Expression of estrogen receptor β in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis

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Abstract The involvement of estrogen receptor beta (ER) in prostate carcinogenesis has been hypothesized. Several reports have shown that ER expression was decreased when prostate cells undergo neoplastic transformation, suggesting that it could play a tumor-suppressor role. By restoring ER expression in prostatic carcinoma cells by adenoviral delivery, we aimed to test this hypothesis. We observed that ER strongly inhibited the invasiveness and the growth of these cells. In addition, ER cells were undergoing apoptosis, as shown by quantification of Bax, Poly(ADP-ribose) polymerase and caspase-3 expression. Our data suggest that ER acts as a tumor-suppressor by its anti-proliferative, anti-invasive and pro-apoptotic properties.
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1. Introduction

Prostate cancer is one of the most common forms of cancer in males in developed countries [1]. The role of sex steroids in the biology of the prostate and prostate cancer remains unclear, despite nearly four decades of research since Charles Huggins dramatic demonstration of orchiectomy and endocrine therapy on prostate cancer [2]. Until 1996, it was thought that estrogen receptors (ER) are present only in a limited number of cells in the prostate, mainly in the stromal cells of the prostatic lobes and the posterior periurethral region, while the epithelial cells were reported to contain very low amounts of the receptors [3]. However, the recent discovery of a novel ERβ, cloned from rat prostate cDNA library, and abundantly expressed in rat and human prostate epithelium [4], suggests that prostate epithelium is also a direct target for estrogen action. Moreover, a recent study has shown that ERβ is the only ER detected at the protein level in the morphologically normal developing human fetal prostate [5]. Since the identification of ERβ, many reports have shown that a loss of ERβ expression is associated with progression of normal prostate epithelium into prostate cancers [6–9]. A similar down-regulation in ERβ expression is also noted in ovarian, breast or colon tumors [10–12]. These data and the observation that older ERβ-null mice develop prostatic hyperplasia [13] have led to the hypothesis that the loss of ERβ may be a mechanism by which prostate epithelial cells escape normal control of proliferation [14]. The goal of this study was to determine whether restoration of ERβ expression in prostate cancer cells had major effect on proliferation, invasiveness and apoptosis of prostate cancer cells.

2. Materials and methods

2.1. Recombinant adenovirus construction and propagation

The complete coding sequences of wild-type hERα and hERβ cDNAs were subcloned in *Bam*HI site of the pACsk12CMV5 shuttle vector. To obtain recombinant viruses, permissive HEK-293 cells (human embryonic kidney cells) were cotransfected with the backbone or recombinant pACsk12CMV5-hER plasmid and with pJM17, which contains the remainder of the adenoviral genome as previously described [15,16]. In vivo recombination of the plasmids generates infectious viral particles Ad5 (backbone virus), Ad-hERα and Ad-hERβ. Titered virus stocks were used to infect DU-145 prostate cancer cells.

2.2. Cell culture and infection

DU-145 cells (ER-negative) were obtained from the American Type Culture Collection (ATCC Rockville, MD). Cells were maintained in MEM with 5% FCS and penicillin/streptomycin (Invitrogen). DU-145 cells were weaned off steroids in phenol red-free MEM supplemented with 10% CDFCS (charcoal dextran-treated FCS) 3 days before experiments. Cells were infected overnight at a multiplicity of infection (MOI) of 50. The next day, the medium was removed and replaced with fresh one.

2.3. Transfections

3×10^5 cells were plated in 6-well plates in phenol red-free MEM 10% CDFCS 24 h before infection. Cells were then infected overnight as specified in Section 2.2. The next day, transfections were performed with lipofectamine according to the manufacturer's recommendations using 4 μg of ERE2-TK-LUC luciferase reporter along with 0.8 μg of the internal reference reporter plasmid (CMV-Gal) per well. After overnight lipofection, the medium was removed and the cells were placed into fresh medium supplemented with control vehicle ethanol or E2 (10^{-8} M). 24 h later, cells were harvested and assayed for luciferase activity on Centro LB960 Berthold luminometer. β-Galactosidase was determined as previously described [15].

2.4. Whole cell extract preparation and Western blot

Cells were lyzed by sonication in 10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol containing protease inhibitors. The resulting whole-cell extract proteins were subjected to SDS–PAGE followed by

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electrotransfer onto a nitrocellulose membrane. The following antibodies were used: Bax monoclonal antibody, B/9 (Santa Cruz Biotechnologies; 1:500 dilution); and Poly(ADP-ribose) polymerase (PARP) monoclonal antibody, 4C10-5 (BD Pharmingen; 1 μg/ml).

2.5. Immunofluorescence

10^6 cells were plated in a 6-well plate and infected with adenovirus at a MOI of 50. Paraformaldehyde fixed cells were incubated with primary antibody against cleaved caspase 3 (Cell Signaling Technologies) for 1 h at room temperature in a humidified chamber. After washing with Tris-buffered saline + 0.025% Tween, staining was performed using biotinylated secondary antibodies (ABC kit, Vector Laboratories), and streptavidin–FITC (1:100, Vector Laboratories). Cell images were analyzed using a Zeiss microscope (Axioskop, Carl Zeiss Inc., Oberkochen, Germany).

2.6. Transwell matrigel invasion assays

Invasion was performed as previously described [15]. Briefly, DU-145 cells were plated 24 h after infection (MOI 50) in the upper compartment filled with MEM 10% CDFCS with 30 μg/ml fibronectin of a 24-well Transwell (Corning-Costar) on a polycarbonate filter (8 μm pore size), which was pre-coated with 30 μg of matrigel (Becton–Dickinson). Cells migrating to the lower side of the filter and control cells in the 24-well plate were quantified using MTT uptake (Sigma Chemical Co., St. Louis, MO).

2.7. Cell proliferation assays

Cells were estrogen depleted for 3 days and then plated in 96-well plates at a density of 5000 cells/well and infected with adenovirus overnight (MOI 50). MEM 10% CDFCS containing either ethanol or 10 nM E2 was added to the cells after infection. Cell proliferation was measured with MTT.

3. Results

3.1. ERβ introduction in prostate cancer cells

To test this hypothesis of the involvement of ERβ in prostate cell growth, we decided to restore ERα and ERβ expression in the ER-negative prostate carcinoma cell line DU-145, by using ERα or ERβ encoding adenoviruses. Cells were mock infected (non-infected) or infected with backbone (Ad5), ERα (Ad-ERα), or ERβ (Ad-ERβ) adenoviruses. No detectable levels of ERα or ERβ proteins were seen by immunoblotting or immunofluorescence in uninfected or Ad5 infected cells, whereas adenoviral delivery enabled a high expression of ERα and ERβ proteins (data not shown). To further assess the functionality of the receptors, virally expressed ERα and ERβ were able to transactivate an estrogen-responsive reporter construct when transfecting DU-145 cells (Fig. 1). Both receptors were active, even though ERβ was a weaker activator than ERα, as previously shown in breast cancer cells [15,17].

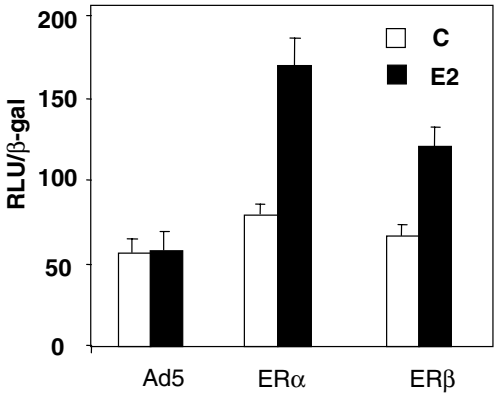

Fig. 1. Adenovirus mediated expression of ERα and ERβ in DU-145 cells. DU-145 cells were either infected with Ad5, Ad-ERα, Ad-ERβ viruses and transfected with ERE_2-TK-LUC and CMV-GAL reporter constructs. Cells were grown for 48 h in the presence of control vehicle ethanol (C) or 10^{-8} M E2. Results represent means ± SEM of three independent experiments.

3.2. ERβ inhibits prostate cancer cell invasiveness and growth

We next examined the consequences of ERα or ERβ expression on the invasion potential of DU-145 cells by using matrigel-coated Transwell (Fig. 2A). Ad-ERα infected cells had the same invasion potential as Ad5 infected cells (Fig. 2A). With the addition of estradiol, a 30–40% decrease in invasion was observed. Interestingly, in ERβ infected cells, in the absence of estrogens, a 50% decrease in invasion was observed. With the addition of E2, an even greater inhibition of invasion was observed, up to 70% of baseline (Fig. 2A).

In addition to invasion, another major goal of these experiments was to determine the effect of ERα and ERβ on proliferation in DU-145 cells. Ad-ERα infected cells in control medium had a proliferation profile similar to that of Ad5 infected cells

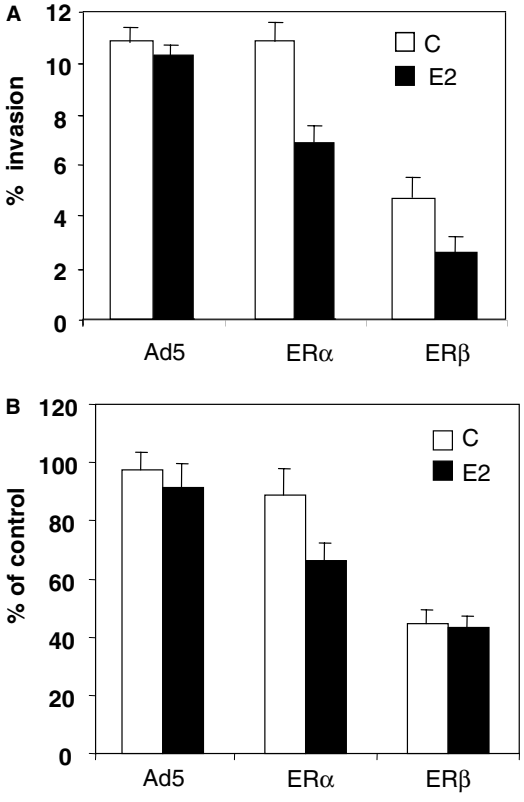

Fig. 2. Ad-ERα and Ad-ERβ inhibit cellular invasion and growth (A) DU-145 cells were infected with Ad5, Ad-ERα, and Ad-ERβ adenoviruses at MOI 50 and were plated on a transwell chamber coated with matrigel. Cells were treated with either ethanol (C) or 10 nM E2 and allowed to invade the matrigel for 24 h. Results represent means ± SEM of three independent experiments. (B) DU-145 cells were plated in a 96-well plate and infected with Ad5, Ad-ERα, or Ad-ERβ adenoviruses overnight at MOI 50. Fresh media containing either ethanol or 10 nM E2 were added to the cells and proliferation was determined 6 days post infection. Results are expressed as % of control non-infected cells and represent mean ± SEM of three independent experiments.

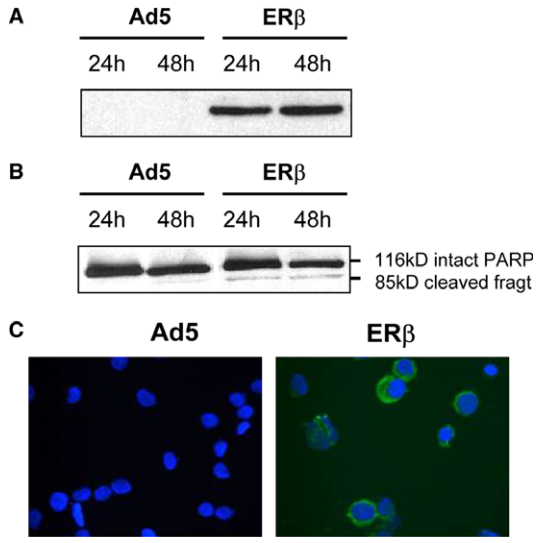

Fig. 3. ERβ induces apoptosis in DU-145 cells. Estrogen depleted cells were infected with Ad5 or Ad-ERβ adenoviruses overnight at MOI 50. Cells were then grown for 24 or 48 h and whole cell lysates were collected. (A) Immunoblotting using anti-Bax monoclonal antibody was performed. (B) Immunoblotting using anti-PARP monoclonal antibody. This antibody recognizes both intact and the 85 kDa cleaved fragment of PARP. (C) Paraformaldehyde fixed cells were incubated with primary antibodies against cleaved caspase 3 and examined by immunofluorescence. Nuclei were stained using DAPI containing mounting medium.

(Fig. 2B). A 30% decrease in proliferation was seen only in Ad-ERα cells treated with estradiol. On the contrary, Ad-ERβ cells displayed a 60% decreased proliferation in both control and estradiol treated conditions. These data suggest that although both ERα and ERβ inhibit proliferation, ERβ is a stronger regulator of cell growth than ERα in prostate cancer cells.

3.3. ERβ is triggering apoptosis of prostate cancer cells

We next hypothesized the potential involvement of apoptosis behind the decrease in proliferation seen in Ad-ERβ cells. To answer to this question, we analyzed the expression of several markers of apoptosis. Introduction of ERβ in DU-145 cells led to a strong increase in pro-apoptotic Bax levels (Fig. 3A). PARP, a caspase target, was also analyzed. Levels of cleaved PARP (85 kDa) were increased in Ad-ERβ infected cells compared to Ad5 infected cells. To further characterize the apoptosis seen in Ad-ERβ cells, immunofluorescence with anti-cleaved caspase 3 antibodies was performed (Fig. 3C). Introduction of ERβ in DU-145 cells led to peri-nuclear expression of cleaved caspase 3 (Fig. 3C). All these data suggest that cells expressing ERβ undergo apoptosis, which could contribute to the reduced proliferation observed.

4. Discussion

Prostate is considered together with ovary as one of the main tissue in terms of ERβ expression [4]. Moreover, several studies have shown a decreased expression of ERβ in prostate carcinoma compared to non-pathological tissues [6–9]. The potential beneficial anti-neoplastic role of ERβ in prostate is thus crucial but remains to be demonstrated. To investigate the roles of ERα and ERβ in prostate, we used a prostate cancer cell line devoid of both endogenous receptors in which ERα and ERβ expression was restored using adenovirus delivery. Interestingly, a potent decrease in invasion (60%) was observed with the introduction of ERβ, which is in agreement with the situation found in breast cancer cells [15]. The results from the invasion assay also correlate with the emerging clinical data. At least three different reports show that the loss or decrease in ERβ expression is associated with higher Gleason grade tumors and prostate cancer with higher metastatic potential [6–8].

Concerning cell growth, the introduction of ERβ in DU-145 cells caused a strong inhibition of proliferation, in agreement with our previous data in breast cell carcinoma [15], and with the more recent studies showing that ERβ can also inhibit the proliferation of ERα-positive breast cancer cells [18,19]. These data correlate well with the phenotype of ERβ knock-out animals (βERKO) studies in which, most breast and prostate epithelial cells express the proliferation antigen Ki-67 [14]. Moreover, 80% of 1-year-old βERKO animals show hyperplastic lesions in prostate, suggesting that the loss of ERβ leads to an uncontrolled cellular proliferation. The mechanisms responsible for the reduced growth of cells exogenously expressing ERβ are beginning to be investigated. We and others have shown that ERβ was able to induce $p21^{Cip\text{-}1}$ and $p27^{Kip\text{-}1}$ expression, while decreasing c-myc, cyclin A, cyclin D1, cyclin E, Cdc25A, and p45 (Skp2) levels [15,18,19]. Moreover, at least in ERα-positive breast cancer cells, growth inhibition would result from an arrest in G2 phase of the cell cycle [18].

Concerning the fact that ERα decreases both the invasion and the proliferation in a ligand-dependent manner, several hypotheses can be raised. We have previously shown by a cDNA array screen and western blot analysis that introduction of ERα in ER-negative breast cancer cells leads to striking changes in gene expression. In particular, levels of $p21^{CIP\text{-}1}$, $p27^{Kip\text{-}1}$, macmarcks and TOB are increased, whereas pRb protein is hypo-phosphorylated, suggesting that these events could account for the estrogen-dependent observed growth inhibition [20]. In the same line, changes in integrin and BMP-4 [20] could also account for the observed inhibition of invasion observed after reintroduction of ERα. For ERβ, the situation is indeed different as this receptor inhibits both the invasion and the proliferation, mainly in a ligand-independent manner. We have previously shown that ERβ could inhibit Interleukin-8 (IL-8) expression in a ligand independent manner [21]. Moreover, IL-8 is able to increase invasion, which suggests that at least part of the effects of ERβ on invasion could involve a decreased expression of IL-8. The mechanisms accounting for the ligand-independent inhibition of growth remain elusive. We cannot entirely rule out the possibility of an endogenous cryptic ERβ ligand. Indeed, Weihua et al. [22] have proposed that the testosterone metabolite 5α-androstane-3β-diol (3βAdiol) could be a potential ligand of ERβ. Therefore, it is possible that prostate cancer cells auto-synthesize sex-steroids, which could function as natural activators of ERβ. In addition, it has also been shown that unliganded ER can transduce growth factor signals [23]. ERβ is able to recruit the coactivator, SRC-1 through phosphorylation of Ser-106 and Ser-124 by MAPK in a ligand independent manner [24]. Perhaps the ligand independent ERβ action in apoptosis involves SRC-1 interaction, or some yet unidentified pathway that is unique to ERβ AF-1 activity.

Of particular interest is our finding that at least part of the growth inhibition triggered by ERβ is due to apoptosis, demonstrating the involvement of ERβ in such events. Apoptosis of the ERβ expressing cells was shown by the increased peri-nuclear expression of cleaved caspase 3. The apoptosis induced by ERβ appears to involve the mitochondrial pathways, as demonstrated by the increase in Bax protein expression and the cleavage of PARP. To our knowledge, this is one of the first demonstration that ERβ is able to trigger apoptosis. Indeed, a recent study has shown that ERβ could increase apoptosis in colon cancer cells [25]. A detailed analysis of this phenomenon will need to be undertaken in the future.

The fact that exogenous expression of ERβ in prostate cancer cells leads to an inhibition of proliferation and invasion and to an increase of apoptosis is in good agreement with clinical studies showing that ERβ expression is lost in prostate, breast, ovary, and colon tissues when they undergo dysplastic transformation [7,10,12]. The reasons underlying ERβ loss remain poorly understood. Human ERβ promoter has been cloned recently [26] and could help to study this phenomenon. The promoter is active only in ERβ expressing cells and displays a high GC-content. Interestingly, another study has shown that ERβ promoter was methylated in about 80% of cancers but not in normal tissues [27], which could explain the down regulation of ERβ observed in cancer cells. The understanding of ERβ expression regulation will definitely constitute a challenging issue in the future, as it could lead to the development of new therapies.

In conclusion, our data strongly support the hypothesis that the loss of ERβ could be one of the key elements leading to an uncontrolled growth of prostate epithelial cells. In this model, ERβ would play a ''gatekeeper'' role by inhibiting invasion, proliferation and stimulating apoptosis, preventing the rapidly differentiating prostatic epithelial cells from undergoing oncogenic transformation.

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References

[1] Schaid, D.J. (2004) Hum. Mol. Genet. 13 (Suppl. 1), R103-21.
[2] Huggins, C. (1967) Cancer Res. 27, 1925–1930.
[3] Prins, G.S. and Birch, L. (1997) Endocrinology 138, 1801–1809.
[4] Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.A. (1996) Proc. Natl. Acad. Sci. USA 93, 5925–5930.
[5] Adams, J.Y., Leav, I., Lau, K.M., Ho, S.M. and Pflueger, S.M. (2002) Prostate 52, 69–81.
[6] Leav, I., Lau, K.M., Adams, J.Y., McNeal, J.E., Taplin, M.E., Wang, J., Singh, H. and Ho, S.M. (2001) Am. J. Pathol. 159, 79–92.
[7] Horvath, L.G. et al. (2001) Cancer Res. 61, 5331–5335.
[8] Fixemer, T., Remberger, K. and Bonkhoff, H. (2003) Prostate 54, 79–87.
[9] Latil, A., Bieche, I., Vidaud, D., Lidereau, R., Berthon, P., Cussenot, O. and Vidaud, M. (2001) Cancer Res. 61, 1919–1926.
[10] Pujol, P., Rey, J.M., Nirde, P., Roger, P., Gastaldi, M., Laffargue, F., Rochefort, H. and Maudelonde, T. (1998) Cancer Res. 58, 5367–5373.
[11] Campbell-Thompson, M., Lynch, I.J. and Bhardwaj, B. (2001) Cancer Res. 61, 632–640.
[12] Roger, P., Sahla, M.E., Makela, S., Gustafsson, J.A., Baldet, P. and Rochefort, H. (2001) Cancer Res. 61, 2537–2541.
[13] Krege, J.H. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 15677–15682.
[14] Weihua, Z. et al. (2001) Proc. Natl. Acad. Sci. USA 98, 6330–6335.
[15] Lazennec, G., Bresson, D., Lucas, A., Chauveau, C. and Vignon, F. (2001) Endocrinology 142, 4120–4130.
[16] McGregory, W.J., Bautista, D.S. and Graham, F.L. (1988) Virology 163, 614–617.
[17] Cowley, S.M. and Parker, M.G. (1999) J. Steroid Biochem. Mol. Biol. 69, 165–175.
[18] Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G.R., Firestone, G.L. and Leitman, D.C. (2004) Cancer Res. 64, 423–428.
[19] Strom, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J. and Gustafsson, J.-A. (2004). Proc. Natl. Acad. Sci. USA, 0308319100.
[20] Licznar, A., Caporali, S., Lucas, A., Weisz, A., Vignon, F. and Lazennec, G. (2003) FEBS Lett. 553, 445–450.
[21] Freund, A., Chauveau, C., Brouillet, J.P., Lucas, A., Lacroix, M., Licznar, A., Vignon, F. and Lazennec, G. (2003) Oncogene 22, 256–265.
[22] Weihua, Z., Lathe, R., Warner, M. and Gustafsson, J.-A. (2002) Proc. Natl. Acad. Sci. USA 99, 13589–13594.
[23] Newton, C.J., Buric, R., Trapp, T., Brockmeier, S., Pagotto, U. and Stalla, G.K. (1994) J. Steroid Biochem. Mol. Biol. 48, 481–486.
[24] Tremblay, A., Tremblay, G.B., Labrie, F. and Giguere, V. (1999) Mol. Cell 3, 513–519.
[25] Qiu, Y., Waters, C.E., Lewis, A.E., Langman, M.J. and Eggo, M.C. (2002) J. Endocrinol. 174, 369–377.
[26] Li, L.C., Yeh, C.C., Nojima, D. and Dahiya, R. (2000) Biochem. Biophys. Res. Commun. 275, 682–689.
[27] Sasaki, M., Tanaka, Y., Perinchery, G., Dharia, A., Kotcherguina, I., Fujimoto, S. and Dahiya, R. (2002) J. Natl. Cancer Inst. 94, 384–390.