Flow Cytometric Analysis of Androgen Receptor Expression in Human Prostate Tumors and Benign Tissues

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ABSTRACT

Androgen receptor (AR) plays an important role in growth and hormonal therapy of human prostate tumors. Immunohistochemical analysis of AR expression, a non-quantitative technique, is currently used for screening of receptor expression in prostate tissues. The present report describes a laser flow cytometric method for monitoring AR expression in human cell lines and in archival formalin-fixed paraffin-embedded prostate tissues and tumors. Multiparametric flow analysis can be used for simultaneous detection of other cellular markers (e.g., DNA aneuploidy), and by gated analysis, AR expression in subpopulations of a tumor can be quantitatively determined.

INTRODUCTION

Biological activity of the male sex hormone (androgen) is mediated by the AR, a key element in the androgen signal transduction cascade and a target for antiandrogen therapy of human prostate tumors. Androgen deprivation remains the primary therapy for patients with metastatic prostate cancer, and initial response to hormonal therapy is variable and often followed by the emergence of hormone-refractory disease (1–3). The value of AR expression as a prognostic marker for hormone therapy and time to tumor progression (after hormone therapy) and as an indicator of tumor aggressiveness has been discussed previously (3–6). AR expression has been detected in the nuclei of both benign and malignant prostatic epithelial cells by IHC, and several reports indicate that high-grade and advanced-stage prostate tumors have reduced AR immunoreactivity compared with that of BPH and normal prostate tissues (7–14). Extensive variability in AR expression increases with tumor grade (Gleason score) and stage and may account for the variable response of prostate tumors to endocrine therapy (11).

In view of its importance as a possible clinical marker, a precise and reliable assessment of AR expression in prostate cancer cells may be important for diagnostic and prognostic studies in human prostatic disease. Biochemical methods for quantitation of AR expression in cytosolic or nuclear fractions of frozen tissue homogenates cannot discriminate between AR expression of the tumor cells and that of the nonmalignant epithelial and stromal cells. As a result, studies correlating AR expression with response to hormonal therapy have been limited and controversial and cannot assess the heterogeneity of receptor expression in a mixture of tumor and nonmalignant cells. Recently, immunohistochemical techniques have been developed for the study of AR expression in human prostate tumors (15–17). However, standard IHC methods in general are not quantitative and cannot simultaneously determine the expression of other cellular markers. In some of the recent publications, automated and color video image analysis has been used for quantitation of AR expression and determination of receptor heterogeneity (18–20).

Laser flow cytometry offers a means for rapid analysis of cellular marker expression in a heterogeneous tumor cell population where multiparametric and simultaneous analysis of several markers can be used to correlate marker expression with diagnostic and prognostic indicators. We have recently described flow cytometric methods for the simultaneous determination of ER and PgR expression and DNA content in human breast tumor cells (21, 22). The present study was undertaken with the aim of developing a similar method for multiparametric analysis of AR expression in human prostate tissues by flow cytometry.

MATERIALS AND METHODS

Human Prostate Cell Lines and Tissues. LNCaP (obtained from Dr. Kerry Burnstein, University of Miami, Miami, FL) and PC-3 (CRL-1435; obtained from the American Type Culture Collection, Manassas, VA) cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The PC-3 cell line established from bone metastasis of an adenocarcinoma of the prostate has low testosterone-5α-reductase activity and low AR expression (as determined by IHC). The LNCAp cell line established from a metastatic lymph...
node has high acid phosphatase, prostate-specific antigen, and AR expression.

Monolayer cultures were harvested from T-75 tissue culture flasks by treatment with 0.05% trypsin/0.53% EDTA solution. Cells retrieved by centrifugation at 130 × g for 5 min were resuspended in PBS containing 2% FBS and 2% HEPES buffer and fixed in 0.5% paraformaldehyde at 4°C for 15 min. After centrifugation, the cell pellets were resuspended in 2 ml of 0.1% Triton X-100 at 4°C for 5 min.

Formalin-fixed and paraffin-embedded human prostatic tissues were analyzed in the present study by both IHC and flow cytometry. H&E-stained sections were used to confirm the presence of tumor or BPH cells in the block and to select areas to be processed for the flow cytometric analysis. Data included in the present report are from: (a) a patient with BPH; (b) a BPH patient with an atrophied prostate gland; (c) a patient with ductal Gleason grade 4 prostate carcinoma; (d) seminal vesicles of patient 3 with invasive cerviform tumor; and (e) a positive lymph node from a patient with Gleason score 9 prostatic carcinoma. A H&E-stained slide from each paraffin block was used to confirm the presence of tumor cells in the sections processed for IHC and flow analysis.

Specimen Preparation. Sections (25 μm thick) were deparaffinized and rehydrated in a descending ethanol/water series, followed by two washes in distilled water. For nuclear isolation, deparaffinized sections were digested in pepsin [0.05% in normal saline (pH 1.65)] for 60 min at 37°C. After 60 min of incubation, samples were vortexed every 5 min for an additional 30 min. The proteolytic reaction was terminated by the addition of chilled 10% FBS in PBS. The resulting digest was filtered through a 40-μm nylon mesh (Small Parts, Inc., Miami, FL), washed in PBS, and centrifuged at 200 × g for 10 min. Antigen unmasking was achieved by heating the nuclear suspension in citrate buffer for 15 min at 90°C in a water bath (21).

Sections (6 mm thick) were deparaffinized and rehydrated in an ethanol/water series, washed in two changes of distilled water, and incubated in 70% methanol at −20°C for 10 min. Antigen unmasking was performed in a pressure cooker placed in a 600 W microwave oven for 30 min.

Incubation with Antibodies. Cells and nuclei (from suspension cultures or enzyme digests of sections from paraffin blocks) were incubated with 150 μl of anti-AR MAb (F39.4.1; BioGenex, San Ramon, CA) at a 1:25 dilution (in PBS) for 2 h at 37°C. The negative isotype control used for the anti-AR antibody was normal mouse IgG (Sigma, St. Louis, MO) adjusted to the same concentration as anti-AR MAb. After incubation with the primary MAb, cells were washed twice with 2 ml of 0.1% Triton X-100 and stained with 150 μl of FITC-conjugated antimouse IgG antibody (Sigma) at a 1:80 dilution (in PBS) for 35 min at 37°C. After incubation, the cells were washed with 3 ml of 0.1% Triton X-100 and centrifuged at 200 × g for 10 min. The pellets were resuspended in 1 ml of PBS for flow cytometric analysis. For simultaneous monitoring of AR expression and nuclear DNA content, PI (final concentration, 25 μg/ml + 0.5 mg/ml RNase) was added to the AR FITC-stained samples for 15 min at 37°C.

For determining AR immunoreactivity by the immunoperoxidase staining method, the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used. Background nonspecific binding was blocked by incubation with 1.5% normal horse serum (20 min), and endogenous peroxidase activity was quenched by the addition of 3% hydrogen peroxide (4 min). Cytospin preparations and histological sections were incubated with MAb F39.4.1 (BioGenex) at a 1:50 dilution in PBS for 2 h at 37°C. Mouse IgG (Sigma) was used as a negative control. Sections were incubated with the biotinylated secondary antimouse antibody (Vector Laboratories) for 10 min. After washing with PBS, the slides were treated with horseradish peroxidase-conjugated avidin-biotin complex for 5 min. The immunoperoxidase complexes were visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) for 5 min.

Flow Cytometric Analysis. Samples were analyzed on a Coulter Electronics XL/MCL flow cytometer with the standard argon ion laser excitation and filter configuration for the FITC/PI dye combination. The percentage of receptor-positive cells/nuclei was determined by setting electronic gates to exclude 95% of positive cells (fluorescent) in the isotype controls. The relative fluorescence intensity was determined by dividing the MFC value of the antibody-reacted samples by that of the isotype control. Overton’s modified histogram subtraction method was also used for comparison of flow cytometric data (22). Modfit Software (Verity Software House, Topsham, ME) was used for cell cycle analysis.

RESULTS

AR Expression in Human Prostate Cell Lines. The human prostate PC-3 cell line is generally considered to be AR negative, whereas the LNCaP cells are AR positive. In IHC preparations, PC-3 cells did not show any reactivity with the AR antibody. In contrast, the LNCaP cells stained intensely, and most of the immunoreactivity was confined to the nucleus.

To determine the effect of cell density on AR expression, cells were harvested from monolayer cultures at different levels of confluence. Data in Fig. 1 compare the AR expression of the PC-3 cells with that of the LNCaP cells. PC-3 cells, which have a short doubling time (approximately 24 h), show a prominent population of cells with S-phase DNA content (Fig. 1, A–C). In contrast, LNCaP cells are slow growing (with a 2–3-fold longer doubling time than PC-3 cells), which results in the absence of a major and distinct population of cells with S-phase DNA content (data not shown). Histograms in Fig. 1 are of human prostate PC-3 (Fig. 1, A–F) and LNCaP cells (Fig. 1, G–I) stained with PI for DNA content/cell cycle analysis (Fig. 1, A–C) and with the anti-AR/PITC antibody (Fig. 1, D–I). In Fig. 1, D–I, the shaded histograms are of isotype controls, whereas the overlays (unshaded histograms) are of AR-stained cells. Histograms in the top row (Fig. 1, A, D, and G) show cells from highly confluent cultures (80–100% confluence), histograms in the middle row (Fig. 1, B, E, and H) show cells from 50–70% confluent cultures, and histograms in the bottom row (Fig. 1, C, F, and I) show cells from cultures with low confluence (25–40%).

In PC-3 cells, the percentage of cells with S-phase DNA content decreased from 40% to 20% with an increase in confluence (Fig. 1, A–C). As shown in the overlay histogram (Fig.
of AR expression, the decrease in S phase was accompanied by a corresponding increase in the number of AR-positive cells (from 10% to 19%), and the antigen density (as measured by the ratio of the MFC value of the isotype:the MFC value of the antibody-treated sample) increased from 1.38 (Fig. 1D) to 1.65 (Fig. 1F). In contrast to PC-3 cells, the LNCaP cells shown in overlay histograms (Fig. 1, G–I) had a much higher reactivity (38–94% AR-positive cells with a MCF of 2.48–
with atrophied tissue had small acini, and in IHC slides, immunoreactivity was medium (+ +). In flow cytometric analysis (Fig. 4B), 35% of the cells in this specimen had positive AR expression, and the MFC ratio was 3 (approximately one-third of a log).

Prostatic Tumor. In Fig. 5, a DNA distribution histogram (Fig. 5A), two parameter scattergrams (DNA and FITC fluorescence, Fig. 5, B and C), and overlay histograms (isotype versus anti-AR antibody, Fig. 5D) of a prostate adenocarcinoma are shown. As seen in photomicrographs (Fig. 3C), loss of acini in this tumor was accompanied by heterogenous immunostaining with the anti-AR antibody (Fig. 3D). DNA distribution histograms of this tumor and its metastasis in the seminal vesicle (Fig. 5, A and E) indicate the presence of a small population with hypertetraploid DNA content (arrows). This population had a peak channel value of 500 in contrast to the G, and G, peak channel values of 200 and 400, respectively. Scattergrams (Fig. 5, B, C, F, and G) and overlay histograms (Fig. 5, D and H) of the primary tumor (Fig. 5, B–D) and the seminal vesicle (Fig. 5, F–H) show that 13–26% of cells in these samples had positive AR reactivity, with MFC ratios of 1.5 and 2.2. We used electronic gated analysis to determine the AR reactivity of the hypertetraploid population in this tumor. As shown in Fig. 6, the immunoreactivity of this gated population (Fig. 6, A and E) was greater than that of the total (ungated) cell population. The percentage of positive cells increased from 13% to 18.7% in the prostate and from 26% to 44.3% in the seminal vesicle. The MFC values also increased in the gated population from 1.5 to 2.1 (in prostate tumor) and from 2.2 to 4.32 in the seminal vesicle.

In cells from the lymph node of a prostate tumor patient, immunoperoxidase staining showed high AR expression (Fig. 3, E and F). As shown in a flow histogram (Fig. 7E), 90% of cells in this lymph node had positive AR expression, and the ratio of the MFC of the isotype:MFC of MAb-treated cells was 11. In DNA histograms of cells from this lymph node (Fig. 7A), a predominant population of cells with tetraploid DNA content was seen. To determine whether the tetraploid tumor cells had a different AR expression than the diploid cells in this tumor, gated analysis of subpopulations in this specimen was carried out. Fig. 7D shows forward angle scatter (FS) and DNA scattergram of nuclei isolated from this lymph node. In Fig. 7F, AR expression of the R1 gated population in Fig. 7D shows 80% of cells with AR-positive expression and a MFC ratio of 7.7. In contrast, the tetraploid population (R2 gated in Fig. 7D) had 97% AR-positive cells with a higher MFC ratio of 12.2 (Fig. 7G).

DISCUSSION

The predictive value of AR expression as a prognostic marker of response to therapy has been the subject of several previous studies (1–4, 23). Most of the early work on AR expression and its correlation with disease progression was based on human prostate carcinoma cell lines and their xenografts or the murine models of prostate cancer. Recently, the availability of well-characterized xenograft models of prostate carcinoma has made it possible to carry out biological studies seeking to correlate marker expression with growth character-
istics and response to therapy (24–26). In Dunning rat prostate tumors, high levels of nuclear AR expression are seen in tumors that respond to androgen ablation therapy, and with tumor progression, AR levels decrease, and AR-negative cells have higher metastatic activity. The observation that human prostate LNCaP cells that have high AR expression are not as aggressive as PC-3 and DU-145 tumors with negligible AR expression confirmed these studies in human prostate xenografts. de Winter et al. (10) reported that the proportion of cells with positive AR expression and the intensity of staining were decreased in more
aggressive (grade 3) tumors. Brolin et al. (7) observed that the highest proportion of AR-positive cells was found in BPH and in prostate cancer metastasis, as compared with normal tissues. AR expression was significantly higher in well-differentiated adenocarcinoma than in moderately or poorly differentiated tumors. Magi-Galluzzi et al. (11) reported that 85% of prostatic carcinomas had high AR expression (50% stained tumor cells). High-grade PIN present in 42.5% of cases had markedly reduced nuclear staining as compared with low-grade PIN or normal prostate tissue. AR expression became more variable with increasing Gleason score. These data were confirmed by Sweat et al. (5), who observed higher AR reactivity in benign epithelium than in PIN and prostate tumors.

In general, data from the immunohistochemical studies
have shown that BPH and normal prostatic epithelial cells have high AR expression; in advanced PIN and prostate tumors of higher Gleason score and T value, AR expression is reduced, and metastatic prostatic tumor cells in lymph nodes have positive AR expression (27, 28).

The role of mutations and other genetic alterations in hormone-refractory AR-positive tumors has been discussed by several investigators. Culig et al. (9) concluded that the adaptation of prostate tumor cells to a low androgen-containing environment involves mutations that generate receptors with a wider activation spectrum and increased expression and activation by other signaling pathways. Several studies have discussed the “field effect” phenomena in which normal cells in the vicinity of the malignant cells may have altered phenotypes. In a recent study, Olapade-Olaopa et al. (29) found that whereas AR expression was decreased in dedifferentiated tumors, the decrease in immunoreactivity of the stromal nuclei was far more pronounced. They suggested the presence of a unique field effect that resulted in decreased AR expression in adjacent benign glands and its total loss in the surrounding stroma.

Most of the earlier studies on AR expression in human prostate tissues used radioligand binding assays. These assays cannot distinguish between receptor expression of the different normal and malignant subpopulations, nor can one determine the antigen density on individual tumor cells. With the availability of AR-specific antibodies, it has been possible to monitor cellular AR expression by IHC, with the added advantage that one can determine the heterogeneity of receptor expression and correlate expression with histology and tissue architecture. To add a quantitative dimension to IHC data, the use of image analysis has been recently introduced to measure antigen expression and correlate it with histology (18–20). In general, these studies have shown that AR expression in human xenografts of castrated mice is one-half that of intact mice or animals injected with testosterone. In human tumors, epithelial cells have higher average AR immunostaining than BPH cells. In a recent article, Kim et al. (20) used an automatic video color image analysis system to measure AR antigen density in human prostate tumor xenografts in hormone-manipulated (castration/testosterone stimulated) animals and in clinical specimens obtained by transurethral resection of prostate. Their results indicate that this methodology could be standardized for accurate quantitation of AR immunostaining in human prostate tissues. These authors also showed that an average transurethral resection of prostate specimen of BPH or prostate tumors had significantly higher immunostaining intensity than specimens obtained by prostatectomy, thus suggesting that rapid fixation was essential for preserving the AR content.

Although flow cytometry has become a standard and universal method for phenotypic analysis of marker expression in
human leukemia and lymphoma, its use for the analysis of human solid tumors has been hindered by a variety of technical reasons. The difficulty in obtaining a suspension of single cells with intact cell membranes and the antigen-masking effects of the fixatives have contributed to some of these problems. Based on the observation that antibodies currently used for the immunohistochemical determination of ER, PgR, and AR expression specifically stain the nuclear antigens, we have used nuclear isolation methods to prepare specimens for flow cytometric analysis of hormone receptors. In contrast to the isolation of intact single cells, the isolation of nuclei from fresh or paraffin-embedded tissues for flow analysis is relatively easy. Fixation of isolated nuclei from fresh tissues with low concentrations of paraformaldehyde avoids the problem of antigen masking. As shown in the present study, with the use of antigen retrieval methods for processing of nuclei isolated by enzyme digestion from formalin-fixed, paraffin-embedded tissues, one can perform multiparametric analysis of AR expression in human prostate tumors.

In contrast to ligand binding assays and IHC, laser flow cytometry offers several advantages for the analysis of hormone receptor expression in human tumors. As shown in our earlier study on ER and PgR analysis by flow cytometry (21, 22) and the present report on AR expression, laser flow cytometry can provide multiparametric data in which several markers and their coexpression can be determined simultaneously. For example, in breast tumors, one can examine estrogen versus progesterone or estrogen/progesterone versus DNA content of a heterogeneous tumor population. By using electronic gates for the selection of subpopulations (e.g., DNA content of diploid or aneuploid cells), one can determine the expression of a second marker (e.g., ER or AR expression) in a selected population. We have illustrated this useful feature of flow analysis in Figs. 6 and 7, in which the near tetraploid tumor cells in the primary tumor, seminal vesicle, and the lymph node have more positive AR-expressing cells and higher antigen density than the total population. By using electronic gates and comparing the ratio of the MFC value of the isotype control:MFC value of the antibody-reacted cells, we can get a fair quantitative estimate of the percentage of cells with positive expression and the mean antigen density of the subpopulations. An additional advantage of flow cytometric analysis is that one can sort the selected populations for further biochemical or molecular analysis. However, it should be noted that unless one uses a tumor-specific marker (e.g., prostate-specific antigen and cytokeratin), flow cytometric analysis cannot differentiate between the receptor

**Lymp Node**

**DNA Content**

**FITC Fluorescence**

*Fig. 7* DNA distribution histogram (A), two-parameter contour maps of DNA content versus AR expression (B and C), scattergrams of forward angle scatter and DNA content (D), and overlay histograms (E, F, and G; isotype versus anti-AR antibody-reacted cells) of a positive lymph node. In the total sample analyzed (E), 90% of the population was AR positive with a MFC ratio of 11. In the diploid cells (R1 gated in D), 80% of the cells were AR positive with a MFC ratio of 7.7. In contrast, the tetraploid population (R2 gated in D) had 97% AR-positive cells with a MFC ratio of 12.2.
expression of a normal and a malignant cell in a heterogeneous tumor.

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