Co-localization with DJ-1 Is Essential for the Androgen Receptor to Exert Its Transcription Activity that Has Been Impaired by Androgen Antagonists

Takahiro TAIRA,[a,c] Sanae Marta Margarita IGUCHI-ARIGA,[b,c] and Hiroyoshi ARIGA*,[a,c]

[a] *Graduate School of Pharmaceutical Sciences, Hokkaido University;* [b] *College of Medical Technology, Hokkaido University; Kita-ku, Sapporo 060–0812, Japan: and* [c] *CREST, Japan Science and Technology Corporation; 4–1–8 Honcho, Kawaguchi, Saitama 332–0012, Japan.* Received December 24, 2003; accepted January 12, 2004

DJ-1 was first identified as a novel candidate of an oncogene product in cooperation with an activated *ras*, and DJ-1 was later found to be a positive regulator of the androgen receptor (AR) transcription activity that was repressed by PIASxα. DJ-1 was also found to be an infertility-related protein that was reduced in rat sperm treated with sperm toxicants that cause infertility in rats. To determine the roles of DJ-1 in the AR function, the effects of several androgen antagonists, some of which had been identified as endocrine-disrupting chemicals, on AR transcription activity and localization of AR and DJ-1 in Cos7 cells were examined. Co-localization of DJ-1 with the AR as dot-like spots in the nucleus was first found in cells that had not been treated with chemicals. Although all of the chemicals tested inhibited AR transcription activity to an average of 25% of that without chemicals, there were two classes affecting the localization of the two proteins; one changes the AR from dot-like spots to diffuse spaces in the nucleus and the other still keeps the AR in the dot-like spots. The localization of DJ-1, on the other hand, was found to be dramatically changed by all of the chemicals, resulting in loss of co-localization with the AR. These results indicate that DJ-1 is an essential factor for the AR to exert its full activity.

Key words DJ-1; androgen receptor; endocrine disrupter

DJ-1 was identified as an oncogene product that transforms mouse NIH3T3 cells in cooperation with activated *ras*.[1] The human DJ-1 gene is mapped to 1p36.2—36.3, where many chromosome aberrations in cancer have been reported.[2] DJ-1 was found to be more strongly expressed in the testis than in other tissues and also in sperm, suggesting that DJ-1 has at least two functions, one function in somatic cells, especially in germ cells, and one function in sperm.

With regard to the function of DJ-1 in somatic cells, we have identified PIAS (Protein Inhibitor of Activated Stat)xα/ARIP3 (androgen receptor-interacting protein 3) and DJBP as DJ-1-binding proteins.[3,4] PIASxα, a member of the PIAS family of proteins, was characterized at first as a testis-specific androgen receptor coregulator.[5,6] We have shown that PIASxα inhibits the transcription activity of the androgen receptor (AR) by binding to the DNA-binding domain of AR and that DJ-1 antagonizes this inhibition by sequestering PIASXα from the AR in CV-1, Cos1 and TM4 sertoli cells, indicating that DJ-1 is a positive regulator of the AR.[3] DJBP, a novel DJ-1-binding protein, recruited histone deacetylase complex to the AR to repress its transcription activity and DJ-1 restored its repressed activity by absorbing DJBP.[4]

The AR is a member of the nuclear receptor superfamily and plays a role as a ligand-dependent transcription factor. After a ligand binds to the AR, the AR is translocated into the nucleus and binds to the androgen-responsive element, ARE, on the androgen-activating gene that affects development, growth and regulation of male reproductive functions.[7—10] Transcriptional activity of the AR is known to be regulated by various coregulators that bind to the respective domain of AR. More than 30 proteins of such coregulators have been reported,[11] (see homepage at http://ww2.mcgill.ca/androgendb). The AR was located in the nucleus as a dot-like spot, in which coactivators, including TIF2/GRIP, SRC1 and CBP, were colocalized,[12,13] and that this localization of the AR was abrogated to diffuse spaces in the nucleus by chemicals, including OHF, vinclozolin, p,p′-DDE and nitrofen, thereby leading to the repression of the AR transcription activity.[14] These results suggest that the distinct localization of the AR with proper coactivators is essential for exertion of its full transcription activity.

To further characterize the function of DJ-1 in the AR transcription machinery, the transcription activity and localization of the AR and DJ-1 were examined after the cells had been treated with chemicals that rendered AR transcription activity. The results showed that co-localization of DJ-1 with the AR was drastically hampered by the chemicals, irrespective of the proper or improper localization of the AR, suggesting that DJ-1 is a factor determining the AR transcription activity in cells.

MATERIALS AND METHODS

Chemicals Vinclozolin, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p′-DDE) and nitrofen were purchased from Kanto Chemical Industries. Ornidazole, epichlorohydrin (ECH) and dihydrotestosterone (DHT) were purchased from Sigma. Tamoxifen citrate was purchased from Biomol Research Labo. All of the chemicals were handled according to the guidelines of the company and the Ministry of International Trade and Industry. Hydroxyflutamide (OHF) was kindly provided by Nippon Kayaku, Co. Ltd.

Luciferase Assay Monkey Cos7 cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum. Two micrograms of pARE$_2$-TATA-Luc, a reporter plasmid, 0.5 μg of pcDNA3-F-rAR, and 0.5 μg of pCMV-β-gal, a β-galactosidase expression vector, were transfected into cells approximately 60% confluent in a 6-cm dish in the presence of 10^{-8} M DHT by the calcium phosphate method.[15] Chemicals were added to the medium for 12 h from 36 to 48 h after

* To whom correspondence should be addressed. e-mail: hiro@pharm.hokudai.ac.jp

transfection, and whole cell extracts were prepared by the addition of the Triton X-100-containing solution from a Pica gene kit (Wako Pure Chemicals Co., Ltd.) to the cells. About a one-fifth volume of the extract was used for the β-galactosidase assay to normalize the transfection efficiency as described previously,[3] and the luciferase activity due to the reporter plasmid was determined using a Pica gene kit and a luminometer, Lumat LB 9507 (EG & G Berthold). The same experiments were repeated three to five times.

Indirect Immunofluorescence Monkey Cos7 cells were transfected with DNAs and treated with chemicals by exactly the same protocols as those used in the luciferase assay described above. Forty-eight hours after transfection, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with a mouse anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody.[1] The cells were then reacted with a rhodamine-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope.

RESULTS AND DISCUSSION

DJ-1 in the epididymis and sperm of rats and mice exposed to male or testis/sperm toxicants, including ornidazole and epichlorohydrin (ECH), were reduced and that treatment of animals with these chemicals lead to infertility.[19—22] The effects of these chemicals on the AR, however, have not been investigated. Nawata and his colleagues, on the other hand, reported that both OHF, an anti-androgen agent, and androgen-antagonists, including OHF, vinclozolin, p,p′-DDE and nitrofen, repressed AR transcription activity in monkey Cos7 cells by abrogation of the proper localization of the AR in the nuclei.[14] The effects of these chemicals on DJ-1, however, have not been investigated. To first examine or confirm the effects of these chemicals on AR transcription activity, Cos7 cells were transfected with pARE2-TATA-Luc as a reporter and with pcDNA3-F-AR in the presence of 10^{-8} M testosterone. The plasmid pCMV-β-gal was also cotransfected into cells to normalize the transfection efficiency. Chemicals were added to cells for 12 h from 36 h to 48 h after transfection, and then cell lysates were prepared and their luciferase activities were measured (Fig. 1). The results showed that ethanol, which had been used to dissolve the chemicals, had no effect but that OHF, an anti-androgen used as a positive control, repressed the AR transcription activity in a dose-dependent manner and that 10^{-6} M OHF repressed the AR activity to 14.9% of that without OHF (Fig. 1A). Tamoxifen, an anti-estrogen and very weak anti-androgen agent, was found to repress, to some extent, the AR activity. The three chemicals vinclozolin, p,p′-DDE and nitrofen, which have been identified as endocrine-disrupting (ED) chemicals, were found to repress the AR transcription activity in a dose-dependent manner as previously described (Fig. 1A).[14] Ornidazole and ECH, on the other hand, were also found to repress AR transcription activity in a dose-dependent manner, and 10^{-6} M ornidazole and ECH repressed AR activities to 25.9% and 28.8%, respectively, of those without chemicals (Fig. 1B). These results indicate that ornidazole and ECH can be classified as androgen antagonists.

It has been reported that the AR was localized in the nucleus as a dot-like spot and that this localization was disrupted by chemicals used in the experiment for which the results are shown in Fig. 1A.[14] To determine the localization of the AR and DJ-1 in cells exposed to these chemicals, monkey Cos7 cells were transfected and treated with chemicals under exactly the same conditions as those in the experiments on AR transcription activity as shown in Fig. 1. Two days after transfection, the cells were stained with anti-FLAG and anti-DJ-1 antibodies, and the proteins were detected by rhodamine- and FITC-conjugated secondary antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 2). Three independent experiments were carried out and the typical results were shown. Endogenous DJ-1 (green) and the AR (red) were found to be localized in nuclei as dot-like spots and that these two proteins were co-localized as shown by the yellow color in a merged figure (Fig. 2, Merge). Cell nuclei were identified by DAPI staining (data not shown). Although the number of dots in the nuclei of

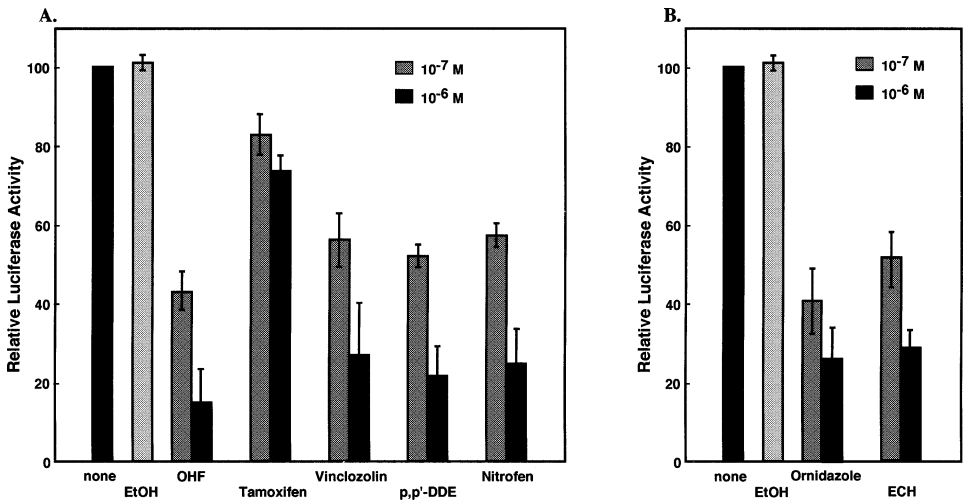

Fig. 1. Effects of Chemicals on Transcriptional Activity of the AR

(A) Cos7 cells were transfected with an expression vector for FLAG-AR together with pARE$_2$-TATA-Luc, a reporter plasmid for AR transcription activity. At 36 to 48 h after transfection, 10^{-7} or 10^{-6} M OHF, tamoxifen, vinclozolin, p,p′-DDE and nitrofen were added to the cells, and then cell extracts were prepared and the luciferase activity was measured as described in Experimental procedures. (B) Cos7 cells were transfected with DNAs and treated with 10^{-7} or 10^{-6} M ornidazole and ECH under exactly the same conditions as those described in (A), and the luciferase activities were measured as in (A).

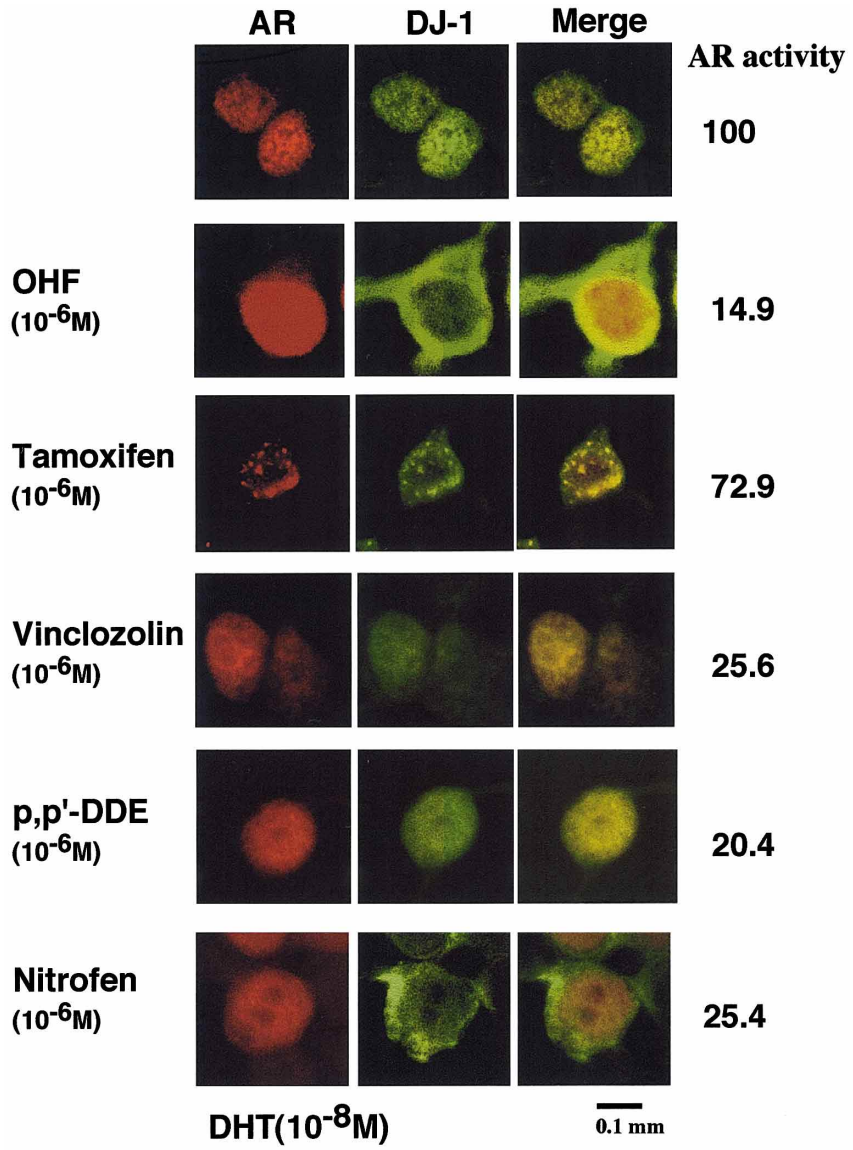

Fig. 2. Abrogation of Co-localization of DJ-1 with the AR by an Anti-androgen Agent or Androgen Antagonists

Cos7 cells were transfected with an expression vector for FLAG-AR together with $pARE_2$-TATA-Luc, a reporter plasmid for the AR transcription activity. At 36 to 48 h after transfection, 10^{-6} M OHF, tamoxifen, vinclozolin, p,p′-DDE and nitrofen were added to the cells. The cells were then fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody,[1] and visualized with a rhodamine-conjugated anti-mouse IgG and an FITC-conjugated anti-rabbit IgG, respectively. The two figures have been merged. The average AR activities determined by the luciferase assay as described in the legend to Fig. 1 are indicated on the right sides of the figures.

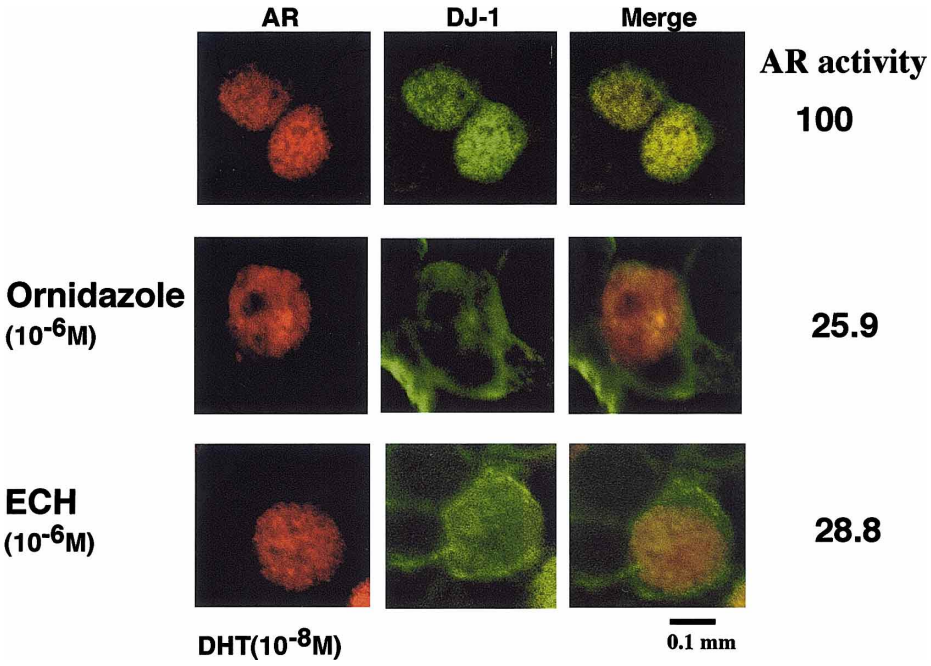

Fig. 3. Abrogation of Co-localization of DJ-1 with the AR by Ornidazole and ECH

Cos7 cells were transfected with an expression vector for FLAG-AR together with $pARE_2$-TATA-Luc, a reporter plasmid for the AR transcription activity. At 36 to 48 h after transfection, 10^{-6} M ornidazole and ECH were added to the cells. The cells were then fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody,[1] and visualized with a rhodamine-conjugated anti-mouse IgG and an FITC-conjugated anti-rabbit IgG, respectively. The two figures have been merged. The average AR activities determined by the luciferase assay as described in the legend to Fig. 1 are indicated on the right sides of the figures.

cells treated with 10^{-6} M tamoxifen decreased, DJ-1 and the AR were found to be still co-localized. In cells treated with 10^{-6} M OHF, vinclozolin and nitrofen, the localization of the AR changed from dot-like spots to diffuse spaces in the nucleus as described previously.[14] The localization of DJ-1 in cells treated with these chemicals, on the other hand, was found to be dramatically changed; both OHF and nitrofen pushed DJ-1 into the cytoplasm and co-localization of the AR with DJ-1 was abrogated; p,p′-DDE localized DJ-1 to the diffuse spaces in the nucleus but these proteins were still co-localized. These results suggest that the proper localization of the AR in the nucleus as dot-like spots is not sufficient for the AR to exert its full transcription activity and that co-localization of the AR with DJ-1 is important.

These possibilities were supported by the results of experiments in which Cos7 cells were treated with 10^{-6} M ornidazole and ECH, and the typical results were shown of three independent experiments (Fig. 3). Although these two chemicals repressed the AR transcription activities to degrees similar to those with OHF, nitrofen and p,p′-DDE, the AR was found to be still localized as dot-like spots in the nucleus. Localizations of DJ-1, on the other hand, were changed to the cytoplasm or both the cytoplasm and the diffuse spaces in the nuclei of cells treated with ornidazole or ECH, respectively, thereby resulting in loss of co-localization of the AR with DJ-1 (Fig. 3). Since the transfection efficiency of plasmids into the cells were 10—20% and almost all of the cells transfected showed the tendency of the localization of DJ-1 and the AR, these results clearly indicate that the co-localization of the AR with DJ-1 as dot-like spots in the nucleus is essential for the AR to exert its full transcription activity.

Several studies have shown that the AR is complexed with other proteins, including coactivators and corepressors and that the proper localization of the AR with these proteins in the nucleus is necessary for its transcription activity. TIF2/GRIP and SRC1 of p160 cofactors and CBP of a general coactivator were found to be co-localized with the AR as dot-like spots in the nucleus, and abrogation of this localization of the AR with an anti-androgen agent or androgen antagonists led to the repression of AR transcription activity.[12—14] We reported that DJ-1 played a role as a positive regulator of the suppressed AR but did not respond to the AR that had already been activated.[3] In this study, we further characterized the relationship between DJ-1 and the AR by using chemicals as tools, and we found that ornidazole and ECH, which are known to be sperm or epididymis toxicants, also repressed AR transcription activity to the degrees similar to those of other androgen antagonists, thereby indicating that they can be classified as androgen antagonists. While OHF, vinclozolin, p,p′-DDE and nitrofen changed the localization of the AR to diffuse spaces in the nucleus, ornidazole and ECH kept the AR in dot-like spots in the nucleus. Moreover, the AR was co-localized with DJ-1 in diffuse spaces but not in dot-like spots in the nucleus in cells treated with vinclozolin and p,p′-DDE, and other chemicals were found to localize DJ-1 to the cytoplasm, thereby resulting in a loss of co-localization of DJ-1 with the AR. These results clearly indicate that the localization of the AR in dot-like spots in the nucleus is not sufficient and that the co-localization with DJ-1 in proper compartments in the nucleus is necessary for the AR to exert its full transcription activity, at least in these systems. What are the mechanisms of translocation of DJ-1 from the nucleus to the cytoplasm? DJ-1 has been reported to be induced in cells treated with paraquat or lipopolysaccharide, which induce the production of activated oxygen species,[20,21] and DJ-1 was strongly expressed and secreted from cells to serum in about half of breast cancer patients.[22] Since some chemicals are known to induce the production of activated oxygen species in cells, it is possible that the chemicals used in this study also produce the activated oxygen species, leading to a change in the components of the DJ-1–protein complex that determine the localization of DJ-1 in cells. Alternatively, proteins associated with DJ-1, which determines the localization of DJ-1 in cells, may change adter treatment of the chemicals.

Taken together, our findings indicate that DJ-1 is an essential component in determination of AR activity.

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