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Short communication

The effects of natural ligands of hormone receptors and their antagonists on telomerase activity in the androgen sensitive prostatic cancer cell line LNCaP

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Abstract

The effects of the 17β-estradiol, dihydrotestosterone and hormone antagonists tamoxifen and bicalutamide on telomerase activity and expression of cell cycle related proteins in the androgen-sensitive prostatic cancer cell line LNCaP were studied. The cell line was grown in RPMI supplemented with 2.5% charcoal-stripped FBS for 72 hr. The IC_{50} of tamoxifen and bicalutamide and the optimal stimulatory concentrations of 17β-estradiol and dihydrotestosterone were determined by means of the cell-viability assay, the activity of telomerase was measured by the telomere repeat amplification protocol (TRAP) and the expression of proteins was analysed by the Western blot technique. 17β-estradiol stimulated cell growth more effectively than dihydrotestosterone whereas hormone antagonists tamoxifen and bicalutamide caused a significant decrease in cell viability. The treatment of cells by a combination of low doses of 17β-estradiol and dihydrotestosterone stimulated cells stronger than treatment by a single hormone. Only 17β-estradiol, in concentration of 10 nM, increased strongly the expression of $p21^{Waf1/Cip1}$ and increased slightly telomerase activity in the LNCaP cells. 50 μM of bicalutamide down-regulated the levels of the androgen receptor, the proliferating cell nuclear antigen and telomerase activity, and up-regulated the expression of $p27^{Kip1}$. We hereby describe the first observation of the influence of bicalutamide on telomerase activity and a positive correlation between the effect of 17β-estradiol and the induction of both the endogenous cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$, and telomerase activity in a prostatic cancer cell line LNCaP. These findings can shed a new light on the steroid-signaling pathway in prostate cancer cells.

Keywords: 17β-Estradiol; Dihydrotestosterone; Tamoxifen; Bicalutamide; Telomerase; LNCaP

1. Introduction

Prostate cancer is one of the most common neoplastic diseases. Androgen deprivation is the only effective systemic therapy available for metastatic prostate cancer. Prostate cancer progression to the androgen insensitive phenotype is accompanied by a shift in reliance on endocrine to paracrine/autocrine controls. However, the molecular mechanisms involved in the development of androgen-independent prostate cancer are not completely understood [1]. Much evidence exists that mechanisms such as androgen receptor mutations, gene amplifications, aberrant co-regulators and signal transduction could be involved. Therefore, the study of relationships between nuclear hormone receptors, their ligands and regulatory pathways could be important.

In the treatment of prostate cancer androgen antagonists such as bicalutamide have recently been used [2]. There is also evidence that different ligands can bind the same receptor [3]. In the prostate, besides dihydrotestosterone, the significance of 17β-estradiol as a second natural ligand of androgen receptor [4] and/or estrogen receptor α and β has been described [5,6]. With respect to this finding, the significance of tamoxifen, the anti-estrogen widely used in therapy of breast cancer, should be analysed for therapy of androgen sensitive prostate cancer. The importance of telomerase in prostate cancer and derived cell lines has

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Abbreviations: AR, androgen receptor; ER, estrogen receptor; IC_{50}, concentration inhibiting cell viability to 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCNA, proliferating cell nuclear antigen.

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recently been reported [7,8]. Since the prostate cancers with an androgen insensitive phenotype have an increased viability, one can speculate about a role of telomerase in this process. This suggestion was confirmed by a recent report of the telomerase activity decrease after tamoxifen treatment in the breast cancer cell lines MCF-7 and MDA-MB-231 [9].

The aim of this study was to test our hypothesis that the above mentioned agents can elicit significant changes in both telomerase activity and cellular regulatory pathways, and if substantiated, it could contribute to an explanation of the hormone insensitivity found in some prostate cancers.

2. Material and methods

2.1. Cell culture and MTT cell viability assay

The LNCaP cells were obtained from the ATCC and maintained in RPMI with 10% (v/v) FBS. Experiments were performed in RPMI with 2.5% (v/v) charcoal-stripped FBS. The MTT cell viability assay [10] was used to determine both the IC_{50} of bicalutamide and tamoxifen and the optimal stimulatory concentrations of dihydrotestosterone and 17β-estradiol. In brief, cells were exposed to test compounds for 3 days in 96-well plates, then incubated with MTT dye which was converted by viable cells to blue formazan. The absorbance was measured at 540 nm after lysis by 10% (w/v) sodium dodecyl sulphate. In a 3-day experiment, LNCaP cells were treated with 50 μM of bicalutamide or 7 μM of tamoxifen with or without 1 nM of dihydrotestosterone or 10 nM of 17β-estradiol. The morphology of cells was documented photographically.

2.2. Telomerase activity

Telomerase activity was assayed by TRAPeze kit (Intergen) based on the TRAP method [11]. In brief, the concentration of proteins in cell lysates was measured by the Bradford method and aliquots of the lysate containing 1 μg of protein were loaded for PCR. After separation on 12% polyacrylamide gel, telomerase ladder was visualized by Sybr Gold and CCD camera DIANA 2 (Raytest).

2.3. Immunobloting

Following a 3-day exposure to the test compounds, cultured cells were detached by trypsin, immediately re-suspended in sodium dodecyl sulphate loading buffer and boiled for 5 min. After electrophoresis, proteins were transfered to nitrocellulose membranes (Amersham). The membranes were stained with Ponseau Red (Sigma) to control sample loading. After blocking in buffer containing 5% (w/v) milk, the membranes were treated overnight at 4° with mouse anti-AR monoclonal antibody (clone AR411, Dako), mouse anti-p21 monoclonal antibody (clone 118, kindly provided by Dr. Vojtesek, Masaryk Memorial Cancer Institute Brno), mouse anti-p53 monoclonal antibody (clone DO-7, Dako), mouse anti-p27 monoclonal antibody (clone SX53G8, Dako), mouse anti-PCNA monoclonal antibody (clone PC10, Dako), mouse anti-α-tubulin monoclonal antibody (clone DM 1A, Sigma). Following a second incubation with peroxidase-conjugated anti-mouse antibody (USOL), proteins were visualized by an enhanced chemiluminescent detection system (Amersham). The experiments were repeated three times.

3. Results

LNCaP cells, derived from androgen sensitive metastatic prostate cancer, were treated with the synthetic hormone antagonists bicalutamide and tamoxifen, and with the androgen receptor (AR) natural ligands dihydrotestosterone and 17β-estradiol. The analysis of cell viability, activity of telomerase, expression of AR, PCNA, p53, inhibitors of the cyclin-dependent kinases $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ was performed.

The optimal concentrations stimulating LNCaP cells were 1 nM of dihydrotestosterone and 10 nM of 17β-estradiol. The average stimulation, which was determined from seven experiments, was 125% for 1 nM of dihydrotestosterone and 150% for 10 nM of 17β-estradiol. The strongest stimulation was observed by a combination of low doses of dihydrotestosterone and 17β-estradiol (Fig. 1). The IC_{50} was 50 μM for bicalutamide and 7 μM for tamoxifen (Fig. 2).

Both bicalutamide alone and in combination with hormones, induced in cells regressive changes such as vacuolization and fragmentation (Fig. 3) and inhibited telomerase (Fig. 4A). The same treatment also slightly increased the level of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, and significantly decreased the levels of both the AR and proliferating cell nuclear antigen (PCNA) (Fig. 4B). Regressive changes induced both by tamoxifen alone and in combination with hormones were not associated with changes in the studied proteins (Figs. 3, 4A and B). In cells treated only with 17β-estradiol, there was found a slight increase in telomerase activity (Fig. 4A) and a large increase in the level of the cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$ (Fig. 4B). This last effect was not observed after stimulation by dihydrotestosterone which also increased telomerase activity. With respect to the involvement of the AR in the regulation of $p21^{Waf1/Cip1}$ transcription [12], we also tested other concentrations of dihydrotestosterone (from 10^{-5} to 10^{-8} M) but none of them altered $p21^{Waf1/Cip1}$ expression in our 3-day experiment (data not shown). None of the various treatments elevated the p53 level, which was very low (data not shown).

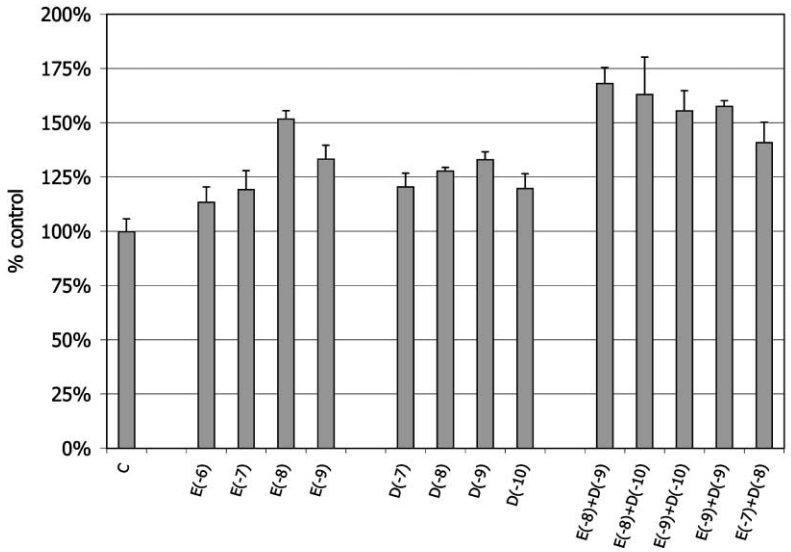

Fig. 1. Stimulation of LNCaP cell viability by 17β-estradiol [E(−6) to E(−9) = concentration 10^{-6} to 10^{-9} M; C: control cells], dihydrotestosterone [D(−7) to D(−10) = concentration 10^{-7} to 10^{-10} M] and by their combinations [E(−7) to E(−10) + D(−8) to D(−10) = concentration 10^{-7} to $^{-10}$ M). Bars represent the mean ± SD of four determinations. Broader concentration range was used for both hormones (from 10^{-6} to 10^{-13} M; data not shown) giving bell-shaped response curves with no concentration inhibiting cell viability.

4. Discussion

The LNCaP cell line contains only wild type p53, carries a mutation in the hormone-binding domain of AR, and expresses moderate levels of androgen responsive AR [13,14]. Reports concerning expression of estrogen receptor (ER) mRNA in LNCaP are controversial [5,6,15]. Up to now, this cell line has not been used for the study of a possible relationship between telomerase activity and hormone responsiveness. A study concerning the inhibitory effect of the estrogen antagonist, tamoxifen, on the telomerase activity in breast carcinoma cell lines, was performed by Aldous et al. [9]. They described the fact that tamoxifen inhibits the telomerase activity in MCF-7 and MDA-MB-231 cells.

In our experiment the treatment, both by bicalutamide and tamoxifen, decreased cell viability but only bicalutamide inhibited telomerase activity. It was accompanied by a decrease in the AR and PCNA levels. This result is compatible with the findings of Perry and Tindall [16] who revealed that androgens regulate the expression of PCNA post-transcriptionally in LNCaP cells. Bicalutamide also slightly increased the expression of the cyclin dependent inhibitor, $p27^{Kip1}$, which is probably related to a decreased

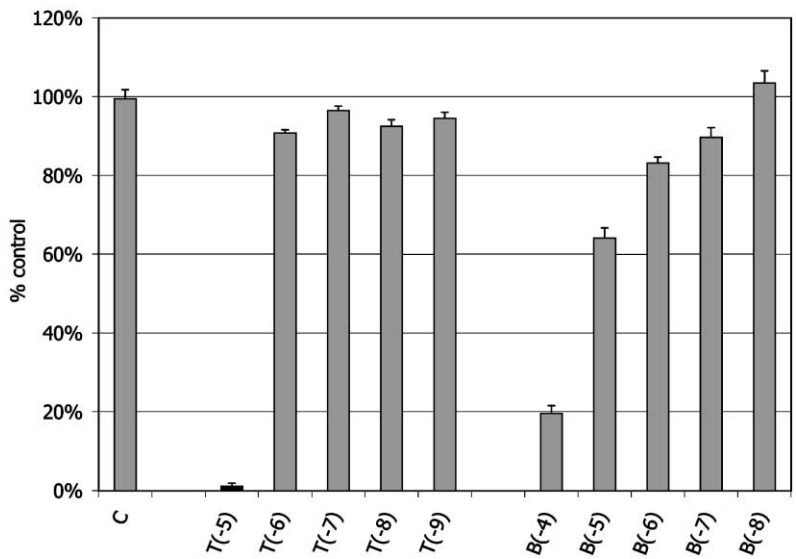

Fig. 2. Inhibition of LNCaP cell viability by tamoxifen [T(−5) to T(−9) = concentration 10^{-5} to 10^{-9} M; C: control cells] and bicalutamide [B(−4) to B(−8) = concentration 10^{-4} to 10^{-8} M]. IC_{50} for tamoxifen (7 μM) and bicalutamide (50 μM) was calculated in experiments with more minute concentration range (data not shown). Bars represent the mean ± SD of four determinations.

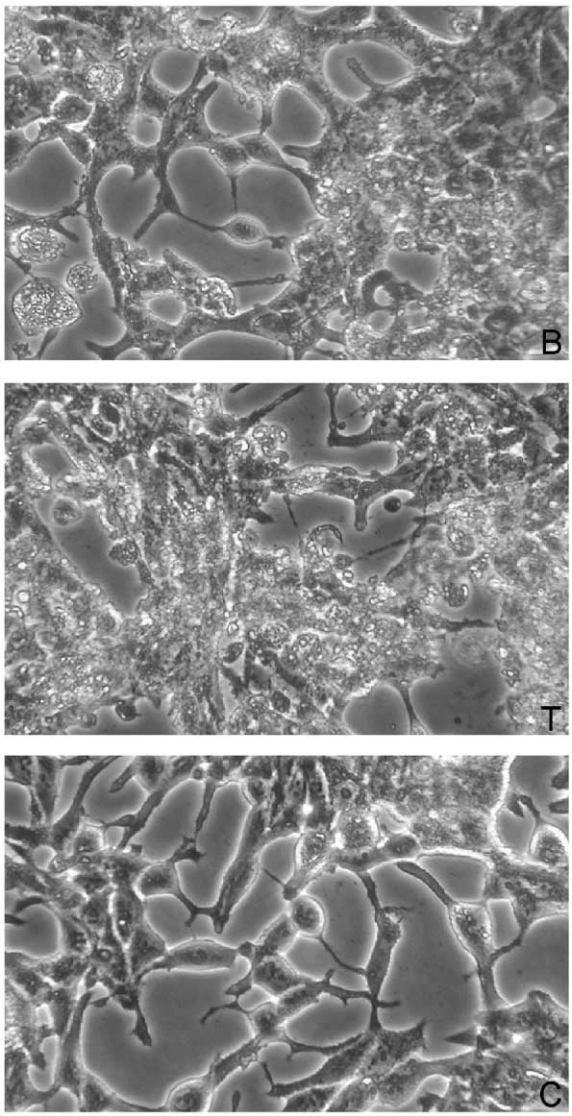

Fig. 3. Morphology of LNCaP cells after treatment with bicalutamide (B) and tamoxifen (T). In comparison with control cells (C) regressive changes are visible (vacuolization and cell fragmentation).

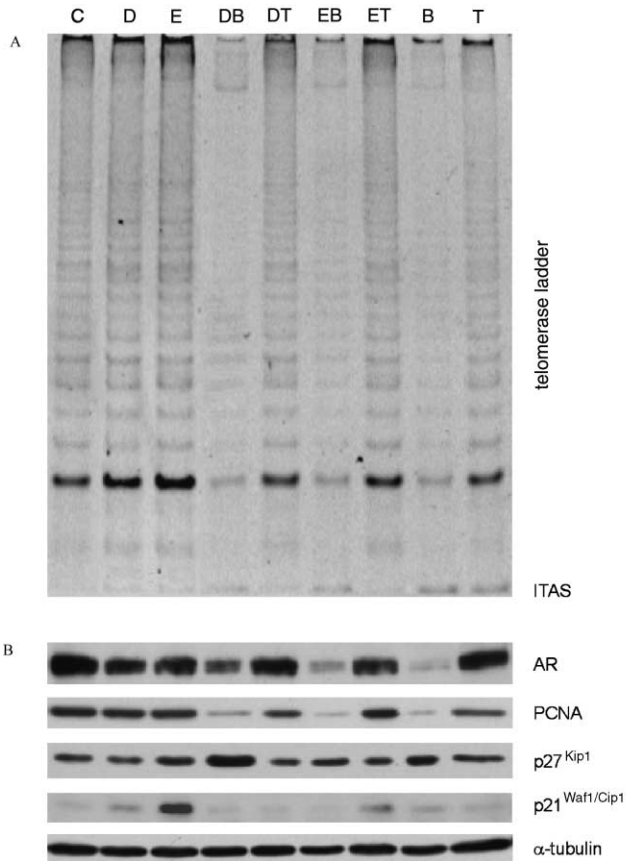

Fig. 4. (A) Telomerase activity in LNCaP cells after treatment with dihydrotestosterone (D), 17β-estradiol (E), bicalutamide (B), tamoxifen (T) and their combinations. (B) Expression of AR, PCNA, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ after treatment with D, E, B, T and their combinations; α-tubulin was used as a loading control.

cell viability. The combinations of bicalutamide with hormones, dihydrotestosterone and 17β-estradiol, were unable to restore the levels of AR, PCNA and telomerase activity. These results may reflect the fact that bicalutamide, in the used concentration, binds to the AR more intensively than the natural ligands, dihydrotestosterone and 17β-estradiol. The effect of tamoxifen, which did not influence the expression of the above mentioned proteins, could be hypothesized as due to its influence on the ER beta [5,17].

Our results confirm the previous finding of Soda et al. [18] who found an increase of telomerase activity in LNCaP cells after dihydrotestosterone treatment. We also revealed an increase of telomerase activity after treatment with 17β-estradiol, which is not accompanied by changes of AR, PCNA and p53 levels, but by a substantial increase in the $p21^{Waf1/Cip1}$ level. In our opinion the elevation of $p21^{Waf1/Cip1}$ level after treatment with 17β-estradiol could be in agreement with the recently described novel function of $p21^{Waf1/Cip1}$ in the activation of the estrogen-signaling pathway in ER negative breast cancer cells [19]. The existence of an estrogen-mediated signaling pathway is also supported by our finding that stimulation by a combination of 17β-estradiol and dihydrotestosterone is stronger than by a single hormone. This could be explained by activation of Src/Raf-1/Erk-2 pathway by AR-ERβ-Src complex [6]. Another explanation for the increased level of $p21^{Waf1/Cip1}$ after 17β-estradiol treatment could be connected to the role of $p21^{Waf1/Cip1}$ in the coordination of stimulatory signals mediated by 17β-estradiol and the starvation conditions of the medium with 2.5% charcoal-stripped FBS [20]. Finally, we cannot support the recent hypothesis that $p21^{Waf1/Cip1}$ causes a direct inhibition of telomerase [21].

In summary, we report the first observation of the inhibitory influence of bicalutamide on telomerase activity and a direct correlation between stimulation by 17β-estradiol and induction of the endogenous cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$, in the prostatic cancer cell line, LNCaP. This finding can shed new light on the steroid-

signaling pathway in prostate cancer cells and the development of androgen insensitivity.

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