Estradiol Inhibits Growth of Hormone-nonresponsive PC3 Human Prostate Cancer Cells

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ABSTRACT

Significant inhibition of proliferative activity in PC3 human prostate cancer cells by estradiol is reported, accompanied by experimental evidence for a specific estrogen receptor (ER). Radioligand-binding assays revealed the presence of high affinity sites of estrogen binding in the nuclear compartment of PC3 cells. In addition, using a reverse transcriptase-polymerase chain reaction system, we obtained evidence of either normal or a variant ER mRNA; the latter, which lacks the entire exon 4, is coexpressed with normal ER mRNA and has been recently characterized in our laboratories. The likelihood that the inhibitory effect exerted by estradiol could be mediated by an increase of transforming growth factor β (TGFß) production was also investigated. Use of monoclonal antibodies against TGFß1 produced a 3-fold increase of growth rate in PC3 cells; this clearly speaks for high levels of endogenous TGFß. This effect was almost completely abolished after addition of 100 nM estradiol. However, we failed to demonstrate any increase of TGFß mRNA after estradiol administration using Northern blot analysis. Further studies are needed to ascertain whether the estradiol-induced growth inhibition of PC3 cells is either mediated by other TGFß species or exerted via alternative mechanism(s).

INTRODUCTION

It is commonly recognized that gonadal androgens are essential in both development and function of the human prostate (for review see Ref. 1). However, whether androgens represent the principal determinants in either induction or maintenance of hyperplastic and cancerous prostate growth remains to be verified. Previous reports have suggested that peptide growth factors may be primarily involved in both initiation and promotion of abnormal prostate growth via either paracrine or autocrine loops (2). Production of TGFα and -ß, insulin-like growth factor II and platelet-derived growth factor has been shown to be regulated by estrogens in some hormone-sensitive human breast cancer cells in culture (3–5). On the other hand, only little information exists on the potential role of estrogens in growth control of either androgen-sensitive or insensitive prostate cancer tissues and cells. Recent studies (6) have shown evidence for ER in malignant prostate tissues using immunohistochemistry; others, however, using biochemical assay also, have failed to demonstrate detectable amounts of ER in both human prostate cancer tissues and cells (7). To further explore this issue, we have investigated the growth effects of steroid hormones, particularly estradiol, and ER status in PC3 human prostate cancer cells, as well as the possible involvement of estrogen-regulated growth factors, such as TGFß.

MATERIALS AND METHODS

Cell Culture. PC3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely maintained in RPMI 1640 containing 5 µg/ml phenol red and supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 µg/ml; amphotericin B, 0.25 µg/ml).

DNA Growth Assay. Cells were seeded onto 6-well tissue culture plates at a density of 5–10 × 10⁴ cells/well in routine medium. After 24 h, cells were washed twice with PBS-A and medium was replaced with phenol red-free RPMI medium, supplemented with 10% dialyzed, heat-inactivated, stripped fetal calf serum. Cells were therefore incubated with either increasing concentrations of androgens testosterone and DHT or with estradiol, from 1 × 10⁻¹¹ up to 1 × 10⁻⁷ M, in the presence or absence of a anti-TGFß neutralizing antibody (chicken, polyclonal; 50% neutralizing dose, 2–3 µg/ml; British Biotechnology, Oxon, United Kingdom) at a final concentration of 9.2 µg/ml. Parallel wells were incubated with the anti-TGFß antibody alone; control wells received vehicle (ethanol, 0.1%) only. After different incubation times (24, 72, 144 h) medium was discarded, cells were solubilized for 30 min at 37°C in 0.1% SDS, and DNA estimation was performed using a Hoechst 33258 microassay, as extensively described elsewhere (8).

Steroid Receptor Assay. Estrogen receptor content of PC3 cells was determined by means of radioligand-binding assay (9, 10), using dextran-coated charcoal and filtration methods to separate bound from unbound ligand in soluble and nuclear fractions, respectively. As routinely carried out in our laboratories for both ER and androgen receptor assay in either breast or prostate tissues, cell homogenates were spun at 3600 × g for 5 min at 4°C to separate the soluble (supernatant) from the nuclear (pellet) cell fraction. Aliquots of each fraction were therefore incubated overnight at 4°C against increasing concentrations (from 0.1 up to 5 nM) of tritiated estradiol, as radioligand. A 100-fold excess of unlabelled DES was used for competition studies. After incubation, aliquots of each cell fraction were finally counted in a LS1801 β-counter (Beckman Analytical SpA, Milan, Italy) for radioactivity.

Receptor data were analyzed and processed using Scatchard analysis and a least square fit routine, yielding both dissociation constant (Kd) and concentration values; the latter were expressed as either fMol/µg DNA or number of sites/cell for any cell compartment.

Reverse Transcriptase-PCR of ER. A reverse transcriptase-PCR system for ER in the ER mRNAs has been recently established in our laboratories (for methodological details see Ref. 11). Briefly, reverse transcription was carried out on total RNA extracted from mammary carcinoma cell lines ZR75–1, MCF7, and MDA-MB231 and from prostate cancer PC3 cells. For PCR amplification, a sense primer complementary to a sequence in exon 3 of the ER and an antisense primer corresponding to a sequence in exon 6 were used as template. Amplification products were separated on a 1.4% agarose gel and blotted onto nylon membranes. For Southern hybridization experiments, a human estrogen receptor cDNA, generously gifted by Pierre Chambon (Strasbourg, France), was cut from pSG5 plasmid, 32P-labeled and used as a probe.

Northern Blot Analysis. For experiments on TGFß mRNA expression and regulation by estradiol, cells were grown to near confluence in 10-cm plastic dishes in routine medium. After two washes with PBS-A, cells were trypsinized and plated at appropriate densities in 75-cm² tissue culture flasks. After 24 h, medium was replaced with serum-free, phenol red-free RPMI medium, supplemented with 20 µg/ml transferrin (Sigma-Aldrich, Milan, Italy) and 400 µg/ml bovine serum albumin fraction V (Sigma-Aldrich), containing 10⁻¹¹ to 10⁻⁷ M estradiol in ethanol (0.1%); duplicate flasks received...
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ethanol only. After varying incubation times (6, 24, and 72 h), cell monolayers were washed three times with ice-cold PBS and lysed directly into the culture flasks using 3 ml/dish of RNAzol B (Biotecx Laboratories, Houston, TX); RNA was thereafter extracted from cells following the recommendations of the supplier. Ten μg of total RNA were electrophoresed in 3-(N-morpholino)-propanesulfonic acid buffer at 30 V for 16 h in a cold room through a 1.2% agarose gel containing 6.6% formaldehyde. The filters were hybridized using DNA probes to human TGFβ1 (British Biotechnology) consisting of chemically synthesized single stranded oligonucleotides. The latter were a cocktail of antisense sequences complementary to exons 6, 7A, and 7B. Probes were end-labeled by T4-polynucleotide kinase using [γ-32P]dATP. Hybridization was carried out at 60°C for 16 h in 1 M NaCl-50 mM Tris-HCl (pH 7.5)-10% dextran sulfate-1% SDS. Filters were subsequently washed for 5 min at room temperature in 2X standard saline citrate and for 15 min at 60°C in 2X standard saline-citrate/0.1% SDS. Filters were finally autoradiographed for 20 h at −70°C using Kodak X-OMAT AR film.

RESULTS

Fig. 1A shows the effects of increasing concentrations of estradiol on growth of PC3 cells, after 6 days of exposure. As it can be seen, PC3 cells displayed a significant, dose-related inhibition of growth, with a maximal effect at 10⁻⁷ M estradiol (55.2% with respect to control; P < 10⁻⁶); this effect was also evident after 72 h at concentrations higher than 1 nM estradiol (not shown). This negative growth regulation is cognate to that observed in PC3 cells after addition of 1 ng/ml TGFβ1 (54.4% of control) under exactly the same experimental conditions. On the other hand, either testosterone or DHT did not affect growth of PC3 cells at any concentration used (not shown).

Incubation with polyclonal antibody which neutralizes the biological activity of TGFβ1 produced a noticeable increase of growth of PC3 cells, which was near 300% of control after 6 days (see Fig. 1B). This effect was opposed by addition of estradiol (10⁻¹¹ to 10⁻⁷ M), being almost completely reversed at the dose of 100 nM.

Fig. 2. Typical Scatchard plot and tracer ligand saturation curve of nuclear ER in PC3 cells. High affinity site (type I) of estrogen binding is here illustrated. Kd = 0.21 nM, ER = 187.9 fmol/mg DNA, r = 0.867. BpM, bound picomolar; B/F, bound over free. •, total; □, specific; ◦, nonspecific binding of tritiated estradiol, as calculated from competition studies using a 100-fold excess of unlabeled DES.

ER content and status of PC3 cells were evaluated by means of radioligand binding method. Soluble fraction did not show any detectable site of estrogen binding. By contrast, presence of both type I (high affinity, lower capacity) and type II (reduced affinity, greater capacity) binding was revealed in the nuclear fraction (see Fig. 2); in particular, type I ER exhibited Kd values ranging from 0.15 to 0.26 nM and mean fm concentrations of 209.7 ± 28.9 (SD) fmol/mg DNA (4037 ± 556.3 sites/cell).

The presence of mRNA coding for ER was evaluated by means of reverse transcriptase-PCR and Southern hybridization of the amplification products. Fig. 3 (left) shows reverse transcription-PCR of total RNA obtained from ZR75–1, MCF7, MDA-MB231, and PC3 cells. Two major bands of 659 and 323 base pairs were detectable in ZR75–1, MCF7, and PC3 cells, whereas no product was observed in MDA-MB231 cells. Southern hybridization analysis, using the complete ER-cDNA as a probe, revealed hybridization of the two major bands and some minor bands in between (Fig. 3, right). The 659-base pair band corresponds to the expected length for the normal ER mRNA and the 323-base pair band derives from a variant ER mRNA which lacks the entire exon 4; the latter, which is likely to be a product of alternative splicing, has recently been detected in our laboratories (11). The bands of intermediate length are of unknown origin, although one amplification product shows a length compatible with a putative variant messenger lacking exon 5 which would also be amplified by the primer set used; using a nested primer set with an
antisense primer in exon 4 a single band was observed in all cases (not shown). Although reverse transcriptase-PCR is by no means precisely quantitative, the relative amount of the ER messengers was reproducibly different in the cell lines studied. The two mammary carcinoma cell lines showed comparable expression level, which was, however, slightly higher in MCF7 than in ZR75-1 cells. The RNA isolated from the PC3 prostate cancer cell line gave rise to low levels of amplification products. Under the same conditions, however, no reaction could be observed in the estrogen-nonresponsive human breast cancer cell line MDA-MB231. Therefore, expression of the ER mRNA in PC3 cells is clearly significant.

Northern blot analysis of TGFβ1 mRNA in PC3 cells after treatment with estradiol (72 h) is shown in Fig. 4. As it can be seen from ethidium bromide staining of rRNA (Fig. 4a), Lanes 3 and 4 reveal less amount of total RNA loaded on gel. The relative level of TGFβ1 mRNA is shown in Fig. 4b. No significant difference was observed between Lane 1 (control) and Lanes 2, 3, and 4 (estradiol 0.01, 1 and 100 nm, respectively) when compared to the ethidium bromide picture. Human fibroblasts (Lane 5) showed higher level of TGFβ1 mRNA expression.

**DISCUSSION**

In the present paper we report that estradiol shows a clear inhibitory effect on growth of human prostate cancer PC3 cells; this is true also at lower concentrations, within physiological range. At higher doses (1 nm or more) this effect becomes evident well before 6 days of exposure. Conversely, PC3 cells, which apparently lack functional androgen receptors, did not respond to androgens, such as DHT or testosterone; this is in agreement with the existing literature (12).

Multiple experimental evidence for ER in PC3 cells is also provided. Firstly, we assessed soluble and nuclear ER content of PC3 cells by means of a radioligand-binding assay; the nuclear fraction showed both type I and II, while the soluble compartment did not display any detectable site of estrogen binding. It is generally agreed that type I ERs are essential intermediaries of the biological actions exerted by estradiol (8, 13). In order to establish the expression of ER at the mRNA level, in PC3 cells, we used the highly sensitive reverse transcriptase-PCR; the combined Southern blot analysis enhances the sensitivity and confirms the identity of the amplification products. This approach clearly shows expression of ER mRNA in PC3 cells, although at a much lower extent than that found in estrogen-responsive mammary carcinoma cell lines; this may account for the apparent lack of ER in the soluble fraction, using a biochemical assay. We also report that the ER mRNA variant lacking exon 4, which we have recently documented in human breast cancer cell lines (11), is also expressed in PC3 cells. It is noteworthy that this variant is jointly expressed with the normal messenger only in ER-positive, estrogen-responsive MCF7 and ZR75-1 mammary carcinoma cell lines, while both mRNAs are absent from ER-negative, nonresponsive MDA-MB231 cells (11).

The possibility that the estradiol-induced growth inhibition of PC3 cells is mediated by TGFβ is sound and has been consequently investigated. Addition of neutralizing anti-TGFβ antibody in stringent experimental conditions provoked a remarkable increase (close to 300% of control) of cell proliferation, revealing the presence of high levels of endogenous TGFβ1 in PC3 cells; estradiol produces a reversal of this effect, which is almost complete at 10^{-7} M dose. Evaluation of possible regulation of TGFβ1 mRNA by estradiol indicated that its expression in PC3 cells is unaffected by different doses (10^{-11} to 10^{-7} M) of estradiol; this was true at any incubation time (6, 24, or 72 h) used. Previous work (14) revealed that antiestrogens, such as tamoxifen, did not affect TGFβ1 mRNA levels in the estrogen-dependent MCF7 breast cancer cells, despite TGFβ1 production increased (from 8- to 27-fold) in this cell line after tamoxifen treatment. Recent experimental evidence has also indicated that estradiol did not modify levels of TGFβ1 mRNA either in ER-positive breast cancer cell lines (MCF7, ZR75-1) or in ER-negative cancer cells, like mammary MDA-MB231 (15) and endometrial HEC-50 (16). It is of interest to note that just these ER-negative human cancer cells have been reported to be exquisitely sensitive to TGFβ1 (17).

Since the early 1940s, the high response rates (70–80%) achieved in prostate cancer patients using a synthetic estrogen, the orally active DES, have been ascribed to the fact that after DES administration circulating testosterone falls to the levels found in castrates (18). Recent experimental data have, however, emphasized that i.v. stilbestrol diphasophate and, more in general, estrogens may exert a direct cytotoxic effect on prostate tumors; this could explain the high resistance of prostate tumors to antiestrogens and androgens.

**Fig. 3. Left.** PCR amplification products, ethidium bromide staining of agarose gel. Lane 1, ZR75-1; Lane 2, MCF7; Lane 3, MDA-MB231; Lane 4, PC3 cells; Lane 5, reverse transcription-PCR of a RNA-free control sample. Marker lane, 4X-174-HaeII digest fragments (length is given in base pairs). **Right.** Southern blot analysis of the PCR-amplified DNA from the left panel. Samples were hybridized using a 32P-labeled human estrogen receptor cDNA as a probe.

![Fig. 3.](cancerres.aacrjournals.org)
sponse rates observed in patients having metastatic, hormone-refractory disease (19). To our knowledge, this is the first report by which clear evidence that estradiol inhibits growth of epithelial prostate cancer cells is provided; this may represent an alternative explanation for the efficacy of the estrogen therapy in advanced prostatic cancer, wherein androgen-independent tumor cells are likely to be largely prevalent.

Further studies are needed to ascertain whether or not the growth inhibition induced by estradiol in PC3 cells involves different TGFβ species. Whether estradiol acts through a direct, receptor-mediated pathway or by alternative paracrine/autocrine mechanisms should also be queried.

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