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The Role of Epigenetic Modifications in Retinoic Acid Receptor β2 Gene Expression in Human Prostate Cancers

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SUMMARY: The *retinoic acid receptor (RAR) β* gene is a putative tumor suppressor gene on chromosome 3p24, where a high incidence of loss of heterozygosity is detected in many types of tumors. Retinoic acid suppresses cancer cell growth through binding to RARs, especially RARβ, indicating a critical role in mediating anticancer effects. Selective loss or down-regulation of RARβ mRNA and protein has been reported in prostate cancers (PCas), although the mechanisms remain unclear. We investigated the role of epigenetic modification in *RARβ2* gene silencing. Aberrant methylation was detected in 11 of 14 (79%) primary PCas, 9 of 10 (90%) hormone-refractory PCas, and 2 of 4 (50%) PCa cell lines, but not in any normal prostate samples. Chromatin immunoprecipitation assay showed that all RARβ2-negative cells (LNCaP, PC3, and DU145) were hypoacetylated at both histones H3 and H4. After exposure to 5-aza-2'-deoxycytidine treatment, Trichostatin A and all-trans retinoic acid induced partial demethylation, increased accumulation of acetylated histones, and markedly restored the expression of RARβ2 in RARβ2-negative cells. These data suggest that the *RARβ2* gene may be one of the frequently silenced genes by epigenetic modifications in PCa. (*Lab Invest 2001, 81:1049–1057*).

Retinoids are inhibitors of tumorigenesis, with effects mediated by binding to nuclear retinoid receptors. Nuclear retinoid receptors comprise two different families: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), with three subtypes for each (α, β, and γ) (Chambon, 1996). Each subtype has several isoforms resulting from different promoter usage and alternative splicing. Among these receptors, RARβ, or more specifically, the isoform β2, is decreased or down-regulated in a number of tumors, including lung, breast, and esophageal cancers, and squamous cell carcinomas of the head and neck (Lotan et al, 1995; Picard et al, 1999; Qiu et al, 1999; Xu et al, 1997). Additionally, *RARβ* mRNA and protein are selectively lost in prostate cancer (PCa) tissues (Lotan et al, 2000). The level of *RARβ* transcripts increases in many cell types in response to all-trans retinoic acid (ATRA). This is caused by the presence of several retinoic acid responsive elements (RAREs) within the *RARβ* promoter region, where the nuclear hormone receptor heterodimer RAR/RXR binds (de The et al, 1990). Exogenous expression of the *RARβ* gene in RARβ-negative cancer cells increases their responsiveness to growth inhibition and induction of apoptosis by retinoic acid (RA) (Sun et al, 2000). However, little is known about the mechanisms underlying the silencing of RARβ expression in tumor cells.

The *RARβ* gene is characterized by two different promoters and transcripts, which are produced by alternative splicing. Most human cells express RARβ2 as the major isoform. The *RARβ2* promoter is characterized by a CpG (cytidine phosphate guanosine)-rich region, the CpG island (Gardiner-Garden and Frommer, 1987), which is located in the 5'-untranslated region, along with several motifs that are potential binding sites for transcription factors such as AP-1, AP-2, and Sp1. Additionally, RAREs, βRAREs, and a TATA box are located near the transcription initiation site (Baust et al, 1996; van der Leede et al, 1992).

DNA methylation, especially in the CpG-rich promoter regions, inhibits transcription by interfering with initiation or by reducing the binding affinity of sequence-specific transcription factors (reviewed in Bird and Wolffe, 1999). Recently, it was demonstrated that methyl-CpG binding proteins recruit transcription repressors such as histone deacetylase (Jones et al, 1998; Nan et al, 1998; Ng et al, 1999; Wade et al, 1999).

To clarify the epigenetic mechanism of *RARβ2* gene regulation in PCas, we detailed the methylation status of the *RARβ2* promoter region using the bisulfite PCR method and histone acetylation status associated with promoter region by chromatin immunoprecipitation (ChIP) assay.

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Results

Methylation Analysis of the RARβ2 Promoter Region

Using bisulfite PCR direct sequencing and methylation-specific PCR (MSP) methods, approximately 1 kb of the *RARβ2* promoter region (Fig. 1) (−477/+392, GenBank accession numbers S82362 and M96016) containing 38 CpGs was examined for its methylation status. In four PCa cell lines, LNCaP cells were densely methylated in the entire region around the CpG island. PC3 cells were heterogeneously methylated in regions 2 and 3, but not in region 1 (Fig. 2, A and B). The most commonly methylated CpG sites were numbers 21, 22, and 23 in region 2 (Fig. 2B). We constructed specific MSP primers to detect the methylation status of these CpG sites. In DU145, TSU-Pr1, and all normal prostate samples, aberrant methylation was not detected by bisulfite direct sequencing in these regions (Fig. 2B). MSP analysis indicated hypermethylation in LNCaP and PC3, and no methylation in normal prostate samples, DU145, or TSU-Pr1 (Fig. 2C). These results were consistent with bisulfite sequencing data.

Using a laser-capture microdissection (LCM) system, three foci were microdissected in each clinical sample and DNA was extracted from each focus. When methylation was detected in more than one focus by MSP, the clinical specimen was classified as methylation-positive (Fig. 3). In tumor specimens, 11 of 14 primary PCas (79%) and 9 of 10 refractory PCas (90%) had hypermethylation of the *RARβ2* promoter region. All samples were confirmed by bisulfite sequencing (Fig. 2B). The relationships between methylation and clinicopathologic factors are summarized in Table 1.

Expression of RARβ2 in Normal Prostate Samples, PCa Samples, and PCa Cell Lines

Only one of four PCa cell lines (TSU-Prl) expressed *RARβ2* mRNA, the other three cell lines (LNCaP, PC3, and DU145) were essentially negative for *RARβ2* mRNA (Fig. 4A). In normal prostate tissues, RARβ2 was expressed at various levels. In primary PCas, loss or down-regulation of RARβ2 expression was observed in five of eight cases (Fig. 4B). Regarding the methylation status of the *RARβ2* promoter, positive PCa samples (PC5, 7, and 8) were not methylated. All normal prostate samples expressed RARβ2 and were not methylated.

RARβ2 Re-expression with Demethylating Agent and Histone Deacetylase Inhibitor Treatment

To clarify the role of epigenetic suppression of the RARβ2 gene, we treated PCa cell lines with 5-aza-2'-deoxycitidine (azaC), Trichostatin A (TSA), and ATRA. With single agents, RARβ2 re-expression was not detected in RARβ2-negative cells (data not shown). However, combined treatment with azaC and ATRA induced slight *RARβ2* gene re-expression in LNCaP and PC3 cells, and after azaC treatment, the combination of TSA and ATRA caused a marked increase in *RARβ2* gene re-expression. In DU145 cells, re-expression of RARβ2 was not detected with azaC and ATRA treatments, but was observed with addition of TSA and ATRA after azaC treatment (Fig. 5A). To determine the effects of the demethylating agent, we examined the methylation status in LNCaP and PC3 cells after azaC treatment. In both cell lines, partial demethylation was detected by MSP (Fig. 5B).

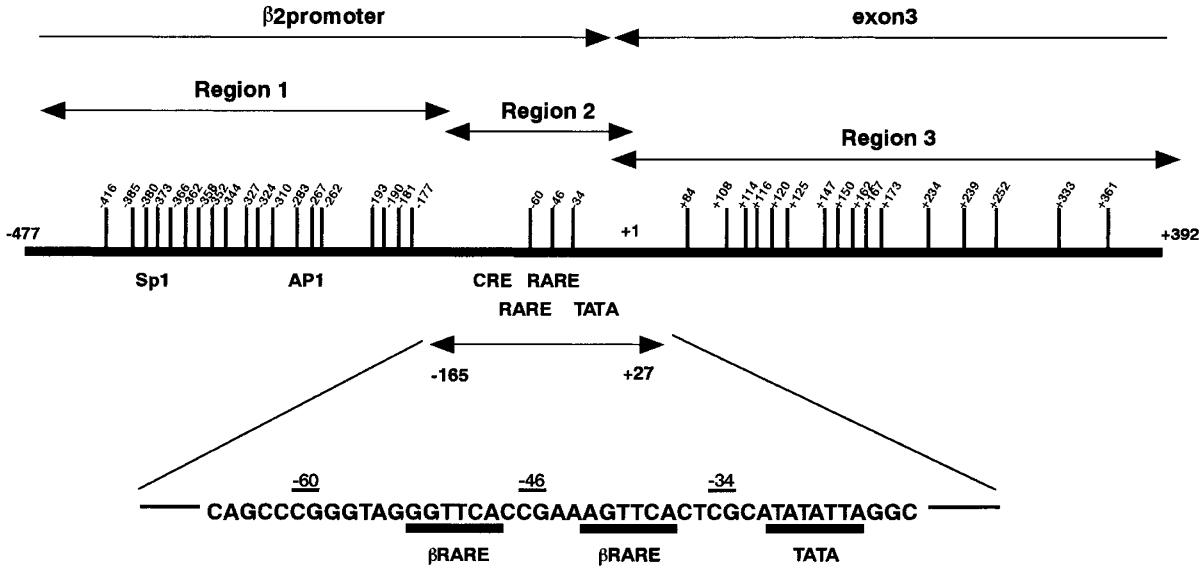

Figure 1.

5'-untranslated region of the retinoic acid receptor (RAR) β2 gene. Top, The cytidine phosphate guanosine (CpG) island covers approximately 1 kb of the RARβ2 gene 5'-untranslated region, containing 38 CpGs (numbered 1 to 38). Several transcription factor binding sites are also indicated. Bottom, Sequence of β retinoic acid responsive element (RARE) region including three CpG dinucleotides.

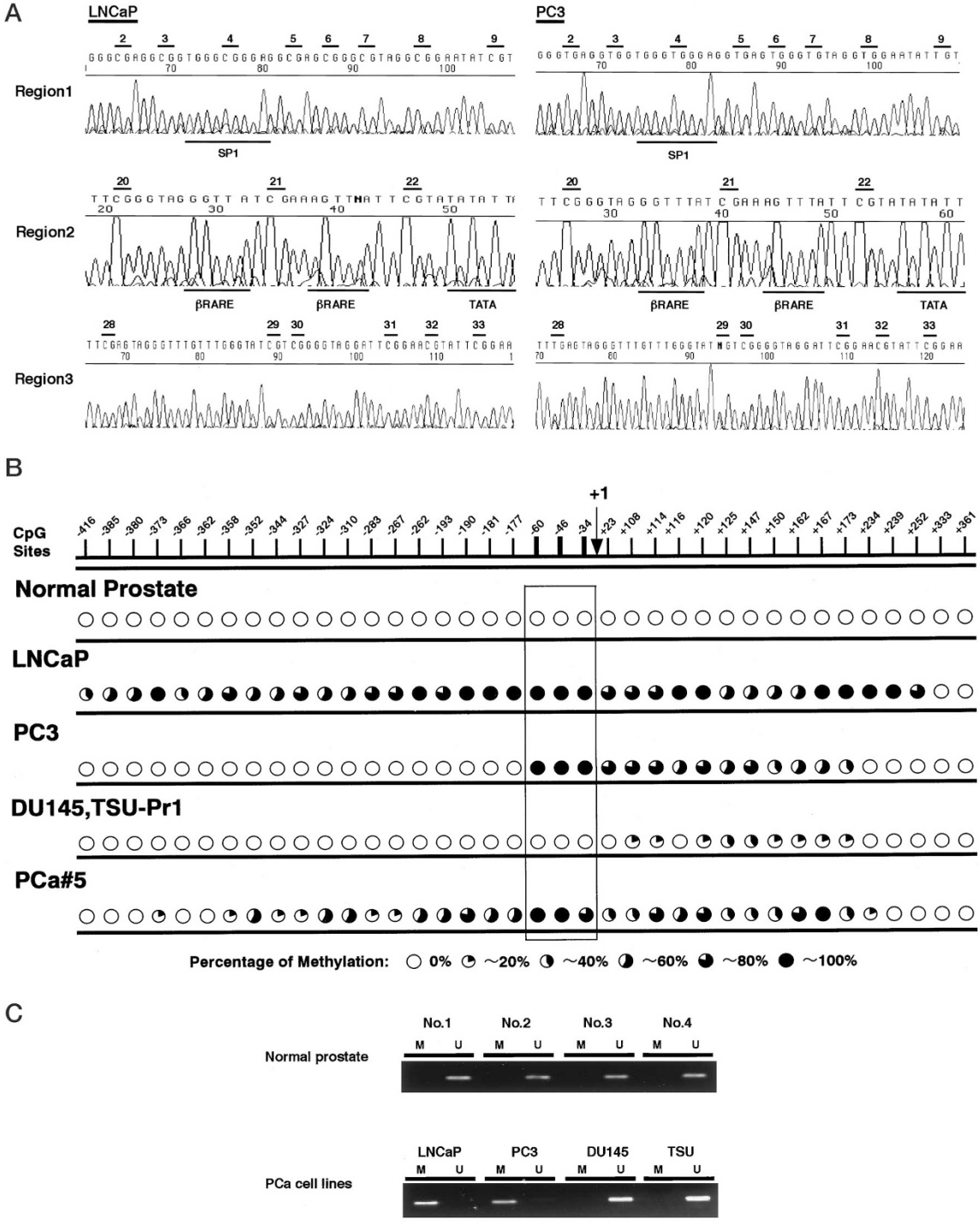

Figure 2.

A, Bisulfite sequencing. Partial sequencing data for LNCaP and PC3 cells are presented. Direct sequencing data revealed that single "C" peaks at the corresponding CpG sites were complete methylations, single "T" peaks were nonmethylations, and overlapping "C" and "T" peaks were partial or heterogenous methylations. B, Summarized bisulfite sequencing of the RARβ2 promoter region in prostate cancer (PCa) cell lines and tissues. At least 10 clones from each cell line and tissue were analyzed. C, Methylation-specific PCR (MSP) analysis. MSP analysis was performed using specific primers for bisulfite-modified DNA. Reactions specific for methylated DNA (M) or for unmethylated DNA (U) are indicated.

ChIP Assay

Using a ChIP assay, we examined the local histone acetylation in the chromatin associated with the *RARβ2* core promoter region (−165/+27), which includes TATA and RAREs sequences and three of the common methylated CpGs of the RARβ2-negative LNCaP and PC3 cells. Figure 6 shows the results of the acetyl-histone H3 and H4 immunoprecipitation assay with or without drug treatment. In TSU-Pr1, RARβ2-positive cells, 192 bp of unmethylated *RARβ2* promoter region was amplified after immunoprecipitation. However, in all RARβ2-negative cells, this region

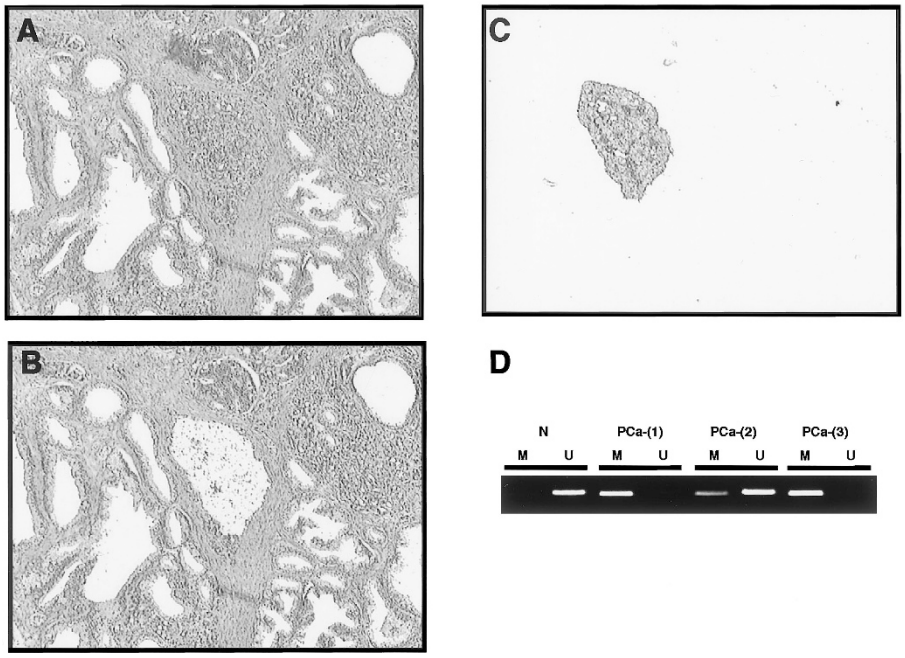

Figure 3.
Laser-capture microdissection (LCM). A, Human PCa specimens before microdissection. B, Human PCa specimens after microdissection. C, Microdissection of cancerous focus. D, In PCa samples, three foci were microdissected and analyzed by MSP. Reactions specific for methylated DNA (M) or for unmethylated DNA (U) are indicated.

Table 1. Summary of Clinicopathological Findings in Prostate Samples

Samples	Age (average)	Clinical staging				Gleason score (average)	RAR β2 methylation
		B1	B2	C	D		
Normal prostate (n = 10)	52–72 (65.8)	—	—	—	—	—	0/10 (0%)
Primary PCa (n = 14)	55–75 (64.1)	4/5 (80%)	5/6 (83%)	2/3 (67%)	0	3–10 (6.6)	11/14 (79%)
Hormone-refractory PCa (n = 10)	64–89 (69.3)	0	0	0	9/10 (90%)	7–10 (8.3)[a]	9/10 (90%)

RAR β2, retinoic acid receptor β2; PCa, prostate cancer.
[a] Scoring of the hormone-refractory tumors was at primary onset before treatment was performed.

failed to be amplified, but after the combination of azaC, TSA, and ATRA treatments, accumulation of acetylated histones was found. Regardless of the drug treatment, the 166 bp of the *GAPDH* promoter region was amplified after ChIP.

Discussion

The results of the present study demonstrated that the 5' CpG island in the *RARβ2* gene promoter region was methylated in two RARβ2-negative PCa cell lines and in more than 80% of human PCa samples. Hypermethylation of the *RARβ2* gene has been reported in breast and lung cancers (Sirchia et al, 2000; Virmani et al, 2000; Widschwendter et al, 2000). Arapshian et al (2000) concluded that methylation of the RARE region may be particularly important in *RARβ2* gene silencing. Here, we show for the first time that the *RARβ2* gene is one of the genes involved in aberrant methylation in human PCas. Additionally, the methylated promoter region of the RARβ2-negative PCa cell lines (LNCaP and PC3) was associated with hypoacetylation of both histones H3 and H4.

DNA methylation is an important mechanism in PCas, and is involved in the inactivation of various essential genes such as *E-cadherin*, *glutathione S-transferase P1*, *the endothelin B receptor*, and *p16/CDKN2* (Graff et al, 1995; Jarrard et al, 1997; Lee et al, 1994; Nelson et al, 1997). In the *RARβ2* gene, the percentages of samples that showed hypermethylation were 79% (11 of 14) in primary PCas and 90%(9 of 10) in hormone-refractory PCas, but 0%(0 of 10) in normal prostate samples. In our samples, no correlations were found between methylation status and clinicopathologic factors. We conclude that hypermethylation of the *RARβ2* gene leading to loss of RARβ2 expression may be an early event during malignant progression.

In the present study, we used a LCM system for avoiding contamination with normal cells, such as lymphocytes and stromal cells. Figure 3 shows heterogeneous methylation in a clinical sample. We speculate that these heterogenous CpG methylation patterns were mainly caused by heterogenous cell populations of tumor cells.

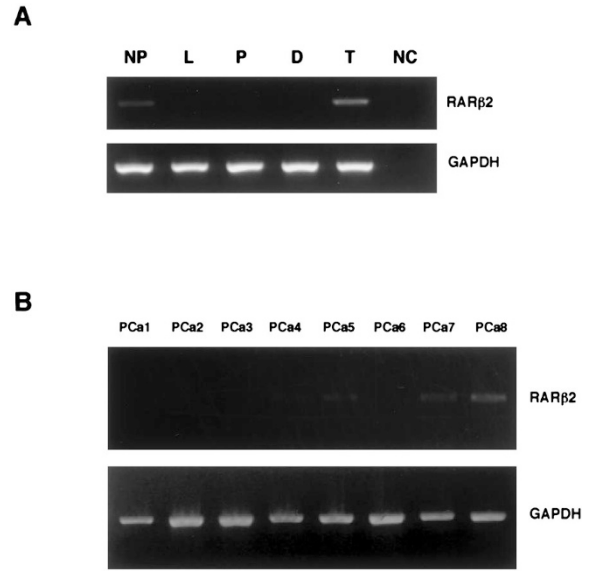

Figure 4.
RARβ2 expression. A, RT-PCR analysis of RARβ2 expression in normal prostate tissue and four PCa cell lines. B, RT-PCR analysis of RARβ2 expression in eight PCa samples. GAPDH was used as an internal control. *NP*, normal prostate tissue; *L*, LNCaP cells; *P*, PC3 cells; *D*, DU145 cells; *T*, TSU-Pr1 cells; *NC*, negative control.

Bisulfite sequencing data indicated that three CpG sites (numbers 20 to 22) near the βRARE region are consensus regions of methylation in PCas. We speculate that methylation of these CpGs may be critical for the silencing of the gene by blocking access of liganded RAR/RXR heterodimers and other cis-acting transcription factors to their binding sequences. In two of methylated, RARβ2-negative PCa cell lines (LNCaP and PC3), azaC and ATRA induced RARβ2 re-expression and partial demethylation. Hypomethylated DU145 cells were not restored. However, in combination with TSA, RARβ2 expression was markedly increased in all negative cells.

The mechanisms of epigenetic change, especially the chromatin structural changes during the silencing of the genes, is not fully understood. Acetylation and deacetylation on lysine residues of histone amino-terminal tails have profound effects on gene transcription (reviewed in Strahl and Allis, 2000). Transcriptional repression is induced by deacetylation of the core histones H3 and H4 (reviewed in Wolffe et al, 2000). Thus, we examined histone acetylation associated with *RARβ2* promoter region. ChIP assay detected a loss of acetylated histones H3 and H4 in all RARβ2-negative cells. A recent study demonstrated that synergistic effects of local histone deacetylation and DNA hypermethylation are crucial factors for chromatin structure alteration leading to transcriptional suppression (Jones et al, 1998; Nan et al, 1998; Ng et al, 1999; Wade et al, 1999). Methylated DNA can be silenced by methyl-CpG binding proteins, such as MeCP2-recruiting Sin3A/histone deacetylase complex. Cameron et al (1999) demonstrated that histone hypoacetylation occurs in the aberrantly methylated promoter. In our previous experiments, we showed that the androgen receptor gene was hypermethylated in an androgen receptor-negative PCa cell line (DU145) and that re-activation was induced by combined azaC and TSA treatments (Nakayama et al, 2000). In the chromatin of the hypermethylated androgen receptor minimal promoter region, both histones H3 and H4 were hypoacetylated (T. Nakayama, M. Watanabe, unpublished data). The *RARβ2* gene may be also silenced by methylation-dependent epigenetic mechanisms in some RARβ2-negative cells. In our experiments, expression of RARβ2 was restored in hypomethylated DU145 cells by inhibition of histone deacetylases but not by demethylation, suggesting that a silencing mechanism such as methylation-independent histone deacetylation may be important in this case. Such inactivations were previously reported for *p21/Waf1* gene inactivation in human cancer cells (Richon et al, 2000; Shin et al, 2000).

We demonstrated that there was a loss of acetylation of histones H3 and H4 associated with *RARβ2* promoter methylation, and that combined TSA and ATRA treatment after azaC treatment increased the accumulation of acetylated histones leading to re-activation of methylated *RARβ2* promoter. These data indicate that promoter hypermethylation may be secondary to the transcriptional repression and may lead to a stable inactive chromatin state, similar to the inactive X chromosome, the imprinted gene locus, and *BRCA1* gene inactivation (Jeppesen and Turner, 1993; Rice and Futscher, 2000; Saitoh and Wada, 2000). Further study is necessary to clarify the precise mechanisms whereby DNA hypermethylation and histone deacetylation are involved in the alteration of chromatin structure and promoter inactivation.

In conclusion, we demonstrated aberrant methylation of the *RARβ2* gene in a majority of clinical PCa samples and in PCa cell lines. Such methylation seems to be specific to cancer because it was not detected in normal prostate samples, and therefore, could potentially serve as a good molecular marker for early cancer detection. We also demonstrated that RARβ2 might be silenced not only by DNA methylation but also by histone deacetylation. Combined treatment with azaC, ATRA, and TSA may be an effective therapeutic strategy to treat PCa by restoring *RARβ2* mRNA expression.

Materials and Methods

Cell Culture and Drug Treatment

PCa cell lines (LNCaP, PC3, and DU145) were obtained from the American Type Tissue Culture Collection (Rockville, Maryland). TSU-Pr1 was kindly provided by Dr. W. B. Isaacs (Johns Hopkins University School of Medicine, Baltimore, Maryland). All cell lines were cultured routinely in RPMI1640 with 10% FBS or 10% charcoal-dextran–stripped FBS at 37° C with 5% CO_2. AzaC (Sigma Chemical Company, St. Louis, Missouri) treatment was performed at 1 μM for 72 hours, TSA (100 ng/ml; Sigma) and ATRA (1 μM; Sigma) treatments were for 24 hours. For the combi-

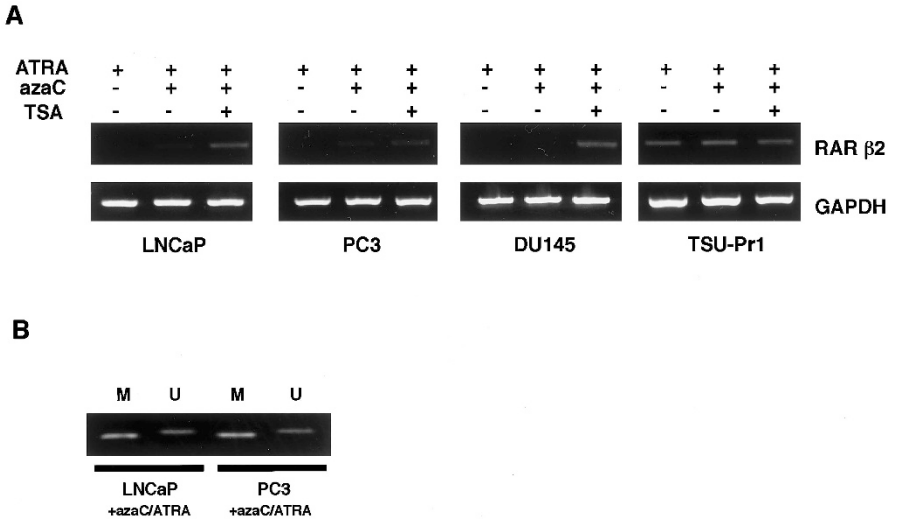

Figure 5.

RARβ2 re-expression. A, RARβ2 re-expression by the treatment of chromatin remodeling drugs. The treatment of 5-aza-2'-deoxycitidine (azaC), Trichostatin A (TSA), and all-trans retinoic acid (ATRA) is described in "Materials and Methods". B, Demethylation after combined drug treatment. MSP analysis showed partial demethylation of the RARβ2 promoter region after azaC and ATRA treatment. These experiments were performed three times, each with similar results.

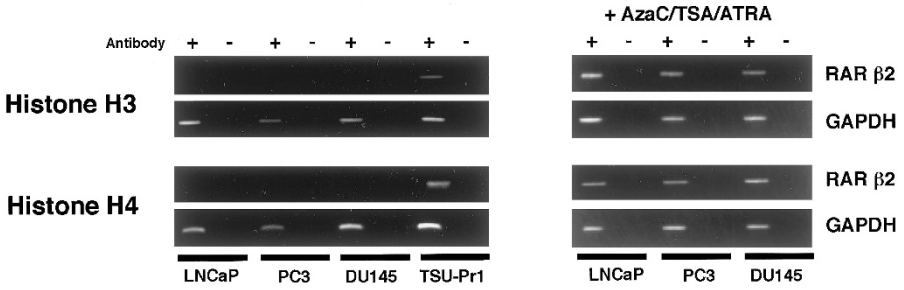

Figure 6.

Chromatin immunoprecipitation (ChIP) assay. ChIP, with the use of antibodies to acetylated histone H3 and H4, detected both histones acetylations of the RARβ2 promoter in the RARβ2-positive TSU-Pr1 cell line and of the GAPDH promoter in all cell lines. After the combined treatment with TSA and ATRA after azaC exposure, histone H3 and H4 acetylation was increased in all RARβ2-negative cell lines. These experiments were performed three times, each with similar results.

nation of azaC, TSA, and ATRA treatments, azaC was introduced for an initial incubation of 48 hours, and ATRA or TSA/ATRA were added for an additional 24 hours.

Tissue Samples

Ten samples of normal prostate tissue were obtained at autopsy. All samples were examined by histopathology and determined to have no evidence of cancerous lesions. Fourteen primary PCa specimens were obtained at radical prostatectomy at Mie University Hospital, Mie, Japan, snap-frozen and stored at −80° C. Additionally, ten hormone-refractory tumors were obtained from distant organ site metastases at autopsy at Chiba University Hospital, Chiba, Japan, and genomic DNA was immediately extracted by a standard protocol. All ten of the hormone-refractory tumor patients had experienced a new onset of cancer under hormonal therapy and died. For RNA extraction, we selected eight of the primary PCa samples and microdissected parts of the specimens composed of more than 80% tumor cells. The clinicopathologic findings for the examined samples are summarized in Table 1.

Tissue Preparation and Sampling by LCM

Frozen tissues were cut into 4 to 6 μm-thick sections with a cryostat. Sections were placed onto glass slides that had been baked at 230° C for 4 hours. The sections were immediately fixed with 70% ethanol for 10 minutes and then washed with diethyl pyrocarbonate-treated water for 5 seconds. Sections were stained with hematoxylin for 15 seconds, washed with diethyl pyrocarbonate-treated water for 10 seconds, dehydrated with an ethanol gradient, and counterstained with an alcoholic Eosin Y solution for 30 seconds. Sections were washed three times with 100% ethanol and three times with xylenes. The sections were air dried with a fan for 20 minutes and stored in a plastic container with silica gel at −80° C until use.

Frozen tumor/or adjacent normal tissues were microdissected using a Pixcell LCM system (LM200; Arcturus Engineering Inc., Mountain View, California).

Sections were covered with LCM transfer film (Capture TF-100; Arcturus Engineering Inc.), and specific portions of the histologic section were affixed to the capture film by brief laser pulses. DNA and RNA were extracted as described previously (Goldsworthy et al, 1999; Hayes et al, 2000).

Bisulfite Modification

Genomic DNA (approximately 0.5 μg) was treated with sodium bisulfite as described previously (Frommer et al, 1992). After denaturation in 0.3 M NaOH at 37° C for 15 minutes, sodium bisulfite was added to a final concentration of 3.1 M, and hydroquinone was added to a final concentration of 0.5 mM. The reaction was performed at 55° C for 16 hours, and desalted using the Wizard DNA purification resin (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Bisulfite modification was completed by 0.3 M NaOH treatment at 37° C for 15 minutes. Modified DNA was precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 50 μl of distilled water.

Bisulfite Sequencing and Methylation-Specific PCR (MSP)

The methylation status of the 5'-regulatory region of *RARβ2* in PCa cell lines was analyzed by bisulfite PCR sequencing and MSP as described previously (Frommer et al, 1992; Herman et al, 1996). Bisulfite genomic sequencing was performed with the following primers: Region 1 (product size, 355 bp): forward, 5'-GTA TGT GTT TTT TTT GGA GTG G-3', reverse, 5'-AAC TTA AAA ACT CCC AAC AAC C-3'; Region 2 (product size, 154 bp): forward, 5'-TGG GAG TTG GTG ATG TTA GA-3', reverse, 5'-ACC CTC CTA ACC TCT AAA CA-3'; Region 3 (product size, 391 bp): forward, 5'-TGT TTA GAG GTT AGG GTT TAT T-3', reverse, 5'-AAC TCC ATC AAA CTC TAC CCC TT-3'. No CpG dinucleotide motifs were contained in these primer sequences. PCR conditions were as follows: 95° C for 10 minutes, 30 cycles of 95° C for 30 seconds, 57° C for 30 seconds, 72° C for 30 seconds, and 72° C for 10 minutes. For bisulfite genomic sequencing, total PCR products were gel-purified and directly sequenced using the ABI 310 automated sequencing system (Perkin Elmer, Foster City, California).

For MSP analysis, modified DNA was amplified with specific-primers: 5'-GGG TTT ATC GAA AGT TTA TTC-3' (forward-methylated) and 5'-TTC CGA ATA CGT TCC GAA T-3' (reverse-methylated); 5'-GGT AGG GTT TAT TGA AAG TTT ATT T-3' (forward-unmethylated) and 5'-AAA CCT TCC AAA TAC ATT CCA AAT-3' (reverse-unmethylated). PCR was carried out under the following conditions: 95° C for 10 minutes then 30 amplification cycles (95° C for 30 seconds, 59° C for 30 seconds, 72° C for 30 seconds) and a final extension incubation of 10 minutes at 72° C. PCR products were directly loaded on 2.0% agarose gels and analyzed after ethidium-bromide staining.

RT-PCR for RARβ2

Total RNA was prepared using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Aliquots of 2 μg of total RNA were used for generation of cDNAs using Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, Maryland). The specific primers applied to detect *RARβ2* transcripts (GenBank accession number X07282) were as follows: forward (located in exon 3), 5'-GCA TGG CAG AGT GCC CTA TC-3'; reverse (located in exon 6), 5'-TCC CAG AGT CAT CCC TGC TTC AT-3'. PCR amplification was performed for 30 cycles at 95° C for 30 seconds, 62° C for 30 seconds, and 72° C for 60 seconds. Human *GAPDH* was used as an internal control. The PCR products were subjected to electrophoresis in 2.0% agarose gels and visualized by ethidium bromide staining.

ChIP Assay

ChIP assays using antibodies to acetyl-histone H3 and H4 were performed according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, New York). Cells were cultured and treated with 100 ng/ml of TSA and/or 1 μM ATRA for 24 hours after 1 μM azaC treatment for 48 hours. Formaldehyde was then added to the cells to a final concentration of 1% and incubated at 37° C for 10 minutes. The cells were washed in 1 ml of ice-cold PBS with proteinase inhibitors, scraped, resuspended in 200 μl of SDS lysis buffer, and incubated on ice for 10 minutes. Lysates were sonicated for 10 seconds three times on ice and centrifuged at 15,000 rpm for 10 minutes at 4° C. Supernatants were loaded on 1% agarose gels and determined to have reduced DNA lengths to between 200 and 1000 bp. Sonicated samples were diluted 10-fold with immunoprecipitation buffer and divided equally to prepare negative control (no antibody) immunoprecipitation samples. The samples were precleaned with a salmon sperm DNA/protein A agarose slurry and incubated overnight at 4° C with or without antibodies to histone H3 or H4. Chromatin-antibody complexes were collected using a salmon sperm DNA/protein A agarose slurry and washed according to the manufacturer's protocol. Immunocomplexes were eluted twice with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 minutes at room temperature. To reverse crosslinks, 20 μl of 5 M NaCl were added with incubation for 4 hours at 65° C. Ten microliters of 0.5 M EDTA, 20 μl of 1 M Tris-HCl pH 6.5, and 2 μl of 10 mg/ml Proteinase K were added, and the samples were incubated at 45° C for 1 hour. Immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. The primer pairs used for ChIP analysis of the *RARβ2* promoter region (GenBank accession numbers S82362 and M96016, PCR product length 192 bp) were 5'-CTC TGG CTG TCT GCT TTT GC-3' (forward), 5'-CAG CTC ACT TCC TAC TAC TTC-3' (reverse). The primers used for the *GAPDH* promoter region (GenBank accession number J04038,

PCR product length 166 bp) were 5'-TAC TAG CGG TTT TAC GGG CG-3' (forward), 5'-TCG AAC AGG AGG AGC AGA GA-3' (reverse). PCR was performed for 25 to 30 cycles of 95° C for 30 seconds, 58° C for 30 seconds, and 72° C for 30 seconds. PCR products were analyzed on 2.0% agarose gels and visualized by UV illumination.

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