Increased Heparanase Expression Is Caused by Promoter Hypomethylation and Up-Regulation of Transcriptional Factor Early Growth Response-1 in Human Prostate Cancer

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ABSTRACT

Purpose: Heparanase degrades heparan sulfate and has been implicated in tumor invasion and metastasis. The transcription factor, early growth response 1 (EGR1), is associated with the inducible transcription of the heparanase gene. We hypothesize that CpG hypomethylation in the heparanase promoter coupled with up-regulation of EGR1 levels may induce heparanase expression in human prostate cancer.

Experimental Design: Cultured prostate cancer cell lines (Du145, DuPro, LNCaP, and PC-3) with and without 5-aza-2-deoxycytidine treatment, 177 prostate cancer samples, and 69 benign prostatic hyperplasia (BPH) samples were used. The frequency and level of heparanase promoter methylation were analyzed by methylation-specific primers which covered the core binding motif of EGR1 (GGCG) or SPI (GGGCCG) or both.

Results: In cultured Du145, DuPro, LNCaP, and PC-3 cell lines, mRNA transcripts of heparanase were significantly increased after 5-aza-2-deoxycytidine treatment, suggesting that promoter methylation was involved in the regulation of heparanase mRNA transcript. Significantly higher methylation was found in BPH samples than in prostate cancer samples (P < 0.0001), whereas mRNA transcripts of the heparanase gene were inversely lower in BPH samples than in prostate cancer samples (P < 0.01). EGR1 expression in prostate cancer tissues was significantly higher than in BPH tissues (P < 0.001) and correlated with heparanase expression (P < 0.0001). Moreover, multiple regression analysis revealed that up-regulation of EGR1 contributed significantly more to heparanase expression than did promoter CpG hypomethylation in prostate cancer samples (P < 0.0001).

Conclusions: To our knowledge this is the first comprehensive study demonstrating that increased heparanase expression in prostate cancer tissues is due to promoter hypomethylation and up-regulation of transcription factor EGR1.

INTRODUCTION

In the process of tumor invasion and metastasis, degradation of basement membrane and extracellular matrix is an essential step. Several extracellular matrix degrading enzymes, such as matrix metalloproteinases (1) and urokinase-type plasminogen activator (2), have been reported to affect various types of extracellular components of extracellular matrix (3, 4). Heparan sulfate and heparan sulfate proteoglycans are ubiquitously distributed and are major components of extracellular matrix (3, 4). Heparanase, an endo-β-N-glucuronidase, has the ability to cleave the heparan sulfate chain of heparan sulfate proteoglycans and is actively involved in the process of extracellular matrix degradation. Heparanase activity is detectable in platelets, neutrophils, activated T lymphocytes, and various malignancies including esophageal carcinoma (5), pancreatic carcinoma (6), melanoma (7), bladder cancer (8, 9), and prostate cancer (10, 11). In numerous studies, a significant correlation of heparanase overexpression is coupled with increased metastatic potential and decreased survival rates (6, 8, 9, 12, 13). However, the mechanisms underlying increased expression of the heparanase gene in cancer tissues remain unclear. CpG hypermethylation of gene promoters plays a critical role in regulating transcription (14), such as inactivation of tumor suppressor genes, cell cycle regulatory genes (15, 16), and DNA mismatch repair genes (17). On the other hand, a correlation between hypomethylation of promoter regions and transcriptional activation has also been described for several genes such as MAGE (18), S100A4 (19), urokinase-type plasminogen activator (2), and matrix metalloproteinases (1). In this regard, Sato et al. (20) reported that several genes that were overexpressed in pancreatic cancer but not expressed in normal pancreatic tissue had a high prevalence of hypomethylation.

A recent study has shown that early growth response 1 (EGR1) can induce transcription of the heparanase gene (21). In
addition, EGR1 has been reported to be overexpressed in prostate cancer tissues (22) and involved in the pathogenesis and progression of prostate cancer (22). Therefore, we hypothesized that CpG hypomethylation of the heparanase promoter coupled with up-regulated EGR1 levels is related to increased heparanase expression in human prostate cancer. To test this hypothesis, the methylation status of the heparanase promoter, along with expression of heparanase and EGR1, was analyzed in human prostate cancer cell lines and tissues [177 prostate cancer samples and 69 benign prostatic hyperplasia (BPH) samples].

MATERIALS AND METHODS

Clinical Samples. A total of 177 newly diagnosed prostate cancer tissues from radical prostatectomy (136 retropubic and 41 perineal approaches) and 69 pathologically proven BPH samples from transurethral resection (TUR-P) were obtained from Shimane University Hospital (Izumo, Japan). The pathologic background of the prostate cancer patients included Gleason <4: 33 cases, Gleason 5: 33 cases, Gleason 6: 31 cases, Gleason 7: 47 cases, and Gleason >8: 33 cases; pT2: 118 cases, pT3: 56 cases, and pT4: 3 cases. The pathologic findings of PC samples were decided by the general rule for Clinical and Pathological Studies on prostate cancer by the Japanese Urological Association and the Japanese Society of Pathology (23). The median age of prostate cancer and BPH patients was 69 (49-80 years) and 75 (54-87 years), respectively. Our routine strategy to diagnose prostate cancer included serum PSA level, transrectal ultrasonography, color Doppler ultrasonography (24), and MRI, which enabled us to accurately localize prostate cancer before radical prostatectomy. Each tissue sample was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. Sections (5 μm) were used for H&E staining for histologic evaluation. Fresh tissue samples were frozen and stored at −80°C until analyzed.

Cell Lines. The human prostate cancer cell lines Du145, DuPro, LNCaP, and PC-3 were obtained from the American Type Culture Collection (Manassas, VA). All prostate cancer cell lines were maintained in RPMI 1640 with L-glutamine and sodium pyruvate. The cells were maintained in a humidified atmosphere of 5% CO2/95% air at 37°C.

Nucleic Acid Extraction. Genomic DNA was extracted from prostate cancer and control prostate samples using a Qiagen kit (Qiagen, Valencia, CA) after microdissection (25). Total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati, OH). The RNA pellet obtained after isopropanol and ethanol precipitation was dried and resuspended in 50 μL of RNAse-free water. All RNA specimens were subjected to DNase treatment (Wako Pure Chemicals, Osaka, Japan) and stored in aliquots at −80°C until use. The concentrations of DNA and RNA were determined spectrophotometrically and their integrity was assessed by gel electrophoresis.

5′-Aza-2-Deoxycytidine Treatment. Four prostate cancer cell lines used in this study were treated with 5′-aza-2-deoxycytidine (5′-Aza-dC) to screen for epigenetic alteration. 5′-Aza-dC was added to fresh cell culture medium at a concentration of 5 μmol/L in duplicate. The cultured cells were harvested after 4 days of 5′-Aza-dC treatment. The mRNA transcripts of heparanase before and after 5′-Aza-dC treatment were analyzed by reverse transcription-PCR using cDNAs.

cDNA Preparation and Reverse Transcription-PCR Analysis. The cDNA was constructed by reverse transcription (Promega Corp., Madison, WI) using DNase-treated RNA as a template. Samples were stored at −20°C until use. HepaRT1 and HepaRT2 primers were designed to detect the heparanase gene. For differential reverse transcription-PCR, G3PDH1 primer (150 nmol/L each) and HepaRT1 primer (200 nmol/L each) for cell line cDNA or G3PDH2 primer (100 nmol/L each) and HepaRT2 primer (150 nmol/L each) for clinical sample cDNA were used. An annealing temperature of 55°C was used for PCR reactions, and 26 or 28 cycles was used for cell line or clinical samples respectively. Primers used for reverse transcription-PCR analysis are as follows: HepaRT1: 5′-tgctatccaaaggagactac-3′ (sense), 5′-gtagtgtagctgctgagact-3′ (antisense); HepaRT2: 5′-atggcagctgcctggag-3′ (sense), 5′-aggctgcacattccaggg-3′ (antisense); G3PDH1: 5′-cctcctccaccaaccca-3′ (sense), 5′-catcagaagattctggtg-3′ (antisense); G3PDH2: 5′-caatgcctccccaggg-3′ (sense), 5′-tggaagactggctgtgg-3′ (antisense); and EGR1: 5′-gagccagctctagccagggg-3′ (sense), 5′-agccgcagctaggtgg-3′ (antisense). The PCR products were electrophoresed on 2.0% agarose gel and the expression levels were evaluated with ImageJ software (http://rsb.info.nih.gov/ij) by calculating and analyzing the areas under the curves. The expression level of heparanase was quantified relative to G3PDH expression level and expressed as arbitrary units.

Methylation-Specific PCR. Genomic DNA (100 ng) samples were modified with sodium bisulfite using a commercial kit (Invitrogen Life Technologies, San Diego, CA). Using the functional promoter sequence of the heparanase gene (26), several sets of primers were designed to detect the methylation status of CpG islands of the heparanase gene using MethPrimer (http://itsa.ucsf.edu/~urolab/methprimer; ref. 27). The amplified region of Pan1-S and Pan2-AS, in which 37 CpG sites are present, and the relationship of primer locations to the CpG sites are shown in Fig. 1. As for methylation-specific PCR (MSP; ref. 28), a second round of nested PCR assay (MSP-A, MSP-B and MSP-C) was done using the universal PCR product amplified by either primer sets of Pan1-S and Pan1-AS or Pan2-S and Pan2-AS as templates. The first universal primer set (Pan1-S and Pan1-AS or Pan2-S and Pan2-AS) has no CpG sites, and MSP-C) was done using the universal PCR product amplified by either primer sets of Pan1-S and Pan1-AS or Pan2-S and Pan2-AS as templates. The first universal primer set (Pan1-S and Pan1-AS or Pan2-S and Pan2-AS) has no CpG sites in either the forward or reverse primer. Both HPBMU-S and HPCMU-S primers cover both EGR1 and Sp1 sites. In each assay, absence of DNA template served as negative control. Primers used for the MSP and unmethylation-specific PCR (USP) analysis are as follows: Pan1: 5′-tttaaataggtaggtggtggaggtg-3′ (sense), 5′-ctaaataactcactaactccc-3′ (antisense); Pan2: 5′-ttttttaataggtggtggtggaggtg-3′ (sense), 5′-ctaaataactcactaactccc-3′ (antisense); HPAM (MSP): 5′-ttttttaataggtaggtggtggaggtg-3′ (sense), 5′-caatgacctactgtgctg-3′ (antisense); HPAM (USP): 5′-ttttttaataggtaggtggtggaggtg-3′ (sense), 5′-ctaaataactcactaactccc-3′ (antisense); HPBM (MSP): 5′-agagctgtggtggaggtggtggaggtg-3′ (sense), 5′-tccctccggcacttcactgtgctg-3′ (antisense); HPBM (USP): 5′-ttttttaataggtaggtggtggaggtg-3′ (sense), 5′-ctaaataactcactaactccc-3′ (antisense); and HPCM (USP): 5′-agagctgtggtggaggtggtggaggtg-3′ (sense), 5′-tccctccggcacttcactgtgctg-3′ (antisense). The annealing temperature used for Pan1 and Pan2 was 50°C and that for HPB (MSP...
and USP) was 55°C. Touchdown PCR (annealing at 62-56°C) was used for HPA (MSP and USP) and HPC (MSP and USP). The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. With ImageJ software, relative methylation levels (%) were calculated (29, 30) by using the area under the curve corresponding to each band (MSP and USP).

**Bisulfite DNA Sequencing.** One microliter of bisulfite-modified DNA (5 ng) was amplified using a pair of universal primers (Pan1-S and Pan1-AS; Pan2-S and Pan2-AS) in a total volume of 20 μL. Direct bisulfite DNA sequencing of the PCR products using either universal forward or reverse primer (Pan1 and Pan2) was done according to the instructions of the manufacturers (Applied BioSystems, Foster City, CA).

**Statistical Analysis.** Statistical analysis was done with the χ² test, ANOVA test, multiple regression analysis, and Kendall rank correlation and/or Pearson’s coefficient correlation. A P value of less than 0.05 was regarded as statistically significant.

**RESULTS**

**Heparanase mRNA Expression and Promoter Methylation Status of the Heparanase Gene in Cultured Prostate Cancer Cell Lines.** As shown in Fig. 2A, heparanase mRNA transcripts in four prostate cancer cell lines (DU145, DuPro, LNCaP, and PC-3) were significantly increased after 5-Aza-dC treatment compared with untreated prostate cancer cell lines. Paralleling with the alteration in heparanase mRNA transcripts before and after 5-Aza-dC treatment, the intensity of MSP-C band was significantly decreased after 5-Aza-dC treatment. Typical bisulfite DNA sequencing in the DuPro cell line before and after 5-Aza-dC treatment is shown in Fig. 2B. As shown in Fig. 1, the MSP-C region encompasses the consensus motifs of the EGR1 binding site (GGCG) and Sp1 binding site (GGGCGG). In this cell line the −16 CpG site within the EGR1 binding motif was partially methylated (“C” covered with “T”) before 5-Aza-dC treatment, whereas it became completely unmethylated after treatment. On the other hand, the −6 CpG site within the Sp1 binding motif remained completely unmethylated before and after treatment. This result was absolutely compatible with the methylation status analyzed by the MSP-C and USP-C assays shown in Fig. 2B.

**Promoter Methylation of the Heparanase Gene in Human Prostate Cancer and BPH Samples.** MSP and USP were carried out to investigate the methylation status of the heparanase promoter region using prostate cancer and BPH clinical samples. Typical results of MSP and USP assays are shown in Fig. 3A and B. Out of 177 prostate cancer samples, positive methylation was found in 54 cases for MSP-A (30.5%), in 15 cases for MSP-B (8.5%), and in 54 cases for MSP-C (30.5%). On the other hand, in 69 BPH samples, methylation was observed in 51 cases for MSP-A (63.8%), in 46 cases for MSP-B (66.7%), and in 54 cases (78.2%) for MSP-C. The positive methylation in these three promoter regions was significantly higher in BPH samples compared with prostate cancer samples. In prevalence, the correlation of MSP-C methylation with MSP-A or MSP-B methylation reached statistical significance (m² = 5.02, P < 0.05 and m² = 57.5, P < 0.0001, respectively). Typical bisulfite DNA sequencing is shown in Fig. 3C and D. The MSP-C region encompasses the consensus motif of the EGR1 binding site (GGCG) and the Sp1 binding site (GGGCGG). As shown in Fig. 3C, the CpG islands of these consensus motifs are unmethylated in prostate cancer samples (upper panel), whereas these CpG islands are completely methylated in BPH samples (lower panel). Likewise, the other CpG sites in the heparanase promoter are methylated in BPH samples and hypomethylated in prostate cancer samples (Fig. 3D).
Methylation Level of the Heparanase Promoter and mRNA Expression of Heparanase. As shown in Fig. 4A-C, methylation levels (MSP-A, MSP-B, or MSP-C) are significantly higher in BPH samples as compared with prostate cancer samples ($P < 0.0001$). However, the methylation level of MSP-B and MSP-C did not show any positive correlation with either Gleason grade or pathologic stage. The methylation level of MSP-A showed the highest value in Gleason 7 as compared with a lower or higher Gleason grade. Also heparanase mRNA expression is significantly higher in prostate cancer samples than BPH samples (Fig. 4D).

Relationship of Heparanase Expression with Promoter Methylation and EGR1 Expression in Prostate Cancer Samples. As shown in Fig. 5A-C, in prostate cancer samples no significant inverse correlation of methylation at...
MSP-A, MSP-B, or MSP-C site with heparanase mRNA expression was found. On the other hand, a positive correlation of EGR1 expression with heparanase expression reached statistical significance ($P < 0.0001$; Fig. 5D). In this series, mean EGR1 expression was significantly higher in prostate cancer samples than in BPH samples ($P = 0.0013$; Fig. 5E). In order to determine which variables confer the most significant contribution to heparanase expression in prostate cancer samples, we did multiple regression analysis. The result showed that EGR1 expression was more closely associated with heparanase expression than CpG hypomethylation of the heparanase promoter (Fig. 5F).

**DISCUSSION**

Heparanase degrades heparan sulfate, a major constituent of the extracellular matrix and basement membrane, and thus is actively involved in the processes of tumor invasion and metastasis (31–33). Human prostate cancer cells have been shown to produce heparanase (10, 11). In this study increased levels of heparanase mRNA transcripts were found in prostate cancer tissues as compared with BPH samples. Our data is in agreement with the findings in other types of cancer (5, 6, 8–11), but contradictory to the results of Stadlmann et al. (34) showing that mRNA transcripts of the heparanase gene may be lost during dedifferentiation of prostatic gland cells.
Probably, the major difference between our finding and their result is due to the materials from which RNA was extracted. Stadlman et al. (34) used formalin-fixed paraffin embedded materials as a source of RNA, whereas we used fresh tissue. It may be quite difficult to extract RNA from formalin-fixed materials, which is more than 500 bp in length. In turn, this suggests that some of the samples that were defined as being negative for heparanase expression in their experiments could be false-negatives.

The mechanism underlying increased expression of heparanase in cancer tissues remains unclear. In this regard, CpG methylation of the heparanase promoter has been shown to be associated with functional regulation of heparanase in the rat glioma cell line (35). However, similar studies are lacking in human tissues. In the present study, we investigated the expression and methylation status of the heparanase gene in 177 prostate cancer and 69 BPH samples. Initially, the effect of a DNA methyltransferase inhibitor (5-Aza-dC) on heparanase expression and methylation was studied.

Fig. 4  Methylation level of the heparanase promoter and expression level of the heparanase gene. All methylation levels (MSP-A, MSP-B, and MSP-C) are significantly higher in BPH samples as compared with prostate cancer samples ($P < 0.0001$, each), whereas heparanase expression was inversely higher in prostate cancer samples than in BPH samples ($P < 0.0001$). No significant difference in MSP-B, MSP-C levels, and heparanase expression was found among Gleason grades. Samples with insufficient PCR product were excluded from the analysis; (A) 8 samples, (B) 6 samples, (C) 5 samples, and (D) 12 samples.
expression was investigated using four prostate cancer cell lines. In Du145, DuPro, LNCaP, and PC-3 cancer cell lines, expression of heparanase mRNA transcripts was significantly increased after 5-Aza-dC treatment, paralleling the reduction in intensity of the MSP-C band (Fig. 2A and B). Based on these findings, we hypothesized that the functional role of heparanase in human prostate cancer tissue is possibly regulated by aberrant promoter CpG methylation of the heparanase gene.

Prior studies have shown that the proximal promoter region contains putative binding sites for several transcription factors (21, 26, 36). According to the functional promoter region described by Jiang et al. (26), we designed three sets of primers to detect the methylation status of the heparanase promoter. The primer sequences of MSP-A, MSP-B, and MSP-C cover putative transcriptional factor binding site Sp1 and both Sp1 and EGR1 sites, respectively (Fig. 1). The frequency of promoter methylation

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**Fig. 5** Correlation of heparanase gene expression with methylation level and EGR1 expression. There was no significant correlation of heparanase expression with either MSP-A (A), MSP-B (B), or MSP-C (C) level in prostate cancer samples. D, in prostate cancer samples a strong positive correlation was found between heparanase expression and EGR-1 expression ($P < 0.0001$). E, EGR1 mRNA expression in prostate cancer samples is significantly higher than in BPH samples ($P = 0.0013$). F, Multiple regression analysis was used to determine which variables confer the most significant contribution to heparanase expression in prostate cancer samples. EGR1 expression is more significantly associated with heparanase expression than promoter hypomethylation in prostate cancer samples.
and relative methylation levels of MSP-A, MSP-B, and MSP-C are all significantly higher in BPH samples than in prostate cancer samples irrespective of the location of the primers (Figs. 3A and B and 4). To confirm these results, bisulfite-modified DNA sequencing was done. As shown in Fig. 3, our data indicates that CpG sites within the consensus motifs of SP1 and EGR1 are completely methylated in BPH samples, whereas those in prostate cancer tissues are unmethylated. In addition, other CpG sites are more likely to be methylated in BPH samples than in prostate cancer samples. These findings strongly suggest that promoter CpG methylation of the heparanase gene is more prevalent in BPH samples and less so in prostate cancer samples. Coupled with a higher prevalence of promoter hypermethylation in BPH samples than in prostate cancer samples, the expression level of heparanase mRNA transcripts was inversely higher in prostate cancer samples than in BPH samples. Taken together, these results indicate that increased heparanase expression in human prostate cancer is related to promoter CpG hypomethylation.

Recent publications have revealed that transcription factor EGR1 is related to the inducible transcription of the heparanase gene in T cells (21), whereas the ubiquitous transcription factor Sp1 is associated with its basal transcription (26). Core consensus motifs of these transcription factors are [5′-GGCCGG-3′] and [5′-GGCG-3′], respectively, in which one CpG site is present close to the 3′ prime end of each motif sequence (Fig. 1, underlined). The difference in percent methylation levels in MSP-A, MSP-B, and MSP-C when comparing BPH and prostate cancer is statistically significant (P < 0.0001, each), whereas the difference in methylation levels of MSP-B and MSP-C among Gleason grades is minimal and not statistically significant (Fig. 4). If the analysis is limited to the series of prostate cancer samples, an inverse correlation was not found between gene expression and promoter methylation of heparanase gene. Therefore, in prostate cancer tissues the effect of promoter methylation on heparanase transcription seems to be limited. In turn, this suggests that mechanisms other than promoter methylation might be more actively involved in the regulation of heparanase in prostate cancer tissues. We hypothesize that increased levels of EGR1 are required to induce heparanase in prostate cancer tissues in addition to hypomethylation at the EGR1 consensus motif.

Transcription factor EGR1 has been reported to be frequently overexpressed in prostate cancer tissue (22) and closely linked to the pathogenesis and progression of prostate cancer (37). As mentioned above, a significantly lower level of methylation at the EGR1 binding motif has been observed in prostate cancer samples when compared with BPH samples. Furthermore, the present data suggest (a) a significant positive correlation between expression of heparanase and EGR1 is found in prostate cancer samples (P < 0.0001; Fig. 5D), but not in BPH samples, and (b) EGR1 expression is significantly higher in prostate cancer samples than in BPH samples (P = 0.0013; Fig. 5E). This data suggests that heparanase might be induced in response to increased EGR1 expression in prostate cancer tissues, where the methylation level of the promoter is remarkably low. A recent publication has shown that EGR1 interacts with the androgen receptor and promotes tumorigenesis in prostate cancer cell lines (38). However, increased EGR1 expression might contribute to prostate cancer carcinogenesis not only through interaction with the androgen receptor but also by regulation of heparanase expression. Previous publications have shown that methylation of consensus sequences interfered with binding of several transcription factors (39). Also Zhu et al. (40) reported that whereas methylation within the consensus Sp1 binding site did not significantly affect Sp1/Sp3 binding, methylation outside the consensus motif for Sp1 significantly decreased Sp1/Sp3 binding activity. In the present study, as shown in Fig. 4, CpG hypomethylation was observed throughout the heparanase functional promoter in prostate cancer tissue. In addition, the correlation of MSP-C methylation with MSP-A or MSP-B methylation reached statistical significance (P < 0.05 and P < 0.0001, respectively). With these findings, it is very possible that CpG hypermethylation around the EGR1 binding motif within the heparanase promoter could interfere with EGR1 binding activity. Thus, increased heparanase expression in prostate cancer tissue might be caused by at least two different but concomitant events: (a) escape from epigenetic CpG hypomethylation of the heparanase promoter in prostate cancer tissue, especially at the EGR1 binding site, and (b) significant up-regulation of the EGR1 transcription factor in prostate cancer tissue.

In conclusion, this is the first comprehensive study demonstrating that increased heparanase expression in prostate cancer tissues is caused by promoter hypomethylation of the heparanase gene coupled with up-regulation of transcriptional factor EGR1.

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