Up-regulation of TRPM-2, MMP-7 and ID-1 during sex hormone-induced prostate carcinogenesis in the Noble rat

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Prostate cancer is the most frequently diagnosed malignancy in the Western world and changes in the ratio of testosterone and estrogens with advancing age is one of the potential risk factors in the development of this disease. However, the molecular mechanisms associated with hormone imbalance in prostate carcinogenesis are poorly understood. In this study we induced a high incidence of prostate hyperplasia, dysplasia and adenocarcinoma in the Noble rat using a combination of testosterone and estradiol-17β. Using this animal model, we studied the gene expression profile during sex hormone-induced prostate carcinogenesis using a cDNA array technique; the results were further confirmed by RT–PCR, western blotting and immunohistochemical analyses. We found up-regulation of TRPM-2 (testosterone-repressed prostatic message-2), MMP-7 (matrix metalloproteinase-7) and Id-1 (inhibitor of differentiation or DNA binding) during development of sex hormone-induced prostate cancer. Increased expression of TRPM-2 and MMP-7 was observed in both premalignant and malignant tissues after sex hormone treatment, indicating their role in the early stages of hormone response and prostate cancer development. In contrast, Id-1 was expressed at relatively low levels in all premalignant samples but increased in malignant cells, suggesting its potential roles as a biomarker for prostate cancer cells. Furthermore, expression of Id-1 appeared to be stronger in poorly differentiated lesions than in well-differentiated carcinomas, suggesting that the levels of Id-1 expression may be correlated with the malignancy of tumors. Our results provide the first evidence of up-regulation of TRPM-2, MMP-7 and Id-1 during sex hormone-induced prostate carcinogenesis and strongly suggest their association with the development of prostate cancer.

Introduction

Prostate cancer is the most frequently diagnosed malignancy in the Western world and the second leading cause of cancer-related death in men (1). However, little is known about the genetic events in malignant transformation of prostate cells. It has been suggested that a change in the ratio of testosterone and estrogens with advancing age is an important factor in initiation of benign prostatic hyperplasia and prostate carcinogenesis (2–4).

To study the mechanisms involved in sex hormone-induced carcinogenesis in the prostate gland, Noble and colleagues successfully induced prostate cancer by long-term implantation of a combination of testosterone (T) and estradiol-17β (E2) in Noble rats (5,6). In their animal model short-term hormone treatment (4 months) consistently induced a proliferative lesion, termed dysplasia, whereas long-term treatment produced carcinogenesis in the prostate (7–10). However, using this approach the incidence of adenocarcinoma is rather low, thus it is difficult to analyse in detail prostate carcinogenesis. Recently, using a similar approach with a modified method that involved a substantial increase in the amount of implanted T and E2, our laboratory achieved a much higher incidence of prostate adenocarcinoma in Noble rats in a relatively short period of time (earliest fully developed adenocarcinoma at 7 months) (11). While hyperplasia was observed 2 months after T+E2 treatment, at 12 months 91% showed full-blown adenocarcinoma ranging from highly differentiated to poorly differentiated types (11).

Human prostate carcinogenesis is believed to involve multiple processes from hyperplasia through dysplasia (pre-cancerous) to carcinoma, from low histological grade to high grade and metastatic carcinoma. Therefore, our Noble rat model provides a good tool to study the association between sex hormone imbalance and prostate cancer development, as well as the mechanisms involved in sex hormone-induced carcinogenesis. We previously found that after T+E2 treatment there was a difference in the apoptosis rate and expression of Bcl-2, Ki-67 and PNCA between sex hormone-treated and untreated tissues, indicating that the imbalance between cell proliferation and apoptosis after sex hormone treatment may be an important factor facilitating prostate carcinogenesis (12). In addition, up-regulation of insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) in premalignant lesions after T+E2 treatment may also play positive roles in epithelial growth during prostate cancer development (11). In the present study we investigated the molecular mechanisms of sex hormone-induced prostate carcinogenesis using the Noble rat model. Using the cDNA array method to compare gene expression profiles of the lateral prostate at different stages after T+E2 treatment we identified aberrantly expressed genes during the development of prostate cancer. Here we report the discovery of three genes, TRPM-2, MMP-7 and Id-1, which were up-regulated during development of sex hormone-induced prostate cancer.

Materials and methods

Experimental animal model

Three-month-old male Noble rats were surgically implanted s.c. with Silastic tubing (no. 508-009; 1.96 mm i.d.×3.18 mm o.d.; Dow Corning, Midland, MI) packed with 210 mg testosterone propionate (Sigma, St Louis, MO) and 25 mg 17β-estradiol benzoate (Fluka, Buchs, Switzerland) as previously described (11). The age-matched control group was implanted with empty tubes.
capsules. All implants were replaced with new ones every 3 months. The animals were kept under standard controlled conditions (20 ± 2°C, 40–70% relative humidity, 12 h light/12 h dark) and allowed access to food and water ad libitum.

**Tissue sampling procedures and histopathological examination**

Animals were killed by cervical dislocation at 4 and 12 months post-implantation. The entire prostate gland complexes were dissected out from the rats at autopsy. After removing the anterior and ventral lobes, the lateral lobes were bisected along an axis parallel to the prostatic urethra. Half of the lateral lobe was fixed in 10% buffered formalin and routinely processed for light microscopy. Sections (5 µm thick) were cut and stained with hematoxylin and eosin (H&E) for histopathological examination, using the criteria and classification described previously (11). The lateral lobe was excised from the other half and further separated into two parts, the proximal perirethral and distal glandular regions, both of which were snap frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

**RNA isolation and cDNA expression array**

Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Total RNA (20 µg) was treated at 37°C for 30 min with 2 U DNase I (Boehringer-Mannheim, Indianapolis, IN) in 50 µl of 1X DNase I buffer (40 mM Tris–HCl pH 7.5, 10 mM NaCl, 6 mM MgCl2) containing 2 U RNAse inhibitor (Boehringer-Mannheim). The reaction was stopped by adding 5 µl of 10X Termination mix (0.1 M EDTA, pH 8.0, 1 mg/ml glycogen). After extraction with phenol:chloroform (1:1 v/v) the RNA was precipitated in ethanol and resuspended in DEPC-treated H2O. Total RNA concentration was assessed by absorbency at 260 nm using a UV spectrophotometer (Pharmacia, Uppsala, Sweden) and its integrity confirmed by denaturing agarose gel electrophoresis.

Gene expression was analyzed using an Atlas Rat cDNA Expression Array (Clontech Laboratory, Palo Alto, CA). For cDNA probe synthesis, 3 µg total RNA was mixed with 1 µl of CDS primer (Clontech) and incubated at 70°C for 2 min. Then 8 µl of Master Mix containing 2 µl of 5X reaction buffer (Clontech) 1 µl of 10× dNTP mix (without dATP, Clontech), 3.5 µl of α-[32P]dATP (3000 Ci/mmol, 10 000 c.p.m./ml; Amersham, Little Chalfont, UK), 0.5 µl of 100 mM DTT and 1 µl of MMLV reverse transcriptase (50 U/µl; Clontech) were added and the mixture was incubated at 42°C for 25 min. After purification the labeled cDNA probe was denatured by mixing with a 1/10 vol of 1X denaturing solution (1 M NaOH, 10 mM EDTA, pH 8.0) and incubated at 68°C for 20 min. Afterwards, an equal volume of 2× neutralization solution (1 M NaH2PO4, pH 7.0) and 5 µl of human C3b-I DNA were added and the solution was incubated for another 10 min at 68°C. The denatured cDNA probe was then added to 5 ml of ExpressHyb hybridization solution (Clontech) with 0.1 mg/ml of salmon testes DNA (Life Technologies) to reach a final probe concentration of 2×106 c.p.m/ml and hybridized at 68°C overnight to an Atlas Rat cDNA Expression Array membrane which was prehybridized in 10 ml of ExpressHyb hybridization solution for at least 30 min. Post-hybridization washing was performed for 3×20 min at 68°C with 2× SSC, 1% SDS and 3×20 min at 68°C with 0.1X SSC. 0.5% SDS, respectively. The membrane was then rinsed in 0.1X SSC and exposed to X-ray film for from overnight to 3 days at −80°C. Autoradiographic intensity was analyzed using AtlasImage 1.01 software (Clontech).

**Western blotting**

Cell lysate was prepared by homogenizing thawed tissues in a modified radio-immunoprecipitation (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS) including proteinase inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and protein concentrations were measured using a protein assay kit (Bio-Rad). Equal amounts of proteins (50 µg) were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham). After blocking with 5% non-fat dry milk/2% bovine serum albumin in Tris-buffered saline for 1 h, the blots were incubated with primary antibodies for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham) for a further h. The immunoreactive signals were detected with ECL Plus western blot detection reagents (Amersham) according to the manufacturer’s instructions. Rabbit or goat polyclonal TRPM-2 (Clusterin) (H330, dilution factor 1:200), MMP-7 (C-17, dilution factor 1:200) and Id-1 (C-20, dilution factor 1:200) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-actin monoclonal antibody (dilution factor 1:500) was obtained from Amersham.

**Immunohistochemistry**

Immunohistochemical staining was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min, followed by rehybridization in phosphate-buffered saline (PBS) and incubation with normal goat or rabbit serum for 30 min to bind non-specific antigens. For Id-1 detection, microwave pretreatment (400 W for 3×5 min in 10 mM sodium citrate buffer, pH 6.0) was used. The slides were then incubated with a 1:1000 dilution of anti-Id-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and primary antibodies against TRPM-2, MMP-7 and Id-1 (Santa Cruz) as described in western blotting. After several rinses in PBS they were incubated with biotinylated anti-rabbit or anti-goat IgG at a dilution of 1:200 for 30 min at room temperature, followed by peroxidase-conjugated avidin–biotin complex and 3,3’-diaminobenzidine (Dako, Carpinteria, CA). The sections were then counterstained with Mayer’s haematoxylin and analyzed by standard light microscopy.

**Results**

**cDNA array analysis of differential gene expression during development of T+E2-induced prostate cancer**

Young male adult Noble rats were exposed to T+E2 and killed as described previously (11). Lateral prostate (LP) was collected at 4 and 12 months after first exposure as detailed in Materials and methods. Histopathological studies showed that normal LP consisted of epithelium lined tubules within the loosely organized stromal tissue (Figure 1A). After 4 months of T+E2 treatment, prostatic hyperplasia was observed which showed increased epithelial thickness and infoldling (Figure 1B). At 12 months, dysplasia was predominant in all glands and was characterized by the appearance of multiple layers of epithelial cells and variable degrees of cytological atypia (Figure 1C). At this stage prostate adenocarcinoma could sometimes be detected in the dysplastic region, but the foci were small and are usually referred to as carcinoma in situ. However, fully developed carcinomas (both poorly and well-differentiated) were found in the perirethral region of the prostate (Figure 1D).

In order to study the gene expression profile during T+E2-induced prostate carcinogenesis tissues from normal LP (control LP), hyperplasia (glandular region of LP treated for 4 months with T+E2), dysplasia (glandular region of LP treated for 12 months with T+E2) and prostate cancer (perirethral region of LP treated for 12 months with T+E2) regions were collected and total RNA was isolated. A labeled probe derived from each sample was hybridized to an Atlas Rat cDNA Expression Array membrane containing 588 cDNAs. Each hybridized filter was analyzed and the intensities of individual signals were compared with the normal control LP. The experiments were repeated once and the reproducibility
TRPM-2, MMP-7 and Id-1 in prostate carcinogenesis

Fig. 1. Effect of sex hormones on the histopathology of the rat prostate. (A) Normal lateral prostate: note that the epithelium lined tubules are surrounded by a thin layer of smooth muscle cells. (B) LP 4 months after T+E2 treatment showing a hyperplastic site characterized by an increase in the thickness of the epithelial layer. (C) LP 12 months after T+E2 treatment showing a typical dysplastic site characterized by the presence of multilayers of epithelial cells with enlarged nuclei and irregular morphology. (D) Periurethral region of the prostate 12 months after T+E2 treatment to show a typical prostate carcinoma. The tumors range from highly differentiated to poorly differentiated adenocarcinoma. This figure shows a region of poorly differentiated tumor cells that formed a barely recognizable glandular structure. All sections were stained with H&E and the photos were taken under 400× magnification.

of the results was very good. Comparative differential gene expression analysis of normal LP versus T+E2-treated samples revealed a total of 17 genes that had altered levels of expression by 2-fold or greater, as shown in Table I. The differentially expressed genes demonstrate a broad range of functional activity. Among them, three genes, TRPM-2, MMP-7 and Id-1, showing the greatest levels of up-regulation in response to T+E2 treatment, were selected for detailed study. TRMP-2 (testosterone-repressed prostatic message-2), a gene which was first identified from the regressing rat prostate after castration (15) and recently has been shown to be important in protection against stress-induced apoptosis in many cell types (16), was found to increase by 27-fold in hyperplasia compared with normal LP. Its expression levels remained unchanged between dysplastic and tumor regions (Figure 2A). The MMP-7 (matrix metalloproteinase-7) gene, which belongs to a family of matrix metalloproteinases that has been shown to be involved in early events of human prostate cancer invasion (17), showed the greatest increment of up to 47-fold in all hormone-treated tissues (Figure 2B). There was also an 18-fold increase in Id-1 (inhibitor of differentiation or DNA binding) gene expression in prostate cancer tissues, which is a negative regulator of basic helix–loop–helix (HLH) transcription factors (18), while no significant increase was observed in hyperplasia or dysplasia (Figure 2C).

Analysis of TRPM-2, MMP-7 and Id-1 expression by semi-quantitative RT–PCR

In order to confirm the cDNA array data, tissues of normal, hyperplasia, dysplasia and carcinoma regions were collected from different animals after the same T+E2 treatments and at the same time points as described for the cDNA array studies. Four normal control LP, four LP from rats treated with T+E2 for 4 months (hyperplasia), six LP from rats treated with T+E2 for 12 months (dysplasia) and six samples of the periurethral region from rats treated with T+E2 for 12 months (carcinoma) were used and histopathological studies were first performed to confirm their histopathological features. Total RNA was isolated and RT–PCR was performed using specific primers as described in Materials and methods. Figure 3 demonstrates that the differential expression patterns of TRPM-2, MMP-7 and Id-1 were similar to those observed with cDNA arrays in all of the samples, confirming the expression array profile data. As shown by the cDNA array, expression of TRPM-2 and MMP-7 was significantly increased (up to 7-fold) in all of the T+E2-treated tissues compared with the untreated controls (Figure 3A and B). Compared with the normal control, the level of Id-1 was significantly increased (~4-fold) only in prostate cancer, but not in hyperplasia or dysplasia (Figure 3C). Thus the RT–PCR results confirmed the findings of the cDNA array studies.
Table I. Representative list of differentially expressed genes and their involvement in prostate and/or other cancers

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Description</th>
<th>Fold change</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10862</td>
<td>Id-1, HLH protein</td>
<td>17.9</td>
<td>Inhibitor of DNA-binding protein, correlated with the aggressiveness of several human breast cancer cell lines</td>
</tr>
<tr>
<td>M64723</td>
<td>Clusterin, TRPM-2, SGP-2</td>
<td>33.4</td>
<td>Anti-apoptotic, correlated with tumor grade in human prostate cancer</td>
</tr>
<tr>
<td>L24374</td>
<td>Matrilysin, MMP-7</td>
<td>36.2</td>
<td>A member of the matrix metalloproteinase gene family, up-regulated in human prostate cancer</td>
</tr>
<tr>
<td>M88601</td>
<td>Metalloendopeptidase meprin β-subunit</td>
<td>−16.8</td>
<td>Integral membrane protease, may be involved in tumor cell migration, invasion and metastasis</td>
</tr>
<tr>
<td>U65007</td>
<td>Proto-oncogene c-met</td>
<td>4.9</td>
<td>HGF receptor, involved in embryogenesis, tissue reorganization and tumor progression, overexpressed in prostate cancer</td>
</tr>
<tr>
<td>X70871</td>
<td>Cyclin G (G2/M-specific)</td>
<td>3.6</td>
<td>Transcriptionally activated by p53, may play a role in p53-mediated cell growth control, overexpressed in breast and prostate cancer</td>
</tr>
<tr>
<td>M86389</td>
<td>HSP27</td>
<td>3.6</td>
<td>A member of the heat shock protein gene family, overexpressed in cancer cells from ovary, endometrium, breast, prostate, digestive tract, etc.</td>
</tr>
<tr>
<td>Z27118</td>
<td>HSP70</td>
<td>6.2</td>
<td>A member of the heat shock protein gene family, overexpressed in many types of cancers</td>
</tr>
<tr>
<td>X63594</td>
<td>IκB α-chain</td>
<td>4.1</td>
<td>A cellular inhibitory protein of NFκB, overexpressed in ovarian carcinoma cell line OVCAR3</td>
</tr>
<tr>
<td>D44481</td>
<td>Proto-oncogene c-crk</td>
<td>3.2</td>
<td>Involved in cell differentiation, migration and the induced non-responsiveness of T cells to stimulation of the T cell receptor, overexpressed in diverse tumor types</td>
</tr>
<tr>
<td>D30040</td>
<td>Proto-oncogene c-akt</td>
<td>7.6</td>
<td>Protein serine/threonine kinase, may be a downstream effector of estrogen- and IGF-1-dependent proliferation of breast cancer cells</td>
</tr>
<tr>
<td>M63122</td>
<td>TNF receptor 1</td>
<td>3.8</td>
<td>A member of the ‘death domain’ receptors, but involved in activation of NFκB and thus potentially inhibiting apoptosis</td>
</tr>
<tr>
<td>U03734</td>
<td>Angiotensin-converting enzyme (ACE)</td>
<td>2.7</td>
<td>Expressed in most benign and malignant endothelial tumors</td>
</tr>
<tr>
<td>J03624</td>
<td>Galanin precursor; neuropeptide Y</td>
<td>6.9</td>
<td>Steroid-regulated neuropeptide, up-regulated in diverse tumor types, including breast cancer</td>
</tr>
<tr>
<td>J05029</td>
<td>Acyl-CoA dehydrogenase; LCAD</td>
<td>4.9</td>
<td>May be mediated by nuclear hormone receptor transcription factors</td>
</tr>
<tr>
<td>U62897</td>
<td>Carboxypeptidase D precursor (CPD)</td>
<td>6.4</td>
<td>A member of the regulatory B-type metalloproteinase family</td>
</tr>
<tr>
<td>D29683</td>
<td>Endothelin-converting enzyme</td>
<td>4.8</td>
<td>The activating enzyme of endothelin, involved in endothelial motogenesis and angiogenesis</td>
</tr>
</tbody>
</table>

*Fold change represents genes differentially overexpressed (positive numbers) or underexpressed (negative numbers) in carcinoma relative to normal lateral prostate.

Analysis of TRPM-2, MMP-7 and Id-1 protein expression by western blotting

In order to confirm our data at the protein level we performed western blotting analyses using tissue sections adjacent to those used in the RT–PCR studies. As shown in Figure 4A, two proteins with molecular weights of 60 and 40 kDa were detected with TRPM-2 specific antibodies in all T+E2-treated samples but were much lower or absent in normal LP samples. The 60 kDa TRPM-2 protein represented the cytoplasmically expressed form of TRPM-2, while the 40 kDa protein is thought to be an intracellular, non-secreted form of TRPM-2 located in the nucleus (19). There was increased expression of both forms of TRPM-2 protein in all T+E2-treated tissues and no significant difference was found between hyperplastic and prostate cancer regions.

MMP-7 is described as a 25–27 kDa protein (20) and we found two specific bands in this molecular weight region in all T+E2-treated samples (Figure 4B), while it was undetectable in normal controls. In contrast, expression of Id-1 was found only in malignant tissues and was undetectable in all non-malignant tissue samples, including normal controls (Figure 4C). Expression of actin was also studied as an internal loading control (Figure 4D).

The protein expression profiles of TRPM-2, MMP-7 and Id-1 were consistent with the results found in the cDNA array and RT–PCR studies and thus supported our earlier findings.

Immunohistochemical analyses of the localization of proteins TRPM-2, MMP-7 and Id-1

Sections from normal LP, LP after 4 months treatment with T+E2 (4M LP), LP after 12 months treatment with T+E2 (12M LP) and periurethral regions after 12 months treatment with T+E2 (12M U) were stained with antibodies against TRPM-2, MMP-7 and Id-1. As shown in Figure 5A, TRPM-2-positive staining was found in all hormone-treated prostate epithelial cells, while it was undetectable in the untreated controls. Expression of TRPM-2 protein was found in both the nucleus and cytoplasm of epithelial cells and there was no significant difference in the staining intensities between hyperplastic and carcinoma cells. Control samples processed simultaneously under identical conditions except for the omission of primary antiserum were all negative (results not shown).

MMP-7-positive staining was mainly observed in the cytoplasm of prostate epithelial cells and the extracellular matrix in all hormone-treated tissues. There was no significant difference in staining intensity when the prostate epithelial cells progressed from hyperplasia to carcinoma (Figure 5B), however, the extracellular localization of MMP-7 was markedly increased in prostate cancer regions.

Id-1 expression was found in the cytoplasm of T+E2-treated samples while it was undetectable in untreated controls (Figure 5C). However, the staining intensity was very weak in non-malignant tissues while very strong expression was found in prostate cancers. Furthermore, within the cancer group Id-1 expression was found to be consistently stronger in poorly differentiated than well-differentiated tumors.

Discussion

In this study we found that 17 genes were differentially expressed using the Noble rat model. Of these, three genes,
TRPM-2, MMP-7 and Id-1 in prostate carcinogenesis

**Fig. 2.** Expression of TRPM-2, MMP-7 and Id-1 after T/H11001 E2 treatment. Differential gene expression profiles were detected using an Atlas Rat cDNA Expression Array membrane and the intensities of individual signals were analyzed and compared using AtlasImage 1.01 software. The relative expression levels of TRPM-2 (A), MMP-7 (B) and Id-1 (C) in control LP (CTL LP), hyperplasia (4M LP), dysplasia (12M LP) and carcinoma (12M U) are presented. The arrows indicate the intensities of each gene expression signal and the graphs show the changes in expression levels. Note the significant increase in TRPM-2 and MMP-7 levels from hyperplasia to carcinoma compared with the controls. Id-1, however, is overexpressed only in carcinoma.

**Fig. 3.** Semi-quantitative RT–PCR analysis of expression of the TRPM-2, MMP-7 and Id-1 genes. Total RNA was isolated from four normal LP (control) and hyperplasia (4M LP) samples and six dysplasia (12M LP) and carcinoma (12M U) samples and the individual genes were amplified using specific primers. (Left) The original RT–PCR results. (Right) Relative expression levels of each gene in comparing with expression of GADPH. Each data point was derived from the mean of three independent experiments and the error bars indicate the SE. Note the significant increases in TRPM-2 and MMP-7 levels from hyperplasia to carcinoma compared with the controls. Id-1, however, is only found in tumors.

**Fig. 4.** Western blotting analyses of expression of TRPM-2 (A), MMP-7 (B) and Id-1 (C) proteins. Aliquots of 50 µg protein extracted from normal LP (control), hyperplasia (LP 4 months after T+E2 treatment, 4M LP), dysplasia (LP 12 months after T+E2 treatment, 12M LP) and carcinoma (periurethral region 12 months after T+E2 treatment, 12M U) were analyzed, with expression of actin used as an internal loading control (D). Note that expression of TRPM-2 and MMP-7 was detected in all hormone-treated samples but was absent in untreated controls, however, expression of Id-1 was only found in tumors.

TRPM-2, MMP-7 and Id-1, were found to be most up-regulated during the development of T+E2-induced prostate cancer using the cDNA array technique. These findings were further confirmed by semi-quantitative RT–PCR, western blotting and immunohistochemical studies. This Noble rat model established in our laboratory has been shown to follow a multistep process similar to that of human prostate carcinogenesis (11) and our findings in the present study provide a first step towards understanding the molecular basis of hormone imbalance in the development of prostate cancer.

**TRPM-2**

The role of TRPM-2 (clusterin, sulfated glycoprotein-2 or apolipoprotein J) was originally considered as a marker for prostate regression because castration readily induced enhanced TRPM-2 expression (15). It has also been regarded as a marker for cell death, as TRPM-2 expression is frequently up-regulated...
in various normal and malignant tissues undergoing apoptosis, including prostate tissue after castration (21–25). However, recently a role in the process of inhibition of apoptosis and induction of cell survival has been proposed for this protein. Overexpression of TRPM-2 not only protects prostate cancer cells against androgen depletion-induced apoptosis (26) but also confers chemoresistance to anticancer drugs such as taxol both in vitro and in vivo (27). These studies indicate that TRPM-2 may play important roles in the protection of prostatic cells and serve as a survival factor. In the present study we found that a significant increase in both TRPM-2 mRNA and protein expression occurred in prostatic hyperplasia after T+E2 treatment (Figures 3 and 4), indicating that up-regulation of TRPM-2 may be an early event during sex hormone-induced carcinogenesis. Our results are consistent with previous findings that up-regulation of TRPM-2 occurs in sex hormone-induced dysplasia using a similar animal model but for a shorter period of treatment time (25). In addition, administration of diethylstilbestrol, a long-acting estrogen, to rats increases prostatic TRPM-2 expression more remarkably than does castration (28), suggesting that estrogen may act as a positive regulator of TRPM-2 expression. In this connection it is possible that induction of TRPM-2 by T+E2 treatment in hyperplastic and dysplastic lesions, as in the present study, may protect premalignant cells from cell death, thus contributing to early neoplastic transformation of prostatic cells.

As a secretory protein TRPM-2 expression is mainly observed in the cytoplasm of many tumor types (29), including rat prostate under similar conditions (25). However, we observed strong positive staining not only in the cytoplasm, as reported by Ho et al. (25), but also in the nucleus of prostate epithelial cells in all hormone-treated samples (Figure 5A). The reason for this discrepancy is not known. Differences in hormone dosage (i.e. much higher in the present study) and different periods of treatment (i.e. up to 12 months in the current study) between these two studies might be factors. The fact that our western blotting study shows the presence of two bands of TRPM-2 (60 and 40 kDa) (Figure 4A) indicates that there might be two forms of TRPM-2 protein, one cytoplasmic and the other nuclear. This would support, albeit indirectly, the positive nuclear and cytoplasmic TRPM-2 reactivities. While the 60 kDa TRPM-2 protein has been shown to represent cytoplasmic expression of this protein, the 40 kDa protein has been postulated to be an intracellular, non-secreted form of TRPM-2 (19). In an androgen-dependent mouse mammary carcinoma model (the Shionogi tumor model) progression to an androgen-independent state has also been correlated with subcellular relocation of TRPM-2 from the cell membrane to
the nucleus (30). This suggests that translocation of TRPM-2 from the cytoplasm to the nucleus may be important in cell proliferation or differentiation in response to certain extracellular stimuli. Thus, a high dosage of sex hormones may induce subcellular relocation of TRPM-2. This may explain the presence of TRPM-2 reactivities in both the nucleus and cytoplasm in all premalignant lesions and carcinomas.

MMP-7

MMP-7 (matrix metalloproteinase-7, matrilysin) belongs to a family of enzymes which are capable of degrading extracellular matrix proteins. Increased expression of MMP-7 has been found in many tumor types, such as glioma, colon, esophageal, ovarian and prostate carcinomas (31–36), and up-regulation of MMP-7 has also been associated with an increased depth of tumor invasion, nodal metastasis and advanced tumor stages in these tumors. However, in prostate cancer MMP-7 is detectable not only in cancer cells (33,36) but also in dysplastic lesions (34), while it is rarely present in normal tissues. Increased MMP-7 expression has been correlated with advanced tumor stage (36) and expression of MMP-7 in prostate cancer cell line DU145 has also been shown to enhance the invasiveness of these cells in vitro (17,37). In the present study we have found that expression of both MMP-7 mRNA and protein was greatly enhanced as early as 4 months after T+E2 treatment, i.e. in premalignant hyperplastic tissues, and expression levels remained high after the appearance of prostate carcinoma (Figures 4B and 5B). Interestingly, increased MMP-7 expression occurs in the rat uterus treated with estradiol during the estrous cycle and during days 3–7 of pregnancy (38), suggesting that increased estrogen levels may contribute to the up-regulation of MMP-7.

Most of the MMPs in carcinomas are primarily expressed in stromal cells and are involved in degradation of the extracellular matrix and are associated with tissue destruction in many tumor types, but MMP-7 has been found predominantly in carcinoma cells, including prostate carcinoma (31,34,35). However, in the present study, in addition to epithelial cells, we also found positive reactivity for MMP-7 in the surrounding extracellular matrix after T+E2 treatment and increased expression was associated with progression from hyperplasia to prostate carcinoma (Figure 5B). It is thus possible that as a secretory protein MMP-7 is first produced in epithelial cells and then secreted into the extracellular matrix. This may explain why MMP-7 was detected both intra- and extracellularly. Expression of MMP-7 was also observed in premalignant tissues such as hyperplasia and dysplasia, indicating that, in addition to its well-characterized role in tumor invasion, MMP-7 may also be important in prostate cancer initiation or promotion.

An interesting observation in the western blot study was that MMP-7 appeared as two bands (i.e. 25 and 27 kDa). A question that came to mind was whether one is the pro-enzyme while the other is the active form. In this connection Festuccia et al. (20) have suggested that the molecular weight of the MMP-7 pro-enzyme is 25–27 kDa while the active form of the enzyme is ~19 kDa (39). In the present study, despite prolonged electrophoresis (45 mA, 6.5 h), the two bands remained closely associated. Although the antiserum used for MMP-7 detection in the western blotting studies was polyclonal (Santa Cruz), no specific band at 19 kDa was detected. We therefore came to the conclusion that both the 25 and 27 kDa bands are more likely to be pro-enzyme forms.

Id-1

In addition to TRPM-2 and MMP-7, we also found increased expression of Id-1, one of the HLH proteins that prevents basic HLH transcription factors from binding to DNA and thus inhibits transcription of differentiation-associated genes (18,40). Expression of Id-1 has been shown to inhibit the differentiation of myoblasts (18), trophoblasts (41), erythroid cells (42), B lymphocytes (43,44), myeloid cells (45) and mammary epithelial cells (40). In addition, expression of Id-1 is not only able to restore DNA synthesis ability in senescent human fibroblast cells but also results in immortalization of human keratinocytes (46,47). This suggests its role in the regulation of cell differentiation and proliferation. So far expression of Id-1 has been found in pancreatic and breast cancers (48–50). In the present study our results have provided the first evidence that expression of Id-1 is associated with prostate cancer. In contrast to TRPM-2 and MMP-7, overexpression of Id-1 was only found in prostate tumor cells, while it was expressed at relatively low levels in premalignant lesions such as hyperplasia or dysplasia in our animal model (Figures 4C and 5C). More importantly, it appears that the level of Id-1 expression is correlated with the histopathological phenotype of tumors; the more poorly differentiated types have a higher level of Id-1 expression. In this connection it is pertinent to note that a significant increase in Id-1 expression has been confirmed in human prostate cancer specimens while its expression was either undetectable or very weak in normal prostate and BPH (benign prostatic hyperplasia) (submitted for publication). Our evidence indicates that Id-1 could be used as a potential biomarker for prostate cancer and the levels of expression may be correlated with the malignancy of tumors.

The relationship between sex hormones and Id-1 expression in prostate cancer is at present unknown. It is well established that estrogen is able to stimulate the proliferative and invasive ability of breast cancer cells with E2 receptor (51). It has recently been reported that direct treatment with E2 leads to up-regulation of Id-1 in breast cancer cells, while antisense oligonucleotide treatment that reduced Id-1 protein expression also decreased estrogen-induced cell proliferation (49). Taken together, our present study suggests that Id-1 may be one of the downstream effectors of E2. In addition, our previous studies showed that the incidence of prostate cancer was greatly increased with the combination E2+T, when compared with T alone (unpublished observations). Our observation of Id-1 overexpression in T+E2-induced prostate cancer cells strongly suggests that Id-1 may be important in mediating the effect of E2 in stimulating prostate carcinogenesis.

Summary and conclusion

In summary, using the Noble rat model our results show that increased expression of TRPM-2, MMP-7 and Id-1 proteins may play important roles in the sex hormone response in prostate cells. Our finding of up-regulation of Id-1 in prostate cancer provides the first evidence of its association with prostate cancer and suggests its potential role as a biomarker for prostate cancer cells. Our evidence also provides new insights into the molecular mechanisms of the effects of sex hormones, especially E2, during prostate cancer development and will contribute to our understanding of the involvement of hormone imbalance in the carcinogenic process of human prostate cancer.
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References


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