[CANCER RESEARCH 57, 35–37, January 1, 1997]

Advances in Brief

Methylation of the 5′ CpG Island of the Endothelin B Receptor Gene Is Common in Human Prostate Cancer[1]

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Abstract

Production of the potent vasoconstrictor endothelin-1 (ET-1) by human prostate cancer cells accompanies prostate cancer progression *in vivo*. The predominant endothelin receptor expressed by normal prostate epithelium, ET_B, is not expressed by any of the established human prostate cancer cell lines, and ET_B binding is decreased on prostate cancer tissues. ET_B, which may mediate ET-1 clearance and may inhibit ET-1 secretion, is encoded by a gene that contains a 5′ CpG island encompassing the transcriptional regulatory region. We examined this regulatory region of the ET_B receptor gene (*EDNRB*) to determine whether hypermethylation of cytidine nucleotides accompanies decreased ET_B expression in human prostate cancer. We found somatic methylation of CpG island sequences in *EDNRB* in 5 of 5 human prostate cancer cell lines, 15 of 21 primary prostate cancer tissues, and 8 of 14 prostate cancer metastases (70% of samples overall). Normal tissues contained only unmethylated *EDNRB*. Treatment of human prostatic carcinoma cell line cultures with 5-azacytidine induced ET_B mRNA expression, suggesting that CpG island methylation changes might accompany the apparent transcriptional silencing of *EDNRB in vivo*.

Introduction

ET-1[3] is produced by human prostatic carcinoma cells *in vitro*, and by primary and metastatic prostatic carcinomas *in vivo* (1, 2). The direct and indirect mitogenic effects of ET-1 on prostate cancer cell lines appear to be mediated exclusively through the isotype-selective ET_A receptor (2). In many normal cells, the secretion and action of ET-1 are regulated by the isotype nonselective ET_B receptor. The ET_B receptor has been proposed to mediate a variety of compensatory activities, including ET-1 clearance, inhibition of ET-1 secretion, and activation of signal transduction pathways that counterregulate ET-1 (3, 4). Previous studies have demonstrated diminished ET_B expression in human prostatic carcinomas *in vitro* and *in vivo* (2).

Somatic methylation of CpG dinucleotides in regulatory regions of critical mammalian genes has been frequently associated with diminished transcriptional activity (5). Sequence analysis of the 1261-bp 5′ regulatory region upstream of the *EDNRB* gene (6) reveals a CpG-rich sequence. This study tested the hypothesis that methylation inactivation of *EDNRB* contributes to decreased ET_B expression in human prostate cancer. Therefore, we studied the methylation status of the 5′ CpG island of *EDNRB* in human prostate cancer cell lines, primary and metastatic tumors.

Materials and Methods

Southern Blot Analysis for *EDNRB* Methylation. Genomic DNA isolation, restriction endonuclease digestion, and Southern blot analysis were performed as described previously (7). Briefly, DNA was isolated from growing cultures of each of five human prostate cancer cell lines and from a variety of normal and neoplastic human tissues. Normal tissues were obtained at autopsy, including prostate specimens from men of different ages (18–69 years) who did not suffer from prostatic diseases. Neoplastic prostate tissues and lymph node metastases, along with matched adjacent prostate or seminal vesicle tissues, were dissected from resection specimens obtained from men treated for localized prostate cancer by radical prostatectomy at the Johns Hopkins Hospital. Autopsy tissues were obtained from men who died of widely metastatic prostate cancer (2). For analysis of *EDNRB* promoter methylation, purified DNAs were digested first with *Eco*RI and *Hin*dIII and then with a m^5C-sensitive restriction endonuclease, either *Bss*HII, *Sac*II, or *Hpa*II. The use of these restriction enzymes allowed direct comparison to the known pattern of pervasive *GSTP1* gene methylation in human prostate cancer (positive control; Ref. 7). Digested DNAs were then electrophoresed on agarose gels, transferred to Zeta-Probe membranes, and hybridized with 20–25 ng of a ^{32}P-labeled *EDNRB* cDNA (1350 bp), 757-bp or 395-bp *EDNRB* promoter probes (Fig. 1*a*). The 5′ sequence-specific probes were obtained by PCR using normal genomic DNA obtained from human placenta as template. Primers were obtained from the published sequence of the 5′ region of *EDNRB* (6): 5′ primer 5′-GTA AAC ATT CGG GCT TGG-3′ and 3′ primer 5′-GGA AGG AAG ACA GGA CAC-3′, yielding a 757-bp product. After digestion with *Bss*HII, the 395-bp fragment was generated. The PCR reaction was optimized using Opti-Prime PCR Optimization kit (Stratagene) using buffer 6 and Perfect Match as an adjunct. The PCR was carried out for 95°C for 1 min, 54°C for 90 s, and 72°C for 3 min for 30 cycles, followed by 1 cycle of 72°C for 8 min. The *GSTP1* Southern blot analysis was performed as described previously (7).

RT-PCR. Total RNA was isolated from prostate cancer cell lines by the guanidium thiocyanate-phenol-chloroform extraction method. RT-PCR was performed as described previously (2), with the following minor modification: the PCR reaction was optimized using the Opti-Prime PCR optimization kit (Stratagene) using buffer 1 and formamide as an adjunct. Southern blot analysis was performed using the ^{32}P-labeled *EDNRB* cDNA probe. Human heart cDNA, the positive control for ET_B expression (8), was obtained from QUICK-screen (Clontech, Palo Alto, CA).

Cell Culture. Cells were grown as described previously (1). To assess reexpression, the following supplements were added to the standard growth media for 7 days: 5aza (1.0, 5.0, 10, 50, and 100 μM; Sigma) and procainamide (1 nM, 10 μM, and 1 mM; Sigma). For chronic 5aza exposure, cells were passaged once a week in media supplemented with 5aza (10 μM) for 3 months before RNA extraction.

Results and Discussion

Sequence analysis of the 1261-bp 5′ region upstream of *EDNRB* (6) reveals manifestations of a stereotypical CpG island (9), including the presence of 56% C and G nucleotides, 74 CpG dinucleotides, and a CpG:GpC ratio of 0.7 (Fig. 1*a*). To determine whether *EDNRB*

Received 9/18/96; accepted 11/18/96.

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[1] This work was supported by a grant from the American Foundation of Urologic Disease, Inc. (to J. B. N., a Dornier Scholar), NIH Prostate Cancer Specialized Programs of Research Excellence CA 58236, and CaP CURE.

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[3] The abbreviations used are: ET-1, endothelin-1; ET_A, endothelin A receptor; ET_B endothelin B receptor; *EDNRB*, ET_B receptor gene; m^5C, 5-methylcytidine; *GSTP1*, π-class glutathione *S*-transferase gene; 5aza, 5-azacytidine; RT, reverse transcriptase.

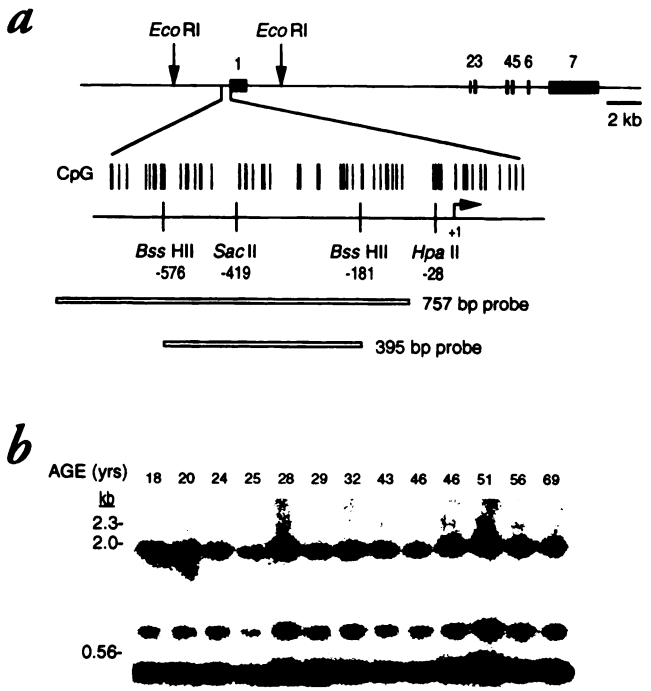

Fig. 1. *a*, restriction map of the human *EDNRB*. The gene spans 24 kb and consists of seven exons (*numbered black rectangles*) and six introns. Exon 1 resides in an *Eco*RI restriction fragment (*arrows*) and is embedded in a CpG island. Several of the m^5C-sensitive restriction enzyme recognition sites in the promoter are indicated (*Bss*HII, *Sac*II, and *Hpa*II). The density of CG dinucleotides in the immediate 5′ region is shown by *vertical bars*. The locations of the 5′-based 757-bp and 395-bp probes are noted. *b*, lack of *EDNRB* promoter methylation *in vivo* in normal prostatic tissues obtained at autopsy from 13 men of different ages. DNA was digested with *Eco*RI, *Hin*dIII, and *Bss*HII and hybridized with the 757-bp probe.

promoter hypermethylation occurs during normal physiological cellular differentiation, DNA isolated from normal human tissues was subjected to Southern blot analysis after digestion with or without the m^5C-sensitive restriction enzyme *Bss*HII, using several *EDNRB*-specific probes. No methylated *EDNRB* promoter sequences were detected in seven different normal tissues, including prostate, seminal vesicle, esophagus, kidney, liver, lung, or spleen (data not shown). Similarly, to assess whether hypermethylation of *EDNRB* promoter sequences arises during aging in normal prostatic cells, DNA from prostatic tissues from 13 men of different ages was also analyzed. No methylation was detected in any of the normal prostatic tissues (Fig. 1*b*).

We have shown previously that no specific ET_B-binding sites could be demonstrated in any established human prostate cancer cell line tested (2). Southern analysis of human prostate cancer cell line DNA cut with m^5C-sensitive *Bss*HII revealed a lack of digestion at both restriction sites in four of the five cell lines, and at the 3′ (−181) *Bss*HII recognition site in the androgen-sensitive human prostate cancer cell line LNCaP (Fig. 2*a*). Similar results were obtained after digestion with the m^5C-sensitive restriction enzymes *Sac*II and *Hpa*II; four of five cell lines were completely methylated, and LNCaP was partially methylated (data not shown). These findings are consistent with the hypothesis that cytidine methylation in the *EDNRB* promoter accompanies decreased ET_B expression in prostate cancer cell lines.

De novo cytidine methylation can occur *in vitro* during cell propagation (10). To determine whether *EDNRB* promoter hypermethylation also occurs *in vivo*, we compared DNA isolated from a number of prostate cancers with matched control DNA prepared from normal tissues. Methylation of the *EDNRB* promoter was observed in 15 of 21 primary prostate cancer specimens (Fig. 2*b*), in 2 of 6 prostate cancer lymph node metastases, and in 6 of 8 distant organ site metastases obtained at autopsy from men dying of prostate cancer. No normal tissues or prostatic tissues

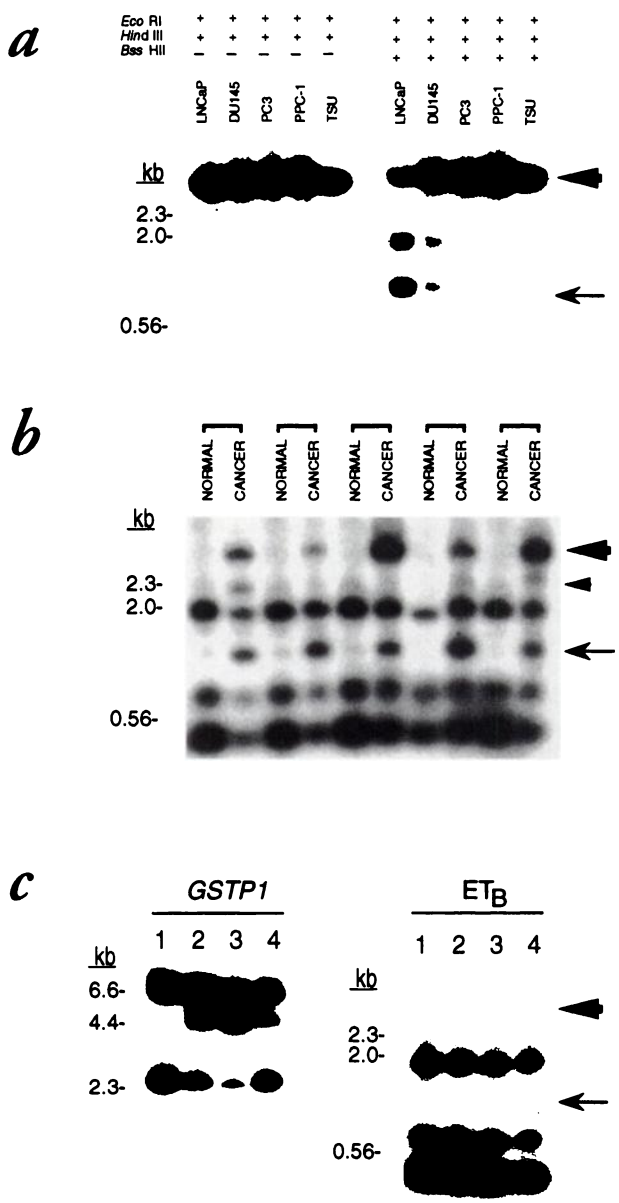

Fig. 2. *a*, methylation of 5′ regulatory sequences at the *EDNRB* locus in human prostate cancer cell lines. Southern analysis of human prostate cancer cell line DNA cut with the m^5C-sensitive restriction enzyme *Bss*HII and hybridized with the 757-bp probe. The *large arrowhead* is the migration position of an *EDNRB* restriction fragment not cut (methylated) by *Bss*HII at either site. The *arrow* is the migration position of an *EDNRB* restriction fragment uncut at the 3′ *Bss*HII site alone. *b*, *EDNRB* promoter methylation in five representative primary prostate carcinomas. DNA was isolated from grossly normal seminal vesicle (*NORMAL*) and prostate cancers (*CANCER*) from the same resection specimen, digested with *Eco*RI, *Hin*dIII, and *Bss*HII, and subjected to Southern blot analysis using the 757-bp probe. The *small arrowhead* is the migration position of an *EDNRB* restriction fragment uncut at the 5′ *Bss*HII site alone. In every case, the 3′ site was more heavily methylated than the 5′ site. *c*, regional methylation in human prostate cancer. Normal prostate tissue (*Lane 1*) is not methylated in *GSTP1* or *EDNRB* (ET_B). In prostate cancer (*Lanes 2–3*), *GSTP1* is methylated (4.4-kb migration band), and *EDNRB* is not. *Large arrowhead* and *arrow* indicate the expected migration positions of *EDNRB* methylation. Tissue was obtained from a radical prostatectomy specimen for primary prostate cancer: normal prostate (*Lane 1*), prostate cancer infiltrating normal tissue (*Lane 2*), an enriched prostate cancer sample microdissected from normal tissue (*Lane 3*), and the bed of normal tissue from which the enriched sample was removed, with residual prostate cancer cells (*Lane 4*). In all cases, complete digestion with *Bss*HII was evident after hybridization with a triosephosphate isomerase probe (not shown).

exhibiting benign hyperplasia were methylated. All blots were also hybridized with a triosephosphate isomerase gene probe (7), which assured complete digestion at the *Bss*HII site (data not shown). In this study, those normal/tumor pairs not showing *EDNRB* methylation consistently retained *GSTP1* methylation (Fig. 2*c*).

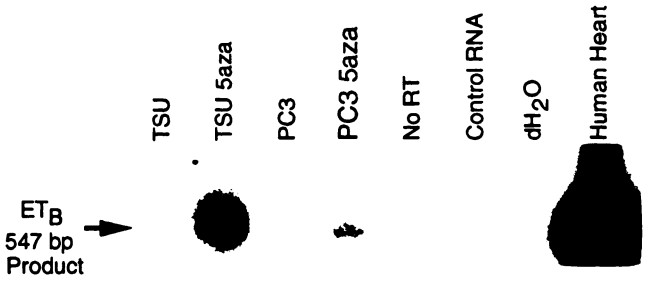

Fig. 3. ET_B mRNA detected in the human prostate cancer cell lines TSUaza and PC3aza after 3 months of exposure to the demethylating agent 5aza. The predicted 547-bp product is also present in the positive control (*Human Heart*), but was not seen in untreated cells (*TSU* and *PC3*) in the negative control lanes (*No RT*, RT excluded from the reaction; *Control RNA*, an amplified cDNA product using non-*EDNRB* primers or water control) after 35 cycles of amplification and hybridization using an *EDNRB* cDNA probe.

To determine the extent of methylation in the 5′ region of *EDNRB*, Southern blot analysis was performed on all samples using two different probes targeting the *EDNRB* promoter. One probe encompassed two *Bss*HII digestion sites (757 bp), and the other annealed between them (395 bp). In every methylated sample, densitometric analysis revealed more frequent methylation at the *Bss*HII site closer to the transcription start site (−181). The more upstream *Bss*HII site (−576), which was never the only site of methylation, was involved in 19 of 28 samples (data not shown). These data do not resolve the mechanistic issue of coincidental *versus* directed methylation, but indicate a pattern of increased frequency of methylation closer to the transcription start site of *EDNRB* in human prostate cancer.

To determine whether 5′ CpG island methylation of *EDNRB* is mechanistically associated with decreased ET_B expression, human prostatic carcinoma cell lines were exposed for 7 days to various concentrations of the methyltransferase inhibitors 5aza and procainamide. No expression of ET_B message, as assessed by RT-PCR, could be demonstrated from any prostate cancer cell line after this acute exposure. However, after chronic, 3-month exposure to 5aza (10 μM), ET_B message was detected in the prostate cancer cell lines TSU and PC3 (Fig. 3), supporting the hypothesis that methylation of the 5′ CpG island of *EDNRB* may be mechanistically involved in decreased ET_B expression. It was our observation that the relatively high dose of 5aza (10 μM) was toxic to the prostate cancer cell lines, although, clearly, some cells continued to proliferate in this environment. Due to rapid decay, 5aza administered once weekly to the culture media may have been effective for only 1 day, contributing to the long time interval required to induce expression.

Somatic CpG island methylation may result in the inactivation of a tumor suppressor gene, leading to the clonal expansion of malignant cells (11, 12). Both ET_A and ET_B receptors are coupled to inositol-1,4,5-triphosphate hydrolysis via G-protein-coupled phospholipase C; the generation of inositol phosphates results in an increased concentration of intracellular calcium (13). Alternatively, ET_B can uniquely mediate divergent responses, such as nitric oxide production and vasodilation (14). The ET_A-mediated mitogenic and synergistic effects of ET-1 on prostate cancer cell lines may be enhanced by loss of the negative regulator ET_B, contributing to androgen-independent prostate cancer progression. Furthermore, as the receptor of ligand clearance and inhibition of ET-1 secretion, ET_B loss may increase ET-1 secretion. High local concentrations of ET-1 can act in a paracrine fashion on surrounding tissues and contribute to the abnormally elevated circulating ET-1 concentrations observed in many men with advanced prostate cancer (1). As a stimulant for osteoblasts, ectopic ET-1 may contribute to the characteristic osteosclerotic response of bone to metastatic prostate cancer (1).

These data do not exclude other mechanisms of *EDNRB* inactivation in prostate cancer; methylation may provide only one of two needed gene-inactivating events (15). *EDNRB* maps to human chromosome 13q22 (16), a region of frequent allelic loss in prostate cancer (17, 18). In 31 advanced prostate cancer tissues studied by comparative genomic hybridization and allelotyping by Cher *et al.* (18), the frequency of loss across 13q21.1-13q31 was "sustained near 60%." Losses in 13q (65%) were second only to 8p (71%) in frequency (18). Furthermore, inactivating mutations in tumor suppressor genes have been strongly associated with CpG islands, acting as "mutational hot spots" in other human cancers (12). Whether these other mechanisms are also operational in prostate cancer is under investigation.

Understanding the nature and consequences of decreased ET_B expression in human prostate cancer may provide new insights into the function of this receptor in both normal and malignant tissues, as well as its role in carcinogenesis. Methylation may be directly "suppressing the suppressor," such as described for *p16* (19, 20), and also may contribute to the morbidity of ectopic ET-1 secretion. These insights will further sharpen the focus for targeted intervention of the endothelin axis.

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