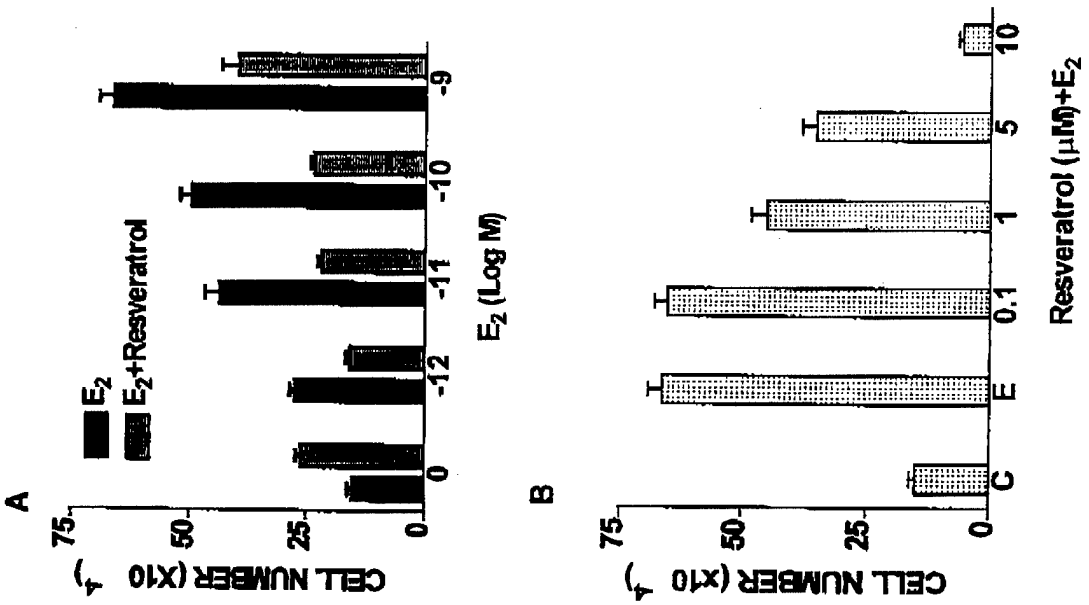
A
B
E_2
E_2+Resveratrol
CELL NUMBER (X10 ^{4})
75
50
25
0
-9
-10
-11
-12
E_2 (Log M)
C
E
0.1
1
5
10
Resveratrol (μM)+E_2

Fig. 3

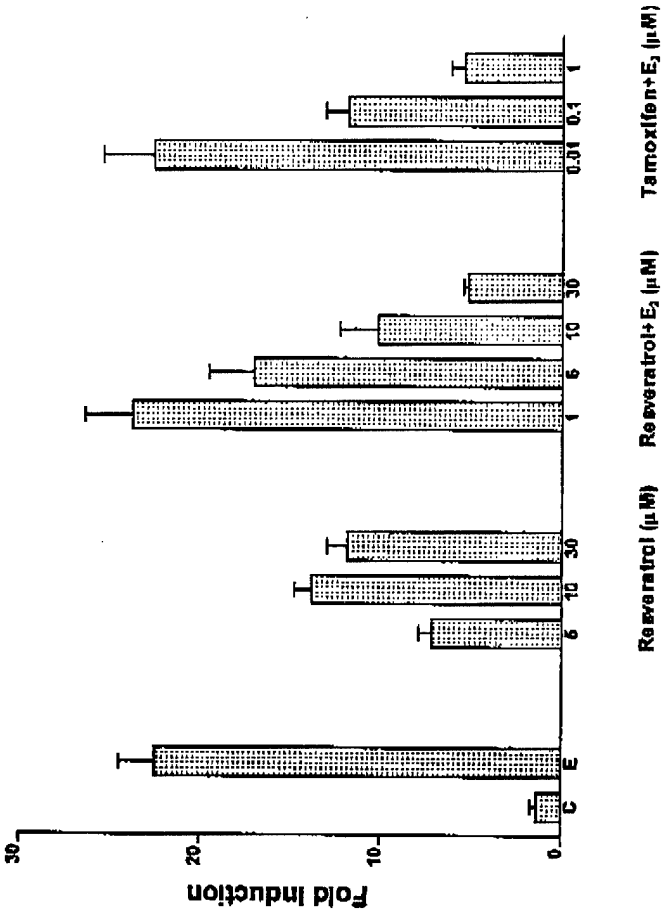
Fold Induction
0
10
20
30
C
E
Resveratrol (μM)
6
10
30
Resveratrol+E2 (μM)
1
6
10
30
Tamoxifen+E2 (μM)
0.01
0.1
1

Fig. 4

A
TGF-α
Fold Inhibition
10
5
0
C
5
10
Resveratrol (μM)

B
IGF-IR
Fold Inhibition
15
10
5
0
C
5
10
Resveratrol (μM)

C
TGF-β2
Fold Stimulation
20
10
0
C
5
10
Resveratrol (μM)

Fig. 5