Prostate cancer, which develops due to androgen and is initially responsive to androgen deprivation therapy, often comes to acquire androgen deprivation therapy resistance in short order. We investigated the role of androgen receptor (AR) protein in an androgen-independent prostate cancer cell line using AR ligands and AR siRNA. Although the androgen-independent cell line scarcely responded to AR ligands, their growth was attenuated by ablation of AR protein by siRNA.

Key words: prostate cancer; androgen receptor; androgen receptor antagonist

Prostate cancer is a major cause of cancer-related deaths in American men and an increasing disease in Japanese men.1) In its initial stages, it is responsive to androgen deprivation therapy achieved by surgical or medical castration, reflecting a dependence on androgen in tumor cell proliferation in the early stage of the disease. But subsequent to androgen deprivation therapy, prostate cancers recur and progress to a terminal stage despite reduced circulating testosterone.2) The molecular mechanisms by which prostate cancer acquires androgen deprivation therapy resistance remains totally unknown. The androgen receptor (AR), a member of the steroid receptor family that is activated by androgens, is the major regulatory transcription factor in normal development of the prostate and in the growth of androgen-dependent prostate cancer.3) Thus AR is assumed to contribute to prostate cancer development during its recurrence in the androgen-deprived patient, but the function of AR in the development of androgen-independent and -dependent prostate cancers remains to be addressed. To test the role of AR protein in prostate cancer, we investigated the effects of androgen ligands and AR expression on androgen-dependent and -independent cell growth.

LNCaP cells were obtained from ATCC (Manassas, VA). LNCaP-Rf cells were kindly donated by Dr. D. Tindall. RPMI1640 and Fetal Bovine Serum (FBS) were purchased from Gibco BRL (Grand Island, NY). DCC-FBS was prepared from FBS by treating it with activated charcoal to remove steroid hormones contained endogenously. Dihydrotestosterone (DHT) was purchased from Fluka (Buchs, Switzerland). Bicalutamide (BIC) were synthesized at Astellas Pharma Inc. (Tokyo, Japan).

Sulfurhodamine B assay was used for growth studies, as described previously.4,5) Briefly, cells were seeded into poly-l-lysine-coated 96-well plates at 10,000 cells/well, and the cells were allowed to attach overnight and then treated with compounds for 72 h. Thereafter, the cell numbers in treated versus control wells were estimated after treatment with 10% trichloroacetic acid and staining with 0.4% sulfurhodamine B in 1% acetic acid.

Two siRNAs were designed from the human AR sequence; they were purchased from Dharmacon (La-fayette, CO). In our experiments to suppress human AR expression in cells, we used the more effective siRNA from among them (target sequence: 5’-AAG GGA AAC AGA AGU ACC UGU dT dT-3’). Scramble II duplex siRNA (Dharmacon) was used as a control. Cells were seeded into poly-l-lysine-coated 24-well plates at 50,000 cells/well. After overnight incubation, siRNA was transfected using oligofectamine (Invitrogen, Carlsbad, CA) as described in the manual. After the incubation period, cell numbers were estimated using Sulfurhodamine B assay, as described above, and expressions of hAR and actin protein were determined by Western blot analysis, which was performed as described previously.6) Cells were lysed on ice in RIPA buffer (1% TritonX-100, 1% deoxycholate, 0.1% SDS,
0.15 M NaCl, 50 mM NaF, 5 mM EDTA, and 50 mM Tris–HCl, pH 7.5) with protease inhibitor cocktail (Sigma, St. Louis, MO). After clearing the cell lysates of debris by centrifugation, protein concentrations were determined by the Lowry method using the Bio-Rad DC-Protein Assay kit. An equal quantity of proteins was separated by 10% polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (Daiichi Pure Chemicals, Tokyo, Japan), and detected with anti-AR polyclonal antibody N-20 or anti-IκBα/C11 polyclonal antibody C-21 (SantaCruz, Santa Cruz, CA) and anti-rabbit IgG conjugated with horse radish peroxidase (Medical and Biological Laboratories, Nagano, Japan).

LNCaP cells originated from lymph nude metastasis. Their growth is androgen-dependent. Several recent reports state that when LNCaP cells are maintained in androgen-deprived circumstances, cell growth stops at once, but after from several weeks to several months, some cells start to grow in those conditions. We used one of that cell line, LNCaP-Rf, as an androgen-independent cell line and LNCaP as an androgen-dependent (parent) cell line in this study.

Initially we investigated the effect of AR ligands on the growth of LNCaP and LNCaP-Rf. While LNCaP cells did not proliferate in the absence of the most potent endogenous androgen, dihydrotestosterone (DHT), as reported previously (Fig. 1A, lanes 1 and 2), proliferation of LNCaP-Rf cells was evident, for 3 d, since the cell numbers of LNCaP-Rf increased by almost 6-fold (Fig. 1B, lanes 1 and 2). DHT stimulated the growth of LNCaP cells in a dose-dependent manner, and a well-known AR antagonist, bicalutamide (BIC), served as an expected antagonist for their growth even in the presence of DHT (Fig. 1A). Unlike LNCaP cells, LNCaP-Rf cells exhibited little response to any tested AR ligands (Fig. 1B), confirming the AR ligand-independent growth of LNCaP-Rf cells. Then, to address whether AR protein is required for the growth of LNCaP-Rf cells, we used siRNAs to abrogate the expression of endogenous AR protein in the cells. Reduced expression of endogenous AR protein by AR specific siRNA in the LNCaP cells and LNCaP-Rf cells was confirmed by Western blot analysis (Fig. 2A). Expression of AR proteins was not affected by non-specific siRNA, Scramble II, and that of IkBα protein was unaltered by siRNAs. In the case of androgen-dependent LNCaP cells, which grew in a medium containing non-treated (androgen-containing) FBS, reduced expression of AR protein resulted in significant loss of cell growth. Interestingly, the cell growth of LNCaP-Rf was also clearly attenuated by AR siRNA, suggesting a pivotal role of AR in the androgen-independent cell growth of a prostate cancer cell line.

A role for AR in recurrent prostate cancer is supported by its expression together with the expression of androgen-regulated genes. Possible mechanisms for AR reactivation in recurrent prostate cancer include altered growth factor-induced phosphorylation and AR mutations that broaden ligand specificity. AR gene amplification was observed after androgen deprivation in 30% of recurrent prostate cancers. AR overexpression is associated with increased sensitivity to the growth-stimulating effects of low androgen concentrations in recurrent prostate cancer-derived cell lines. Our experimental results suggest that the...
quantity and phosphorylation status of AR protein is not
different from LNCaP and LNCaP-Rf. One reason for
androgen-independent growth of LNCaP-Rf might be
mutations that alter the conformation of AR protein
structure which lead to triggering of AR transcriptional
activation androgen independently. Another reason
might be alterations of AR co-regulators that co-activate
or co-repress AR to modulate its transactivation func-
tion.21)

As an analogy to prostate cancer, breast cancer can be
mentioned as an example. Some breast cancers also
express estrogen receptor (ER), a member of the steroid
receptor family as well as AR, and ER antagonist, like
tamoxifen, is initially effective against most of breast
cancers that express ER in the tumor cells. Such breast
cancer patients often become recurrent.22) But a pure ER
antagonist, fulvestrant, has recently been found to show
the growth inhibitory effect on tamoxifen-resistant
breast cancer in a clinical study.23) Since fulvestrant is
known to induce ER protein degradation by binding to
ER,24) suppression of AR protein expression by an AR
ligand is assumed to be effective against prostate cancer,
which is resistant to androgen-deprivation therapy.

References

1) Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward,
2) Feldman, B. J., and Feldman, D., The development of
androgen-independent prostate cancer. Nat. Rev. Cancer,
1, 34–45 (2001).
3) Freedman, L. P., Increasing the complexity of coactiva-
4) Skehan, P., Storeng, R., Scudiero, D., Monks, A.,
McMahon, J., Vistica, D., Warren, J. T., Bokesch, H.,
Kenney, S., and Boyd, M. R., New colorimetric
5) Papazisis, K. T., Geromichalos, G. D., Dimitriadis,
K. A., and Kortsaris, A. H., Optimization of the
6) Furutani, T., Watanabe, T., Tanimoto, K., Hashimoto,
T., Koutoku, H., Kadoh, M., Shimizu, Y., Kato, S., and
Shikama, H., Stabilization of androgen receptor protein
is induced by agonist, not by antagonists. Biochem.
7) Zegarra-Moro, O. L., Schmidt, L. J., Huang, H., and

Fig. 2. Suppression of Androgen Receptor in Cells and Its Effect on LNCaP-Rf Cell Growth.

A, Expression of AR protein in prostate cancer cells. Cell lysates were obtained from LNCaP and LNCaP-Rf treated with siRNA for AR or
control siRNA for 48 h. They were separated by 10% polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes,
and detected with anti-AR or anti-IkBα antibody and anti-rabbit IgG conjugated with horse radish peroxidase. B, Effect of AR siRNA on prostate
cancer cell growth. LNCaP and LNCaP-Rf cells were treated with siRNA for AR or control siRNA. After the indicated periods, cells were
treated with 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The optical density of treated cells to calculate
the cell numbers was determined by means of a fluorescence plate reader.


