Heparanase expression is associated with histone modifications in glioblastoma

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Abstract. In this study we investigated epigenetic modifications such as DNA methylation, histone acetylation and histone methylation in the regulation of heparanase expression in glioblastoma. We found that heparanase promoters are differentially methylated among three glioblastoma cell lines; however, all these cells expressed baseline levels of heparanase. 5-Aza-2'-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor, revoked heparanase expression in all the examined cells. Trichostatin A (TSA), a histone deacetylase inhibitor, activated heparanase expression in promoter unmethylated LN229 and T98G cells but not in promoter methylated U251n cells. To identify the mechanisms of heparanase induction by 5-Aza-dC, heparanase expression-related transcription factors were examined. No detected transcription factors (EGR1, Ets1, GABPα and Sp1) were found to be induced either by 5-Aza-dC or TSA. Furthermore, we found that 5-Aza-dC increased acetylation of histone H3 and di-methylation of histone H3 lysine K4 (H3K4me2) in LN229 and T98G cells. The increased histone acetylation and H3K4me2 were also observed in heparanase-expressing tumor tissues by immunohistochemistry staining. Additionally, we found that nuclear factor κB (NFκB) p65 but not NFκB p50 was correlated with heparanase expression, which could be expressed both by neoplastic cells and angiogenesis-related neovessel cells. However, we did not observe any regulatory mechanism between heparanase and NFκB p65 via transient transfection of their cDNA in T98G and U251n cells. We concluded that heparanase expression is associated with histone modifications and promoter DNA methylation plays a role in the control of gene silencing. Overexpression of both heparanase and NFκB p65 may be the result of excessive histone modifications.

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Introduction

Heparanase is a mammalian endo-β-D-glucuronidase that cleaves heparan sulfate side chains of heparan sulfate proteoglycans in basement membrane and extracellular matrix (ECM) (1,2). Heparanase plays important roles in controlling normal and pathological processes, which include tumor invasion and metastasis (3,4). Over-expression of heparanase has been reported in a variety of metastatic cell lines and human tumor tissues. Moreover, increased heparanase expression was found to be correlated with reduced postoperative survival in patients with pancreatic adenocarcinoma and bladder cancer (5,6). Heparanase has also been shown to elicit an angiogenic response by releasing heparan sulfate-bound angiogenic factors sequestered in the ECM (7). Our previous work indicated that heparanase is over-expressed in gliomas and heparanase expression contributes to the neovessel formation and rapid proliferation of tumor cells (8). We found that not all tumor cells express heparanase, which indicates that there are different regulatory mechanisms of heparanase expression among tumors.

Epigenetic modifications are heritable, reversible changes in gene expression that do not result from DNA sequence alterations (9,10). These alterations are very stable and exert a significant impact on the transcriptional regulation of gene expression (11). Among the various epigenetic modifications, the most important components are believed to be DNA methylation and chromatin remodeling such as histone acetylation and methylation. DNA methylation, mediated by a family of DNA methyltransferases, is processed by adding a methyl group to a cytosine residue followed by guanine (CpG). DNA methylation is a potent gene silencing system that is critical for normal embryonic development but becomes deregulated in nearly all tumor cells (12,13). Acetylation and methylation are major post-translational regulators of proteins. Modification of histones (i.e., H3, H4) disrupts the structure of the nucleosome, which leads to DNA relaxation and a subsequent increase in accessibility to transcription factors (14-16). Mostly, lysine (K) residues of histone proteins are subject to modifications. In general, acetylation of histone lysine is associated with increased gene transcription. Lysine methylation including mono-, di- and tri-methylation may have varying results in gene transcription (17-19).

Heparanase expression and its function in tumor invasion have been well studied; however, little is known about the mechanism governing heparanase transcription. In the current

study we investigate the involvement of DNA methylation and histone modifications in the regulation of heparanase expression and test whether glioblastoma, the highest grade glioma, exhibits different epigenetic modifications between heparanase-positive and heparanase-negative tumors. Nuclear factor κB (NFκB) is a eukaryotic transcription factor involved in a wide range of cellular processes in response to stimuli. A study on gastric carcinoma reported a positive correlation between NFκB and heparanase expression (20). We investigated whether NFκB expression is correlated with heparanase expression in glioblastoma and, if so, how NFκB expression would be regulated.

Materials and methods

Cell culture and plasmid transfection. Resected glioblastoma tissues were collected with written consent from patients in accordance with institutional guidelines and graded pathologically according to WHO criteria. Tumors were processed immediately after surgery as described in the literature and single cells were cultured for further experiments (21,22).

LN229, T98G and U251n glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells including primary tumor cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acid at 37˚C in a humidified atmosphere of 5% CO_2.

Human heparanase cDNA was kindly provided by Dr Ian N. Hampson (University of Manchester, UK). Plasmids expressing human NFκB p50 and p65 were obtained from Addgene, Inc. (Cambridge, MA). Plasmids were transfected using the Lipofactamine 2000 reagent (Invitrogen, Carlsbad, CA).

Drug treatments. LN229, T98G and U251n cells were seeded in T25 cell culture flasks in duplicate. When cell growth reached 60% confluence, 5-aza-2'-deoxycytidine (5-Aza-dC, Sigma) was added at final concentrations of 5 and 10 μM. Cell culture medium was replaced daily and fresh 5-Aza-dC was added. Five days after 5-Aza-dC treatment, total cell RNA and protein levels were collected for analysis. For histone acetylation treatment, cells were treated with trichostatin A (TSA, Sigma) at 2 μM for 6 and 24 h.

Methylation-specific PCR (MSP) and bisulfite DNA sequencing. Genomic DNA was extracted using Qiagen FlexiGene DNA extract kit (Qiagen, Valencia, CA) from cultured cells. Genomic DNA (1 μg) was modified with sodium bisulfite using Qiagen EpiTect Bisulfite Kit (Qiagen). Based on the functional promoter sequence of the heparanase gene (23), several sets of primers were designed to detect the methylation status of CpG sites (24,25). Modified DNA (10 ng) was used for MSP reactions. The amplified region covered 459 bp lengths of the heparanase promoter area in which 37 CpG sites are present. A universal primer (Pan) was designed and used to justify the input of PCR reaction. For sequencing of bisulfite-modified DNA, the entire 459 bp promoter was amplified and then cloned using pGEM-T vector systems (Promega, Madison, WI). The inserts were sequenced by an Applied BioSystem automatic sequencing machine (Foster City, CA, USA).

Reverse transcription (RT) and real-time PCR. Total RNAs were extracted using RNeasy mini kit with DNase digestion (Qiagen, Santa Clarita, CA). The cDNA was constructed by TasqMan reverse transcription kit (Applied Biosystems, Branchburg, NJ) and applied for real-time PCR reaction as described previously (26).

Preparation of nuclear extracts. A nuclear extraction kit from Sigma was applied. Briefly, 5-Aza-dC treated T98G and U251n cells were collected, washed and suspended with a cell extract buffer on ice for 15 min. The swollen cells were treated with 0.6% IGEPAL CA-630 for 10 sec, then centrifuged at 10,000 x g for 30 sec. Nuclei pellets were re-suspended with a nuclear extract buffer. After centrifugation, the supernatant was stored at -80˚C.

Western blot analysis. Cells were collected and lysed at the end of each experiment. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). As described previously (27), 10-20 μg of total protein was subjected to SDS-PAGE. Antibodies against heparanase (H-80), NFκB p65, histone H3 and GABPα (H-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against H3K4me2, H3K9me2 and EGR1 (15F7) were the products of Cell Signaling Technology, Inc. (Danvers, MA, USA); antibodies against NFκB p50, Ets1 and Sp1 were purchased from Thermo Scientific (Rockford, IL, USA), Abcam Inc. (Cambridge, MA, USA) and Millipore (Temecula, CA, USA) separately. Antibodies against heparanase (clone HP3/17) were purchased from Cell Sciences (Canton, MA, USA).

Chromatin immunoprecipitation (ChIP) analysis. A MAGnify ChIP system (Invitrogen) was employed in this study and the ChIP was performed using anti-acetylated histone H3 (06-559) and H4 (06-598) antibodies (Millipore, Temecula, CA). In brief, cells were treated with 1% formaldehyde cross-link histones. Then, cells were washed twice using ice-cold PBS. The cell pellets were resuspended in lysis buffer for 10 min on ice and immediately sonicated to obtain DNA fragments. The sonicated supernatants were incubated with 5 μg antibodies or rabbit IgG and protein A/G magnetic beads for 2 h at 4˚C with rotation. After 6 washings in the wash buffer, the cross-linking was reversed by 55˚C heating. DNA was recovered by DNA purification magnetic beads and applied to PCR amplification using the following primer sets: GAPDH promoter sense, 5'-CTG AGC AGT CCG GTG TCA CTA C-3'; GAPDH promoter antisense, 5'-GAG GAC TTT GGG AAC GAC TGA G-3'; HPSE promoter sense, 5'-GGA AAG CGA GCA AGG AAG TA-3'; and HPSE promoter antisense, TCC CAC TCC TCT TCT GCA TC-3'. PCR product was analyzed using agarose gel with ethidium bromide staining.

Immunohistochemistry (IHC) staining. Formalin-fixed, paraffin-embedded tissues were serially cut at 6 μm and stained with hematoxylin and eosin for histomorphological assessment. Slides were deparaffinized with xylene, and hydrated with series ethanol. Antigen retrieval was performed using citrate buffer (pH 6.0). A 4 plus HRP detection system was applied and the chromogen signal was developed with diaminobenzidine (DAB) (Biocare, Concord, CA).

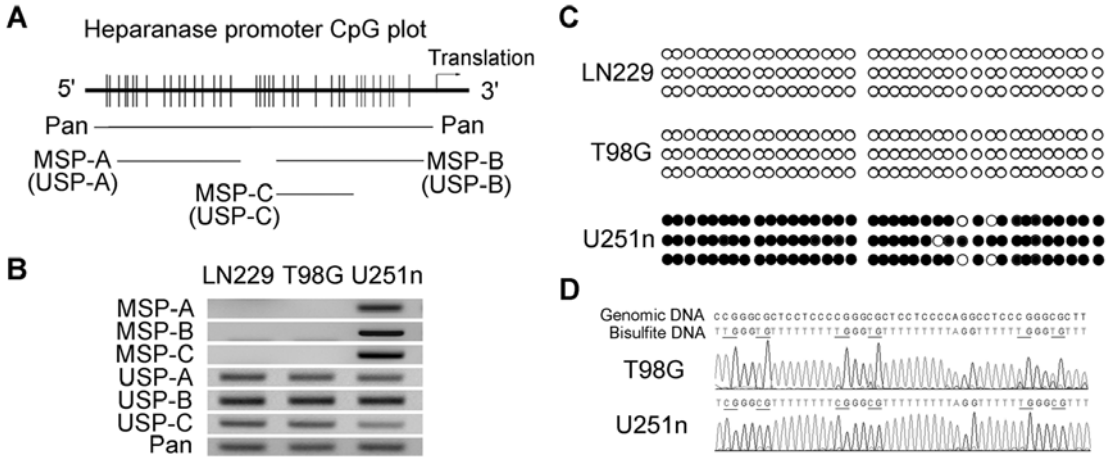

Figure 1. Glioblastoma cells present variant DNA methylation levels at heparanase promoter. (A) The proximal heparanase gene promoter region and its relationship with methylation (unmethylation) detection primers. Vertical marks indicate CpGs in heparanase promoter region. Horizontal lines below CpG plot indicate the location of regions analyzed by methylation and unmethylation-specific PCR (MSP and USP). (B) DNA methylation of heparanase promoter in glioblastoma cell lines as detected by MSP. (C) Detection of DNA methylation at heparanase promoter region by using bisulfite DNA sequencing. Filled circles represent methylated CpGs; open circles represent unmethylated CpGs. (D) Partial typical sequencing results of heparanase promoter using bisulfite genomic DNA of T98G and U251n cells.

Statistical analysis. Data are expressed as the mean ± standard deviation. Each experiment was repeated three times with duplicates. The gene expression data were compared by One-way analysis of variance (ANOVA). A probability value of $p<0.05$ was considered statistically significant between two groups.

Results

Glioblastoma cells exhibit variant DNA methylation at heparanase promoter. To investigate the epigenetic mechanisms in regulation of heparanase expression, we first checked heparanase promoter CpG methylation status in 3 glioblastoma cell lines using MSP. Three sets of PCR primers were designed to cover 14 CpG islands in the heparanase promoter region (Fig. 1A). The MSP results showed that CpG islands in heparanase promoter were highly methylated in U251n cells. No CpG methylations were detected in LN229 and T98G cells. As unmethylation-specific PCR (USP) may amplify both alleles, PCR products could have appeared even if the sense chain was methylated (Fig. 1B). To verify bisulfite PCR results, a 459-bp proximal heparanase promoter was cloned after bisulfite reaction. Three clones of each cell line were selected and applied for sequencing analysis. The sequencing results showed that none of the CpGs in LN229 and T98G cells were methylated (CG→TG) and almost all of the CpGs (2/37, 1/37 and 2/37) in U251n cells were methylated (CG→CG) (Fig. 1C). A typical sequencing result of heparanase promoter is shown in Fig. 1D.

Heparanase expression is restored by epigenetic modifications. To test the possible regulation of epigenetic modifications on heparanase expression, we first treated glioma cells with 5-Aza-dC to decrease DNA methylation. After treating cells for 5 days with 5 μM 5-Aza-dC, heparanase mRNA expression was significantly increased in LN229 and T98G cells. For promoter methylated U251n cells, heparanase expression was only restored when cells were treated with 10 μM 5-Aza-dC for 5 days. In addition to DNA demethylation treatment, we treated cells with TSA, a histone deacetylase inhibitor, to increase histone acetylation. Treatment with 2 μM TSA increased heparanase mRNA expression in promoter unmethylated LN229 and T98G cells, and heparanase mRNA was increased as early as 6 h after TSA treatment. However, TSA treatment did not increase heparanase expression in promoter methylated U251n cells (Fig. 2A). Increased heparanase expression was also detected in protein levels (Fig. 2B). In addition to heparanase induction, we found that 5-Aza-dC and TSA treatment resulted in different outcomes to these tumor cells. TSA treatment caused significant cell death at 48 h, and no cell damage was found in 5-Aza-dC treated cells. Similar results were reported previously in the treatment of cancer cells with epigenetic reagents (28,29).

Decreased CpG methylation of U251n cells by 5-Aza-dC treatment and increased acetylation of histone H3 and H4 by TSA treatment were verified by MSP and ChIP assay. However, TSA treatment did not change DNA methylation at the heparanase promoter (Fig. 2C and D).

5-Aza-dC induced heparanase expression is associated with histone modifications. As heparanase promoter DNA is not methylated in LN229 and T98G cells, 5-Aza-dC induced heparanase expression of these cells is likely through mechanisms other than direct DNA demethylation of heparanase promoter. To address the mechanism of heparanase induction by 5-Aza-dC, we analyzed heparanase-related transcription factors. A literature search revealed that transcription factor Sp1, Ets family member GABP, Ets1, and EGR1 are functionally important in regulating heparanase mRNA expression (23-25,30). We found that only GABPα was induced by 5-Aza-dC, and no transcription factors were actually induced by TSA treatment (Fig. 3A), indicating that increased heparanase transcription by 5-Aza-dC and TSA may not result from up-regulation of transcription factor. We then tested histone modification via 5-Aza-dC treatment. We found that 5-Aza-dC significantly increased acetylation of histone H3 and di-methylation of histone H3 lysine 4 (H3K4me2), indicating

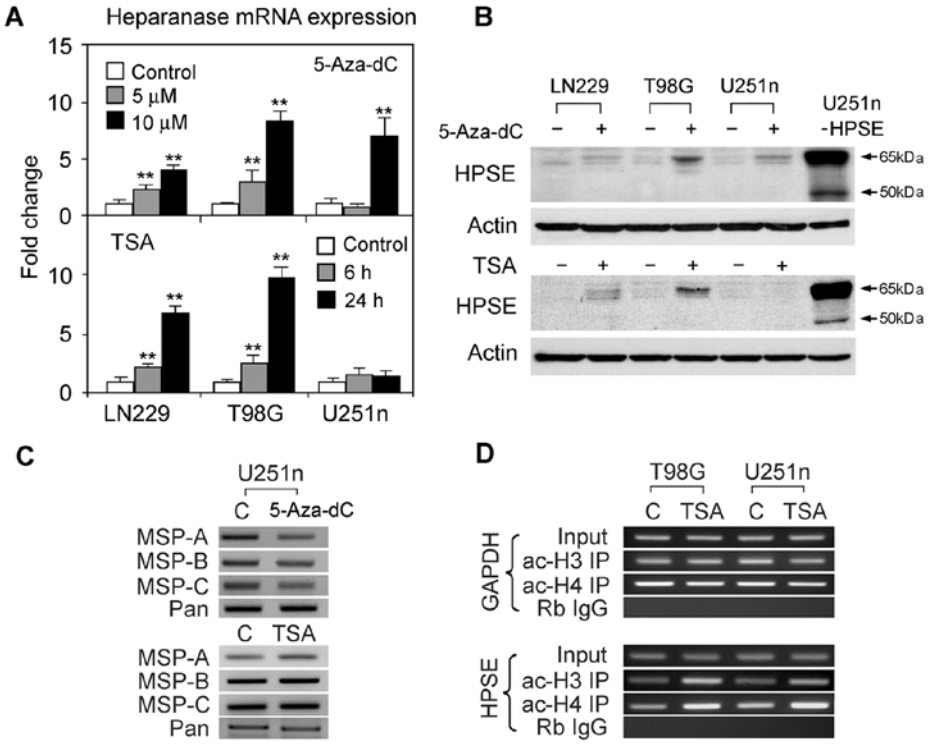

Figure 2. Heparanase expression is restored by epigenetic modifications. (A) Real-time PCR detection of heparanase mRNA expression of glioblastoma cells treated with 5-Aza-dC and TSA. $^{**}P<0.01$, compared with control. (B) Western blot detection of heparanase expression after treatment with 5-Aza-dC and TSA. U251n-HPSE, heparanase cDNA stably transfected U251n cells. (C) Detection of heparanase promoter DNA methylation in U251n cells treated with 5-Aza-dC and TSA using MSP. (D) Analysis of histone acetylation at heparanase promoter in T98G and U251n cells by chromatin immunoprecipitation (ChIP) assay. The above results were obtained from cells treated with 10 μM 5-Aza-dC for 5 days and 2 μM TSA for 24 h.

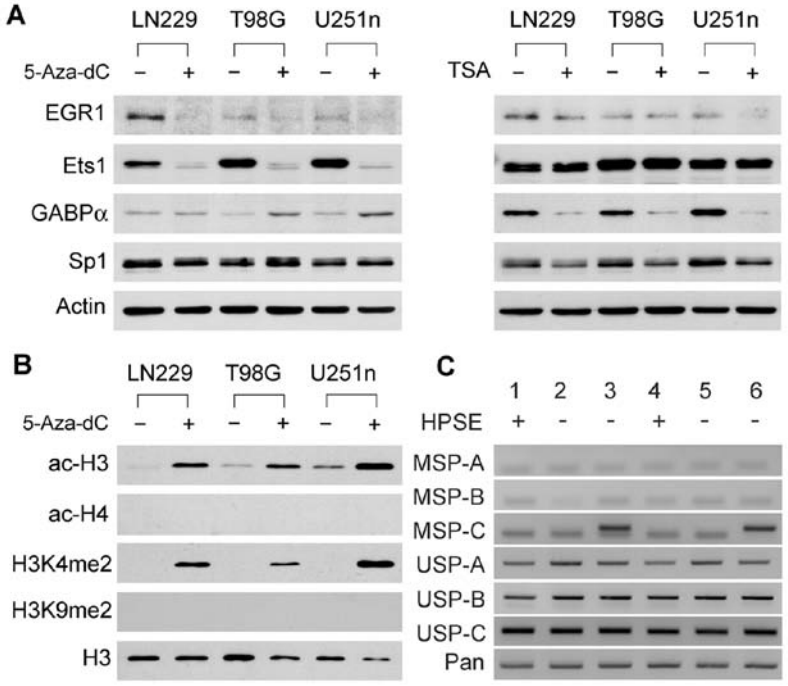

Figure 3. (A) Western blot analysis of heparanase-related transcription factors in glioblastoma cells treated with 5-Aza-dC (10 μM for 5 days) and TSA (2 μM TSA for 24 h). (B) Histone acetylation and methylation analysis of 5-Aza-dC treated tumor cell nuclear extracts by Western blot assay. (C) MSP analysis of heparanase promoter methylation of cultured primary glioblastoma cells within passages 4-7.

that 5-Aza-dC also changes histone modifications and that 5-Aza-dC may induce heparanase through histone modifications (Fig. 3B).

Heparanase promoter DNA methylation and histone modifications in human glioblastomas. To confirm our *in vitro* findings, heparanase promoter methylation was determined in

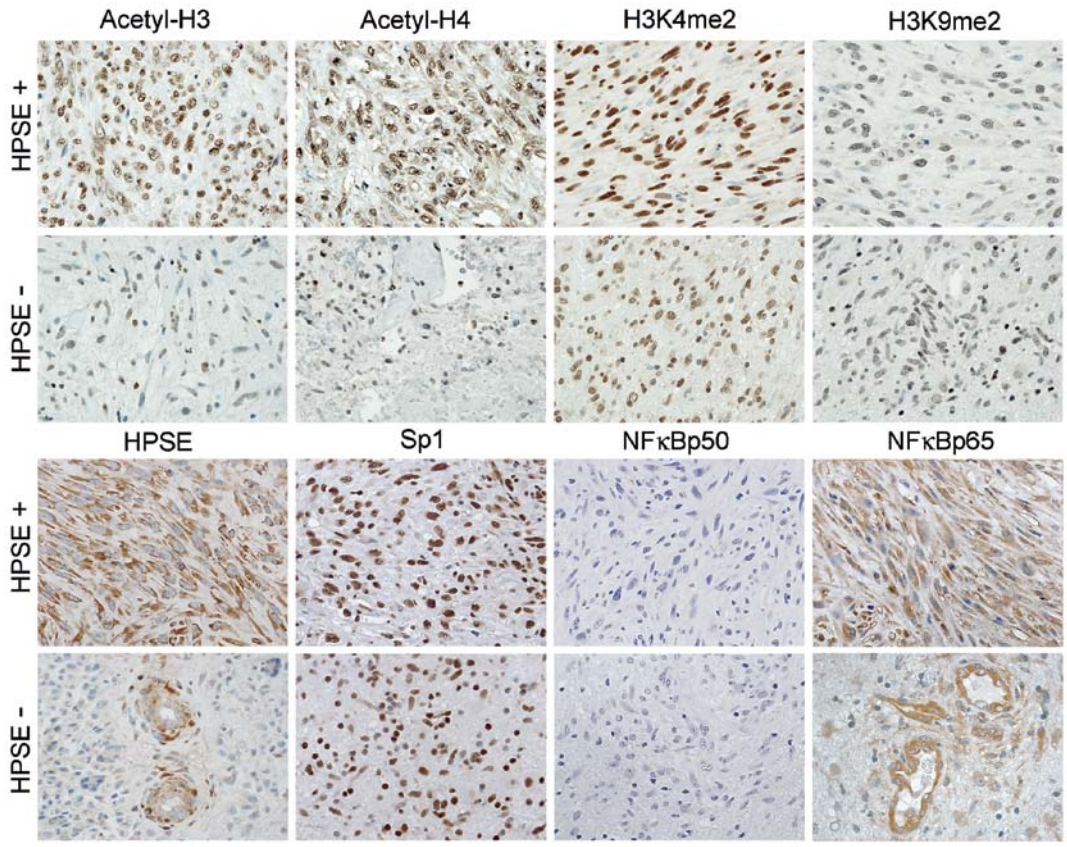

Figure 4. Immunohistochemistry staining of histone modifications and heparanase-related transcription factors in human glioblastoma tissues (x400). $HPSE^+$, neoplastic cells express heparanase; $HPSE^-$, neoplastic cells do not express heparanase.

6 cultured low-passage primary glioblastoma cells (passages 4-7) using the MSP assay. Promoters were partially methylated in samples 3 and 6. Only 2 of the promoter unmethylated tumor cells (samples 1 and 4) express heparanase (Fig. 3C).

Histone modifications were determined by IHC, and staining results were compared between heparanase-expressing tumor and non-heparanase expressing tumor (Fig. 4). Heparanase-expressing tumor cells showed higher staining densities for histone acetylation (acetyle-H3 and acetyle-H4) and H3K4me2 than non-heparanase expressing tumor cells. No difference was observed for H3K9me2 staining between these two tumors. Among the transcription factors, we found only Sp1 expression exhibited a mild increase in heparanase-expressing tumor cells. No significant differences were observed for other examined factors (EGR1, Ets1 and GABPα) (data not shown). Additionally, we found expression levels of NFκB p65 but not NFκB p50 exactly match heparanase expression levels. We previously reported that heparanase could be expressed by neoplastic cells or neovessel cells in glioblastomas. Increased expression of NFκB p65 and heparanase was observed in both of these two cell types.

NFκB p65 expression is not regulated by heparanase but is associated with histone modifications. To investigate the possible regulation between heparanase and NFκB p65, heparanase cDNA was transiently transfected to T98G and U251n cells. We found that expression levels of NFκB p50 and p65 were not increased in heparanase over-expressing tumor cells (Fig. 5A). Although there is no predicted NFκB binding site at the heparanase promoter, we transiently over-expressed NFκB p50 and/or p65 in tumor cells to investigate the possible indirect regulation of NFκB on heparanase expression. The results showed that excessive expression of NFκB p50 and p65 did not induce heparanase expression (Fig. 5B). Interestingly, NFκB p65 but not NFκB p50 was induced both by 5-Aza-dC and TSA treatment in T98G and U251n cells (Fig. 5C).

Discussion

Our results showed that glioblastoma cell lines exhibited variant levels of DNA methylation at the heparanase promoter; however, heparanase expression is not correlated with the methylation status of their promoters. The examined cells evenly express baseline level heparanase *in vitro* including promoter unmethylated LN229 and T98G cells. These results indicate that promoter DNA methylation may effectively control gene silencing but DNA demethylation is insufficient for gene transcription. Compared to DNA demethylation, histone acetylation presented a direct action in activation of heparanase transcription. Nevertheless, TSA treatment failed to induce heparanase expression in promoter methylated U251n cells, suggesting that heparanase expression requires cooperation between DNA methylation and histone acetylation. It has been shown that DNA demethylation and histone modifications play synergic roles in the activation of gene transcription due to the interaction of histone deacetylase (HDAC) with methyl-CpG-binding proteins (MeCP) and DNA methyltransferase (DNMT) (31,32). However, we did not observe that TSA treatment decreased promoter methylation in U251n

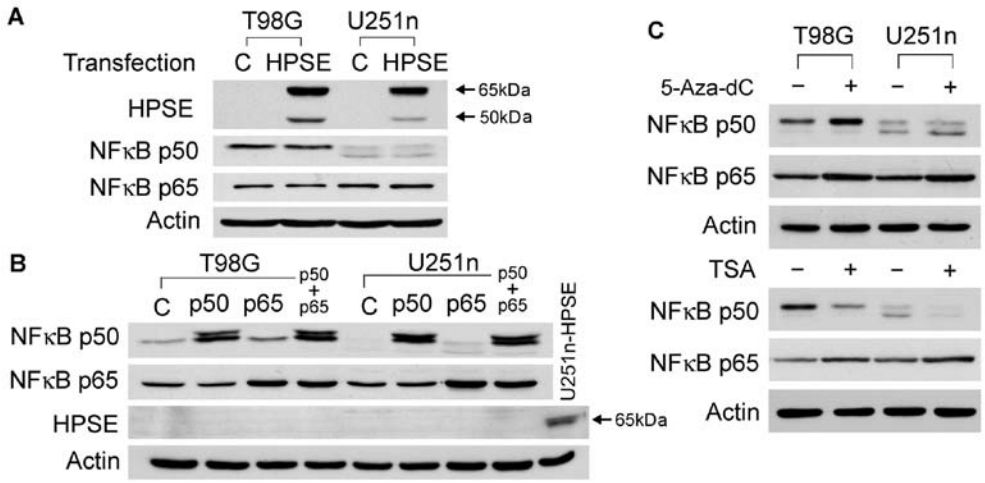

Figure 5. Epigenetic regulation of NFκB p65 expression in glioblastoma cells. (A) Western blot analysis of NFκB p50 and p65 in heparanase over-expressing tumor cells. (B) Detection of heparanase expression in NFκB p50 and p65 transfected T98G and U251n cells. Cells were analyzed 48 h after plasmid transfection. (C) Western blot analysis of NFκB expression of glioma cells treated with 10 μM Aza-dC for 5 days and 2 μM TSA for 24 h.

cells. This may be due to the fact that DNA demethylation is a cell cycle-dependent reaction and 24-h treatment with TSA is not sufficient to reduce DNA methylation, or that TSA-induced cell death interrupted the process of DNA demethylation.

To understand how heparanase transcription was activated especially in promoter unmethylated LN229 and T98G cells by 5-Aza-dC, heparanase-related transcription factors were detected following 5-Aza-dC and TSA treatment. We checked all the transcription factors that have been reported to be correlated with heparanase expression. The transcription factor Sp1 and the Ets family member GABP cooperate in controlling heparanase expression in thyroid tumor cells (23). Two Ets family members (Ets1 and Ets2) were found to regulate heparanase mRNA expression in metastatic breast tumor cells (30). In addition to these *in vitro* studies, human prostate and bladder cancer revealed a correlation between the expressions of EGR1 with heparanase mRNA levels (24,25). We found that only GABPα was induced by 5-Aza-dC treatment and none of the transcription factors was increased after TSA treatment. As GABP regulates gene expression through interaction with the other transcription factors bound to the cognate motifs in the vicinity of the GABP site (23), it is unlikely that 5-Aza-dC-induced heparanase expression is caused by the increased expression of GABPα only. We found that 5-Aza-dC significantly increased acetylation of histone H3 and H3K4me2, indicating that 5-Aza-dC-induced heparanase expression was due to its ability to modify histones. Overall, our findings indicate that histone modification plays a primary role in activation of heparanase expression, while DNA methylation of promoter maintains a dominant role in gene silencing.

Our results from cultured primary glioblastoma cells and immunohistochemistry staining of patient tissues provide supporting evidence that heparanase promoter is differentially methylated among tumors and unmethylated heparanase promoter is not correlated with heparanase expression. Furthermore heparanase-expressing tumor exhibited higher levels of histone modification including histone acetylation and methylation at lysine 4 than non-heparanase expressing tumor, suggesting the importance of histone modification in the regulation of heparanase expression. As a limited number of tumor samples were used in this study, an extensive human glioblastoma study is needed in the future to confirm these findings.

Positive correlation of NFκB p65 and heparanase was first reported in a gastric carcinoma study (20). The increased expression of NFκB p65 and heparanase was also observed in our glioblastoma study. There is no evidence that NFκB can regulate heparanase expression as there are no predicting NFκB binding sites within 3 kb of heparanase promoter. To test the possibility that NFκB may indirectly regulate heparanase expression, two NFκB subunits p50 and p65, which work together to activate gene expression, were transfected in U251n and T98G cells. The results showed that over-expression of NFκB p50 and p65 alone or together did not induce heparanase expression. Similarly, over-expression of heparanase did not induce expression of NFκB p50 and p65 in these cells. However, NFκB p65 but not p50 was up-regulated both by 5-Aza-dC and TSA. The immunohistochemistry staining results showed that over-expressed NFκB p65 is located in cytoplasm but not in nuclei, suggesting that NFκB p65 is upregulated but not activated. NFκB p65 may play a limited role in heparanase-expressing cells.

In conclusion, heparanase expression is associated with epigenetic modification and is cooperatively controlled by histone modifications and promoter DNA methylation. NFκB p65 is correlated with heparanase expression and both heparanase and NFκB p65 may be associated with histone modifications. The different outcomes of 5-Aza-dC and TSA treatment indicate that demethylation and histone acetylation treatment activate different group of genes and pathways. Finding the differences between epigenetic modifications and their interactions will greatly increase our understanding of cancer gene alterations and facilitate the development of epigenetic modification-based cancer therapy.

Acknowledgments

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