The Loss of 5α-Reductase Type I and Type II mRNA Expression in Metastatic Prostate Cancer to Bone and Lymph Node Metastasis

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ABSTRACT

Purpose: Considerable evidence has accumulated demonstrating that the 5α-reduction of testosterone to dihydrotestosterone occurs more efficiently in the normal and benign hyperplastic prostate than in prostate cancer tissues. Efforts have also been channelled into investigating the distribution of 5α-reductase isoenzymes in primary prostate tissues and in "in vitro" cell models of the human prostate. However, no one has, thus far, examined the expression of these isoenzymes in prostate cancer metastasis, although such studies might shed some light on the mechanism(s) responsible for the loss of hormone sensitivity in those tumors. The present report addresses this issue in the hope that this might help to identify the steps leading to the development of prostate cancer metastasis.

Experimental Design: In the present study we used in situ mRNA hybridization of sections from archival paraffin-embedded material to investigate the expression of 5α-reductase type I (5αR-I) and type II (5αR-II) mRNAs in prostate cancer bony (n = 9) and lymph node (n = 13) metastasis, and compared the mRNA distributions with those observed in sections from primary prostate tumors (n = 12). In parallel, sections were investigated for androgen receptor (AR) mRNA expression, and immunostained for AR and prostate-specific antigen.

Results: Neither 5αR-I nor 5αR-II mRNA expression was detected in any of the prostate metastatic lesions, although the same metastatic sites expressed AR mRNA, and stained for cytoplasmic prostate-specific antigen and nuclear AR. In contrast, primary prostate tumors displayed intense staining for 5αR-I and 5αR-II.

Conclusion: These findings suggest that the loss of 5α-reductase mRNA expression in bone and lymph node metastasis may be associated, in part, with the progression of these tumors to androgen insensitivity.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death in men in the Western world, and in the United States alone, 30,200 men will die of this disease in 2002 (1). Prostate cancer has been well characterized at the histopathological level, and can be classified into different stages based on the location and size of the tumor, and into different grades dependent on the degree of differentiation of the epithelial cells in the cancer (2). Additionally, the use, over the last 2 decades, of improved methods of detection and staging including PSA2 testing has considerably facilitated the stratification and management of localized prostate cancer (3). Furthermore, several molecular markers have been proposed as predictors in prostate carcinoma; However, most of these are severely limited by their heterogeneity of expression in prostate cancer (4–6), and the molecular mechanisms underlying the development of the tumor and the progression of the disease still remain, for the most part, unclear.

Attention has focused recently on the enzymes responsible for the conversion of testosterone to the biologically more active DHT as a means of controlling the abnormal growth of the prostate. The formation of DHT is mediated through the action of 5αR, of which 2 isoenzymes exist, 5αR-I and 5αR-II, encoded by separate genes (7–9). Although the respective roles of 5αR-I and 5αR-II in the DHT-forming process are not fully understood, there is now general consensus that both isoenzymes are present in the human prostate and are expressed in a variety of cell types (10–17). Furthermore, lower 5α-reductase activity (measured by monitoring the conversion of testosterone to DHT) was demonstrated in primary prostate cancers compared with benign tissues (18–20); the loss in activity correlated with the histological grade of the tumor (18) and mirrored the reduced hormone sensitivity of the cancer (21). However no one has, to date, measured the expression of 5αR-I and 5αR-II in lymph node and bony lesions of human prostate cancer metastasis.

Therefore, the present study was undertaken to evaluate 5α-reductase isoenzyme expression in prostate cancer metastasis. The presence of metastatic lesions secondary to prostate cancer was confirmed by immunostaining for PSA. The distribution of 5αR-I and 5αR-II mRNAs in bony and lymph node metastasis was studied and compared with the patterns found in primary prostate cancer tumors. In addition we examined, in parallel sections, the coexpression of AR mRNA.

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2 The abbreviations used are: PSA, prostate-specific antigen; DHT, dihydrotestosterone; 5αR, 5α-reductase; AR, androgen receptor; TNM, Tumor-Node-Metastasis; ISH, in situ hybridization; DIG, digoxigenin.
MATERIALS AND METHODS

Archival Material and Tissue Samples. Sections from archival paraffin-embedded primary prostate cancers (n = 12) and prostate cancer metastases (n = 22) were supplied by the Department of Pathology, Western General Hospital. The metastatic lesions were located in bone (9 cases) and lymph node (13 cases). All of the primary tumor cases (M, T, PO, PSA = >10) and all of the lymph node metastatic specimens were from newly diagnosed patients who had not been treated with endocrine therapy. Six patients with bone metastasis had also not received endocrine therapy, whereas the remaining 3 patients in that group were on endocrine therapy but had not been subjected to radical prostatectomy. Serial 5-μm sections were cut, and representative sections from each block of archival tissue were stained with H&E for histopathological evaluation. Suitable blocks for examination were selected by an experienced uropathologist (K. G.), and only sections, which were decalcified using EDTA, were used. Preliminary experiments had demonstrated that decalcification with formic acid inhibited ISH, whereas EDTA treatment had no effect.

Antibodies. A commercial monoclonal anti-AR antibody (NC-2F-AR; Novacastra, Newcastle, United Kingdom) was used. This antibody does not cross-react with estrogen, progesterone, or glucocorticoid receptors. The secondary antibody applied for AR detection was biotinylated goat antimouse IgG (Boehringer Mannheim, Lewes, Sussex, United Kingdom). The PSA staining was detected with a commercial monoclonal anti-PSA antibody (Dako, High Wycombe, United Kingdom) and a biotinylated-labeled goat antimouse secondary antibody (Boehringer Mannheim).

Immunohistochemistry. Human AR immunohistochemistry was performed as described previously (22). Briefly, the archival sections were deparaffinized and dehydrated in xylene and graded alcohols. Before the application of primary antisera, heat-mediated antigen retrieval was performed by boiling slides in 0.01 M citrate buffer in a microwave oven.

Endogenous peroxidase was blocked with 3.0% H2O2 for 10 min, and this was followed by incubation with 10% nonimmune normal goat serum in Tris-buffered saline. Human AR primary antibody (1:25 dilution) was applied to the tissue sections at room temperature for 60 min. After two washes in Tris-buffered saline, the biotinylated secondary antibody (1:500 dilution) was applied at room temperature for 30 min followed by peroxidase-labeled streptavidin-biotin complex (1:800 dilution) for 30 min. For PSA immunohistochemistry, no microwave pretreatment was performed. The primary anti-PSA antibody (1:2500 dilution) was applied for 60 min at room temperature followed by biotinylated secondary antibody as described above. To visualize the immunoreaction a freshly prepared solution of 3',3' diaminobenzidine tetrahydrochloride (0.5 mg/ml; Sigma, Poole, Dorset, United Kingdom) was added for 5 min. Sections were then counterstained, dehydrated, cleared, and mounted. LNCaP cells, which contain high levels of AR protein and PSA, were used as positive control, whereas PC3 and DU145 cells served as negative controls; all of these cells were cultured in the laboratory as described elsewhere (23).

ISH. Expression of mRNAs encoding 5αR-I and 5αR-II was investigated using nonisotopic mRNA ISH as described previously (15). Briefly, archival sections were dewaxed in xylene, refixed, and permethylated by covering the sections with 100 μl 50 mM Tris-HCl (pH 7.6) containing 20 μg proteinase K (Sigma) and 5 mM EDTA for 7.5 min at room temperature. The DIG-labeled cRNA probe used to detect 5αR-I mRNA was synthesized from a plasmid encoding a 780-bp fragment of human 5αR-I cDNA (7) subcloned into pBSII SK. For the cRNA (or “antisense”) probe, the plasmid was linearized with Kpnl and transcription carried out using T3 RNA polymerase. For the “sense” probe, NsiI cleaved plasmid was transcribed using T7 RNA polymerase. The DIG-labeled cRNA probe used to detect 5αR-II mRNA was synthesized in a similar fashion, except that a 365-bp cDNA encoding 5αR-II (9) complementary to the 5αR-II mRNA was used as detailed in our earlier study (15). The specificity of the 5αR-I and -II antisense probes was checked by ISH of Chinese hamster ovary cells, which had been transfected with either the 5αR-I or -II gene. The results showed no cross-reactivity between the probes (15). Hybridization to DIG-UTP-labeled RNA probes (1.6 μg/80 μl) was carried out in a hybridization mixture containing 40 μl formamide, 9.6 μl 5 M NaCl, 1.6 μl 50× Denhardt’s solution, 0.8 μl 1 M DTT, 1.6 μl tRNA (10 μg/ml), and 16 μl 50% dextran sulfate, and proceeded overnight at 50°C in a moist chamber under sealed silane-treated coverslips. Posthybridization washes (30 min each) included 2× SSC (300 mM sodium chloride and 30 mM sodium citrate) at 37°C followed by a single wash in 2× SSC at room temperature before RNase treatment (30 min at 37°C in 2× SSC containing 10 μg/ml RNase-A) then washed in 2× SSC for 20 min, 2× SSC containing 50% formamide at 65°C for 20 min, 2× SSC at 65°C for 20 min, 0.2× SSC at room temperature for 10 min, and finally in 0.1× SSC at 42°C for 20 min. Visualization of the DIG-labeled probes was effected by immersing the sections in DIG-alkaline phosphatase-conjugated antibody (Boehringer Mannheim) followed by overnight immersion in a developing agent (Boehringer Mannheim) to the instruction of the manufacturers. For negative control, hybridization with sense-strand riboprobes was carried out. In addition to establishing the viability of the metastatic specimens for ISH, AR mRNA expression was used as a positive control; earlier studies had demonstrated that distant metastases from prostatic carcinoma expressed ARs (24). The AR probe was made by subcloning an EcoRI generated fragment of 465 bp from the expression vector, pSVAR0 (kindly donated by Dr. A. Brinkman, Rotterdam, Holland), into pGem, which has T7 and SP6 promoter sites producing the antisense and sense AR probes, respectively. The viability of the fragment was established after digestion with SphI restriction enzyme to yield 120- and 345-bp fragments.

RESULTS
None of the metastatic prostate cancer deposits in bone and lymph node entered in this study showed any evidence of either 5αR-I or 5αR-II mRNA expression (data not shown). This contrasted with the strong expression of 5αR-I and 5αR-II mRNAs detected mainly in the glandular lesions of all 12 of the primary prostate tumors examined (Fig. 1, A and B). However, the mRNA expression of 5αR-I and 5αR-II in primary prostate cancers was variable across the tumor sections, and the intensity
of the staining did not correlate with the degree of differentiation of the tumors (Gleason score). mRNAs encoding both isoenzymes were also found in the stroma/fibroblast component of the primary tumors (Fig. 1, A and B), but the staining was significantly weaker than in the corresponding epithelial areas.

Although the metastatic lesions failed to express mRNA for the 5αR isoenzymes, sections obtained from the same bone and lymph node metastatic blocks expressed AR mRNA (Fig. 2, A and B) and PSA (Fig. 4, A and B). The AR mRNA expression in the metastatic lesions was detected in all of the specimens irrespective of the hormonal status of the patients. We also noted that AR immunoreactivity was confined to the nucleus (Fig. 3, A and B), whereas PSA immunoreactivity was, on the other hand, localized exclusively in the cytoplasm of the metastasizing epithelial cells with no PSA immunoreactivity observed beyond the prostatic deposits of the bone and lymph node metastasis.

In common with the metastatic lesions, AR mRNA expression was found in all primary cancers incorporated in this study; immunoreactivity in these primary tumors was exclusively nuclear (results not shown). Additionally all primary prostate cancer specimens demonstrated immunoreactivity to the PSA antibody; the activity was confined to the cytoplasm of the epithelial cells (results not shown).

**DISCUSSION**

This is the first study to examine 5α-reductase expression in prostate cancer metastases. Our findings demonstrated the complete absence of 5αR-I and 5αR-II mRNA expression in bony and lymph node metastases, although these lesions contained substantial prostate deposits, which expressed AR mRNA and were immunoreactive to both the AR and PSA antibodies.

The absence of 5αR isoenzyme mRNA expression in the metastatic lesions contrasted with primary prostate cancers where mRNAs encoding both isoenzymes were present predominantly in the glandular regions but DIG-labeling was also detected in the stroma/fibroblast component of the tumors. These findings confirm earlier reports describing the presence of 5α-reductase enzyme activity in primary prostate cancer (16–21, 25, 26), but unlike the previous studies we were able to identify which isoenzyme was involved. The difference in outcome between the various studies merely reflects the approaches used for detecting 5α-reductase. Although earlier studies (16, 18–20, 25, 26) provided quantitative information on 5αR expression, they were unable to distinguish between the two isoenzymes (16, 18–21, 25, 26), whereas in the present report and in an earlier study (15) we have demonstrated mRNA expression of both 5αR-I and -II, and identified the cellular distribution of both isoenzymes.

The loss of 5α-reductase expression in the secondary sites is striking and in marked contrast to the maintenance of expression in the primary tumors. However, prostate metastatic sites in bone and lymph node do maintain their capacity to express AR as demonstrated by ISH and immunostaining, and the presence of PSA, an AR dependent gene, was detected in all of the specimens, thus confirming the prostate nature of these meta-

**Fig. 1** Representative photomicrographs of ISH studies for 5αR-I and 5αR-II in primary prostate cancers (A and B). Hybridization was carried out with DIG-UTP-labeled 5αR-I (A) and 5αR-II (B) mRNA antisense probes, and visualized by immersion in the DIG-alkaline phosphatase-conjugated antibody. The patterns showed strong staining in the epithelial as well as the stroma/fibroblast for both the 5αR-I (A) and 5αR-II (B). Magnification: ×250.

**Fig. 2** Photomicrographs demonstrating AR mRNA expression by ISH in sections of prostate cancer bone (A) and lymph node (B) metastasis. Hybridization was carried out with DIG-UTP-labeled AR mRNA antisense probes and visualized by immersion in the DIG-alkaline phosphatase-conjugated antibody. Positive labeling for AR mRNA in the prostate deposits of the metastatic lesions (A and B) was noted. Magnification: ×250.
static lesions. The lack of 5α-reductase mRNA in the secondary sites cannot be attributed to a methodological problem, because the positive ISH controls (AR mRNA) testified to the viability of the metastatic specimens, whereas the expression of the 5αR isoforms by primary tumors confirmed that the in situ techniques were working. Furthermore, our earlier report (15) on the distribution of 5αR isoenzymes in benign prostatic hyperplasia testified to the reliability, sensitivity, and isoform specificity of the ISH approach. Therefore, the absence of 5αR mRNA expression in the metastatic lesions must reflect a genuine change in the molecular structure of the invading prostate cancer cells. Significantly, this loss of expression is manifested at a time when the cancer has progressed from a well-differentiated primary tumor to a metastatic lesion, and this transition parallels the development of hormone refractiveness in those tumors. Earlier studies had attributed the development of hormone resistance to an amplification of the AR gene (27), which allows the cells to resume hormone-dependent growth even in the presence of low concentrations of androgen (28). The high levels of AR immunoreactivity detected in the metastatic sites as reported herein are compatible with those from previous immunohistochemical studies (24, 28) and provide a basis for the AR gene amplification theory. Whether the amplification of AR is secondary to the loss of 5αR mRNA expression and to the depletion of the DHT concentrations in the metastasizing cells is not evident. However, the observations made in this report lend support to the view that the loss of DHT-forming 5αR isoenzymes may be of crucial pathogenic relevance to the development of androgen-insensitive growth in prostate cancer.

Thus far, we have been unable to identify the causes for the loss of 5αR expression in prostate cancer metastases. However, mutations of the gene encoding 5αR-II have been described. Lack of 5αR-II causes pseudohermaphroditism (7) in which the prostate remains highly underdeveloped and DHT levels are low. However, these mutations do not appear to be involved in prostatic diseases in adult. More recently, other reports have demonstrated that those genetic variants in 5αR-II correlate with androgen metabolism and prostatic cancer risk. The 5αR gene harbors two frequent polymorphic sites, one in the coding region at codon 89 of exon 1, where valine is substituted by leucine (V89L; Refs. 29–31), and the other in the 3’ untranslated region, where a variable number of dinucleotide TA repeat lengths exist (31). Both polymorphisms are known to increase the activity of this enzyme and may also play a role in predisposition to prostate cancer (29–31). Thus, it is unlikely that any of these genetic variants are responsible for the loss of 5αR-II mRNA expression as seen in the metastatic process. It is more likely that the absence of 5αR-II mRNA expression in prostate bony and lymph node metastasis reflects the loss of a factor in the metastatic lesions, which is essential for 5αR-II expression. A number of studies have recently implicated smooth muscle in the development of prostate function and growth (32–34), and
we have demonstrated that human prostate epithelial cells cocultured with prostate mesenchymal cells maintain their differentiated phenotype, including expression of 5αR-II (34). However, epithelial cells grown in isolation rapidly lose their differentiated features including the expression of 5αR-I and 5αR-II (34). We have additionally demonstrated that a soluble paracrine factor of mesenchymal origin is likely to be responsible for maintaining the differentiated state of the epithelial cell (34). Once prostate cancer epithelial cells invade secondary organs, they lose contact/proximity to prostate stroma, and, as a result, lose their differentiated phenotypic characteristics and fail to express 5α-reductase. We are at present examining this hypothesis using a new model for prostate cancer bony metastasis.

REFERENCES

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