

Decreased expression of heparanase in glioblastoma multiforme

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Object. The authors investigated the presence of endoglycosidase heparanase in human glioblastoma multiforme (GBM) and metastatic brain tumors as well as in healthy brain tissue to explore the relationship between the biological characteristics of GBM and the role of heparanase.

Methods. Heparanase messenger (m)RNA was almost undetectable in GBMs in vivo, whereas it was frequently seen in metastatic brain tumors according to results of reverse transcription–polymerase chain reaction (RT-PCR). Immunohistochemical analysis of paraffin-embedded tissue sections showed that neoplastic cells in metastatic brain tumors, especially in cells that invaded blood vessels, exhibit intense heparanase immunoreactivity. Heparanase was present in two highly invasive glioma cell lines, U87MG and U251MG, in vitro. These cell lines did not have metastatic capability, which was tested in an experimental pulmonary metastases model in mice. The activity of heparanase in these cell lines was almost the same as that in the highly metastatic melanoma cell line B16-F1. After nude mice were inoculated with U87MG cells, however, heparanase was no longer detected in subcutaneous or intracerebral experimental glioma in vivo based on results of immunohistochemical analysis. According to results of real-time quantitative PCR, there was a 10-fold increase in heparanase mRNA in U87MG glioma cells in vitro compared with that in experimental U87MG glioma tissue in vivo in nude mice.

Conclusions. These results indicate that the expression of heparanase was downregulated in GBM in vivo, which rarely metastasizes to distant organs outside the central nervous system. Heparanase is not implicated in the invasiveness of GBM to surrounding healthy brain tissue in vivo.

KEY WORDS • glioblastoma multiforme • metastatic brain tumor • metastasis • heparanase

LOCAL tumor cell invasion and distant metastasis through blood vessels are the hallmark of malignant tumors and the major cause of treatment failure in patients with cancer.^{7,22} The diffuse infiltrative and destructive nature of GBM is one of the major obstacles to successful treatment.¹³ It is generally believed that parenchymal metastatic brain tumors are usually well-defined and circumscribed, and thus the local spreading of these tumor cells is relatively limited compared with that of GBM.⁴¹ Many studies have been directed toward understanding both the steps involved in the invasive spread of GBM and the underlying molecular mechanisms.⁴³ Note that GBM rarely metastasizes to distant organs despite its locally invasive nature in the CNS.¹² In general, the molecular mechanisms for local invasion and distant metastasis of malignant tumor cells are believed to be the same. Glioblastomas multiforme

increasingly express proteolytic enzymes, the urokinase-type plasminogen activator/plasmin system of serine proteases and MMPs,^{41–43} and adhesion molecules that have been implicated in metastatic processes in the other malignant tumors; however, GBMs rarely produce distant metastatic foci outside the CNS. What causes these biological differences between local aggressive invasiveness and distant metastatic potential in GBMs? We sought to determine the factors that affect the low metastatic potential of GBM with its locally aggressive invasive nature. It is important to understand the molecular basis of these particular biological characteristics for the development of novel therapies to improve long-term results for not only GBM but also metastatic brain tumors.

In addition to the structural proteins cleaved by MMPs and serine proteases,^{2,4,18,31} other chief components of the basement membrane and ECM are HSPGs. The degradation of HSPGs can influence a variety of normal and pathological processes such as tissue repair, neurite outgrowth, inflammation, autoimmunity, tumor growth and metastases, and angiogenesis.^{3,37} The human endoglycosidase heparanase degrades the HS side chains of HSPGs. Heparanase is expressed in a variety of cells and tissues, such as placenta, skin fibroblasts, platelets, hepatocytes, and endothelial cells,³⁴ and helps to modulate pathophysiological processes

Abbreviations used in this paper: cDNA = complementary DNA; CNS = central nervous system; ECM = extracellular matrix; FAM = 6-carboxyfluorescein; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; GBM = glioblastoma multiforme; HSPG = heparan-sulfate proteoglycan; mAb = monoclonal antibody; MMP = matrix metalloproteinase; mRNA = messenger RNA; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; TAMRA = 6-carboxytetramethylrhodamine; TBS = Tris-buffered saline.

es,³⁰ such as angiogenesis and the extravasation of inflammatory cells in tissue remodeling. Tumor cells may use the same molecular machinery in cancer cell metastasis.^{6,37,38} The step including the attachment and adherence of tumor cells to the basement membrane and ECM of blood vessels as well as the degradation of these tissue barriers is the most important in the early process of tumor cell metastasis, which enables tumor cells to invade blood vessels. After intravasation, tumor cells adhere to the endothelial cells of target vessels and penetrate the basement membrane and stroma to form a metastatic colony in a distant organ.²² heparanase may accelerate tumor cell dissemination by its enzymatic activity, enabling cells to penetrate through the ECM barrier, and by improving tumor cell adhesion to endothelial cells and the subendothelial matrix.⁴⁴ Human heparanase cDNA has recently been identified and sequenced.^{16,36,38} The upregulation of heparanase correlates well with the metastatic potential of tumor cells in various cancers, such as melanoma and breast, hepatocellular, pancreatic, gastric, and colon cancer,^{8,11,16,20,21,24,26,27,29,35,38,39} as well as increased tumor vascularity and poor patient survival.^{14,20,44} Heparanase plays a key role in degrading the stromal tissue and basement membrane of blood vessels to facilitate the invasion of vessel walls by tumor cells and mediates the extravasation of blood-borne tumor cells to form metastatic foci in target organs.

The pathophysiological function of heparanase remains to be elucidated with regard to the invasiveness and metastatic potential of GBM. If heparanase plays an important role in metastasis, its expression might be downregulated in GBM. In the present study, we examined the expression and distribution of heparanase in human GBM and metastatic brain tumor tissues *in vivo* and in glioma cells *in vitro* to determine its role in the metastatic potential and invasiveness in brain tumors.

Materials and Methods

Surgical Tissue Specimens

Fresh human brain tumor tissues and healthy brain tissue adjacent to the tumor were obtained in patients undergoing therapeutic removal of brain tumors and were used for RT-PCR and immunohistochemical study. Healthy brain tissues were available because their excision was necessary for gross-total removal of the tumors. Some samples were flash-frozen in liquid nitrogen immediately after removal and stored at -80°C . Histological diagnosis was confirmed through standard light microscopy evaluation of sections stained with H & E. The classification of human brain tumors used in this study is based on the revised World Health Organization criteria for tumors of the CNS.¹⁹ All of the tumor tissues in the present study were obtained from primary resections, and none of the patients had been subjected to chemotherapy or radiotherapy before resection.

Informed consent was obtained from each patient. All protocols for obtaining human tissues and tumor specimens were approved by the Institutional Review Board of Fukuoka University School of Medicine.

Cell Lines

The human malignant glioma cell lines U105MG, U251MG, and U373MG were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 20 mM L-glutamine, 100 IU/ml penicillin G, and 100 mg/ml streptomycin in an atmosphere of 95% humidified air and 5% CO₂ at 37°C. Both U251MG and U373MG were obtained from Human Science Resources Bank (Osaka, Japan). The three human malignant glioma cell lines U87MG, A172, and T98G;

the melanoma cell line B16-F1; and the human liver cancer cell line SK-HEP-1 were obtained from American Type Culture Collection (Manassas, VA) and routinely cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin G (100 IU/ml), and streptomycin (100 mg/ml) in an atmosphere of 95% humidified air and 5% CO₂ at 37°C.

Extraction of RNA and RT-PCR Analysis

For RNA extraction, cells were grown in 100-mm plates to 80 to 90% confluence. Total RNA from each cell line was prepared from 3 to 5×10^7 cells by using RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN), according to the manufacturer's instructions.⁴¹ Complementary DNA was synthesized from 2.5 μg total RNA with the aid of a cDNA Cycle kit (Invitrogen, San Diego, CA) with random hexamers.

To eliminate possible contamination by neoplastic cells that might have infiltrated surrounding healthy brain tissue when analyzing bulk samples for genetic and biochemical studies, we analyzed heparanase mRNA expression by using RT-PCR of frozen tissue sections that had been defined histologically as tumorous or healthy.⁴¹ Fresh tumor and healthy brain tissues were frozen in liquid nitrogen after they had been embedded in optimal cutting temperature compound (Miles Scientific, Elkhart, IN). Frozen sections containing healthy tissues or tumorous tissues were selected for RT-PCR analysis following microscopic examination of sequential sections stained with H & E. The mRNA was then extracted from 10 sequential 5- μm -thick frozen sections. Polyadenylated mRNA was extracted using the MicroFast Track kit (Invitrogen) in accordance with the manufacturer's instructions. The RNA was primed with random hexamers and reverse transcribed into first-strand cDNA with a cDNA Cycle kit (Invitrogen).

To amplify the cDNA, 2- μl aliquots (for frozen tissue samples) and 0.5 μg (for cell lines) of the reverse-transcribed cDNA were subjected to 40 cycles of PCR in 50 μl 1 X buffer (10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂; 50 mM KCl; 1% gelatin; and 5% dimethyl sulfoxide) containing 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP; 1 U Taq DNA polymerase (Perkin Elmer, Inc., Boston, MA); and heparanase-specific oligonucleotide primers (50 pmol of the sense primer 5'-TTCGATCCCAAGAAGGAATCAAC-3', corresponding to nt 409-431, and 50 pmol of the antisense primer 5'-GTAGTGATGCCATGTAAGTGAATC-3', complementary to nt 970-993).³⁸ Each cycle consisted of denaturation at 94°C for 60 seconds, primer annealing at 55°C for 60 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 7 minutes in a RoboCycler 96 temperature cyler (Stratagene, La Jolla, CA). The efficiency of cDNA synthesis from each tissue sample was estimated through PCR with GAPDH-specific primers. The GAPDH cDNA was amplified with primers corresponding to nt 27-46 (5'-ACG-GATTTGGTCGTATTGGG-3') and complementary to nt 238-257 (5'-TGATTTTGGAGGGATCTCGC-3')⁴¹ under the same conditions as those used for heparanase. Samples of each heparanase PCR product (10 μl) and GAPDH PCR product (10 μl) were electrophoresed on a 1.5% agarose gel and photographed as ethidium bromide fluorescent bands. The PCR procedure was performed at least three times for each sample. After amplification, 40 μl heparanase PCR product was electrophoresed on a 3% agarose gel. The amplified bands were cut out, eluted, and subjected to direct sequencing to confirm the identity of heparanase transcripts by using an automated DNA sequencer (ABI377; Hitachi, Ltd., Tokyo, Japan).

Immunohistochemical Analysis for Heparanase, HSPGs, and CD34 in Brain Tumor Tissues

Sections (4 μm) of fixed tissues embedded in paraffin were mounted on MAS-coated slides (Matsunami Glass, Inc., Osaka, Japan). The slides were dewaxed with a solution containing 3 parts xylene and 5 parts acetone and were treated in a microwave oven at 95°C for 10 minutes. After pretreatment with 0.3% H₂O₂/MeOH, the sections were blocked with 1% nonfat dry milk. The sections were then incubated with a 1:10 dilution of the anti-human heparanase mouse mAb HP-92 (InSight Biopharmaceuticals, Ltd., Rehovot, Israel) overnight in a humidity chamber at 4°C, a process that has been described previously.³⁸ For immunohistochemical localization of HSPGs and

Heparanase expression in brain tumors

CD34, paraffin-embedded tissue sections were incubated with a 1:100 dilution of the mouse anti-human HSPG mAb MAN458 (Chemicon International, Inc., Temecula, CA) overnight at 4°C and a 1:200 dilution of anti-human CD34 mouse mAb (Immunotech, Inc., Westbrook, ME) for 1 hour at room temperature. The appropriate concentration of these primary antibodies was determined by titration against positive control tissues (lung, colon, and breast cancer tissues). These samples were determined to be positive for heparanase mRNA through RT-PCR analysis. After the sections had been washed three times for 10 minutes each in TBS with 0.1% Tween-20, the tissue samples were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) and then with peroxidase-conjugated streptavidin (Dako Corp., Carpinteria, CA). Peroxidase activity was made visible through a 10-minute exposure to diaminobenzidine. Sections were then counterstained in hematoxylin, dehydrated, and mounted for examination. A pink precipitate in the cell cytoplasm indicated positive immunoreactivity. Negative control studies were performed using a nonspecific normal mouse serum as a primary antibody.

Heparanase expression in cryostat sections of healthy brain tissue, GBM, and metastatic brain tumor tissue was detected using an indirect avidin-biotin complex immunohistochemical method. The sections were sequential to the samples analyzed for the expression of heparanase mRNA by RT-PCR. Cryostat sections from frozen tissues embedded in optimal cutting temperature compound were cut at 4 µm, placed onto MAS-coated glass slides, and fixed in 100% acetone for 15 minutes at -20°C. The sections were then blocked with 1% nonfat milk for 1 hour at room temperature and incubated with a 1:10 dilution of the anti-human heparanase mouse mAb HP-92 (InSight Biopharmaceuticals, Ltd.)³⁸ in 2.5% bovine serum albumin in TBS (50 mM Tris-HCl, pH 7.6, and 137 mM NaCl) overnight in a humidity chamber at 4°C. After the sections had been washed three times for 10 minutes each in TBS with 0.1% Tween-20, the tissue samples were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories) and then with streptavidin-alkaline phosphatase (Dako Corp.). Alkaline phosphatase activity was made visible by adding a substrate solution consisting of naphthol AS-BI phosphate, levamisole, and new fuchsin (Sigma Chemical Co., St. Louis, MO). Sections were then counterstained in hematoxylin, dehydrated, and mounted for examination. A pink precipitate in the cell cytoplasm indicated positive immunoreactivity. Negative control studies were performed using a nonspecific normal mouse serum as a primary antibody.

Immunofluorescence for Heparanase

Cells were plated on slide chambers (Miles Laboratories, Inc., Naperville, IL) 48 hours before the experiment. Cells were fixed for 15 minutes with freshly prepared 4% paraformaldehyde. After nonspecific sites had been blocked using 1% goat normal serum in PBS, cells were incubated with the anti-human heparanase mouse mAb HP-92 (InSight Biopharmaceuticals, Ltd.) in 0.1% bovine serum albumin/PBS for 1 hour at room temperature and then stained with fluorescein-conjugated sheep anti-mouse antibody (1:100) for 45 minutes (Amersham Biosciences, Piscataway, NJ). The chromosomes were counterstained with 4',6'-diamino-2-phenylindole-dihydrochloride hydrate (Sigma Chemical Co.). The slides were mounted in FA mounting fluid (BD, Franklin Lakes, NJ) and evaluated with the aid of a fluorescence microscope fitted with a 60 objective.

Heparanase Activity

Heparanase activity was measured in intact tumor cells (5×10^6 cells/35-mm dish), six glioma cell lines (U87MG, U105MG, U251MG, U373MG, A172, and T98G), B16-F1 melanoma cells, and SK-HEP-1 liver cancer cells by using a heparan-degrading enzyme assay kit (Takara Biomedicals, Shiga, Japan). This test is based on the principle that HS no longer binds basic fibroblast growth factor after it is degraded by heparanase.⁹ Materials extracted from the cells were incubated with biotinylated HS solution for 45 minutes at 37°C. This solution was then incubated for 15 minutes at 37°C in a standard 96-well plate containing basic fibroblast growth factor. The biotin signal from the well surface was detected on incubation with peroxidase streptavidin followed by color development.

The standard activity of heparanase was tested using diluted platelet heparanase.

Tumor Metastasis Assay

To evaluate the ability to produce metastases, B16-F1 melanoma cells and U87MG and U251MG glioma cells were trypsinized, washed three times with Dulbecco modified Eagle medium, and allowed to recover for 1 hour. The cells (5×10^4 cells in 0.2 ml PBS) were then injected into the lateral tail vein of C57/BL6 female mice (6–9 weeks old; Kyudo, Ltd., Kumamoto, Japan) by using a 27-gauge latex-free syringe (Becton Dickinson Bioscience, San Jose, CA). The growth of these cells was determined through histopathological analysis of the lung and other organs 28 days after injection. Lung subpleural tumor nodules were counted with the aid of a stereoscopic magnifier after fixation for 5 hours with Bouin solution.^{1,32}

Experimental Glial Tumor

For subcutaneous tumor, cells from exponential cultures of B16-F1 melanoma and U87MG glioma were detached using trypsin and washed with PBS. Cell suspension (1×10^7 cells/0.2 ml PBS) was subcutaneously inoculated in BALB/c female athymic mice (6 weeks old; Kyudo, Ltd.). The mice were killed by cervical dislocation. Xenografts were resected, frozen in liquid nitrogen, and stored at -80°C or fixed in formalin. Paraffin-embedded 4-µm sections were stained with H & E and immunostained with antiheparanase antibodies.

To produce human brain tumor in the brains of BALB/c female athymic mice (6 weeks old; Kyudo, Ltd.), a cell suspension of U87MG glioma cells in PBS was injected into the murine brain.²⁸ Briefly, animals were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg body weight) and immobilized in a stereotaxic apparatus. The cells (5×10^5 cells/5 µl in 1.5% methylcellulose in PBS) were inoculated into the caudoputamen in the right hemisphere (1 mm anterior and 2 mm lateral to the bregma, 3.5 mm deep) of the nude mouse brain over a period of 5 minutes with a Hamilton syringe. The brains were removed 28 days after inoculation, frozen in liquid nitrogen, and stored at -80°C or embedded in paraffin. The tissue sections (4 mm) were stained with H & E, and the tumor was examined microscopically. Immunohistochemical analysis for heparanase was also performed in the paraffin-embedded sections.

The Animal Subjects Committee Institutional Review Board of Fukuoka University School of Medicine approved all protocols for the animal experiments.

Real-Time Quantitative RT-PCR

Total RNA from U87MG glioma cells, frozen specimens of experimental subcutaneous tumor (U87MG), and frozen specimens of experimental brain tumor (U87MG) were analyzed through RT-PCR and real-time quantitative RT-PCR. Total RNA was prepared from cells and tissue specimens by using RNazol B reagent (Tel-Test), according to the manufacturer's instructions. After DNase treatment, total RNA was reverse-transcribed into cDNA by using random hexamers according to the manufacturer's instructions (Invitrogen). Real-time quantitative RT-PCR analysis was performed with an automated sequence detection system (Prism 7700 Sequence Detector; PE Applied Biosystems, Weiterstadt, Germany) combined with a dual-label fluorogenic detection system (TaqMan Universal PCR Master Mix; Invitrogen).^{15,20} The following primers were used: 1) heparanase mRNA, forward primer 5-TCACCATTGACGCCA-ACCT-3, reverse primer 5-CTTTGCAGAACCCAGGAGAT-3, and fluorogenic probe 5-FAM-CCACGGACCCGCGGTTTCCT-3TAMRA; and 2) 7S rRNA, forward primer 5-ACCACAGGTTG-CCTAAGGA-3, reverse primer 5-CACGGGAGTTTTGACCTG-CT-3, and fluorogenic probe 5-FAM-TGAACCGGCCAGGT-CGGAAT-3TAMRA.²⁰ Optimal concentrations of specific oligonucleotide primers and probes were determined to 300 nM. Thermal cycling was initiated with a 20-second incubation at 50°C for uracil N-glycosylase reaction, followed by a 10-minute reaction at 95°C to activate the AmpliTaq Gold, and 45 RT-PCR cycles at 94°C for 15 seconds and 60°C for 1 minute. Experiments were performed in duplicate for each data point. Each PCR run included five standards,

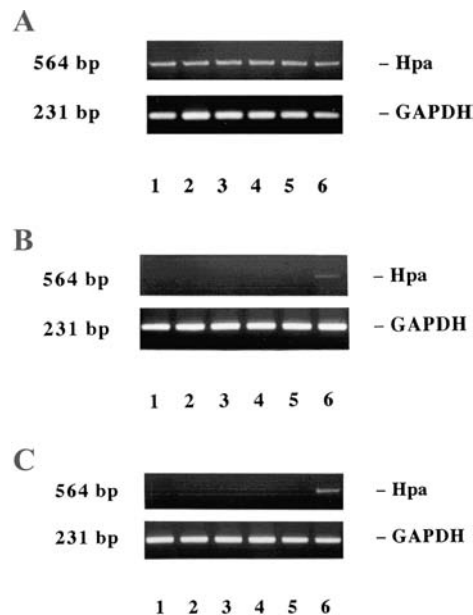


FIG. 1. The expression of heparanase mRNA was analyzed through RT-PCR of frozen tissue sections that had been defined histologically as tumorous. The *upper* gel blot features the results of ethidium bromide staining of a 585-bp PCR product of heparanase mRNA. The *lower* gel blot represents the results of the amplification of GAPDH mRNA. The mRNA was prepared from frozen sections of metastatic lung adenocarcinoma (A, lanes 1–3), metastatic breast carcinoma (A, lanes 4–6), GBMs (B), and healthy brain tissues (C). To evaluate the integrity of cDNA synthesis, GAPDH was amplified separately for each sample by using the same amount of cDNA. Samples of each heparanase PCR product (10 μ l) and each GAPDH PCR product (10 μ l) were electrophoresed on 1.5% agarose gels and photographed as ethidium bromide fluorescent bands. The PCR procedure was performed at least three times for each sample as described in *Materials and Methods*.

one template control, and certain experimental sample points. Standard curves for heparanase were generated using a cDNA synthesized from a serial 1:5 dilution of mRNA from an expression vector encoding full-length heparanase cDNA.³⁸ A relative standard curve was constructed using seven standard Ct (threshold cycle) values as y-axis values and the log of the input standard RNA amount (copy number). The normalized amount of heparanase was determined by dividing the amount of heparanase mRNA by the amount of 7S mRNA for each sample.

Statistical Analysis

The Student t-test was used for a statistical analysis of the real-time quantitative RT-PCR. All of the data were analyzed using a commercially available statistical package (SPSS, version 12.0J; SPSS, Inc., Chicago, IL). A probability value less than 0.05 was taken as the level of significance for all tests.

Results

Detection of Heparanase mRNA by RT-PCR Analysis in Human Brain Tumors In Vivo

The expression of heparanase mRNA was evaluated through RT-PCR of cDNA prepared from frozen sections of histologically defined healthy and tumorous brain tissues. This method circumvented any possible problems with tumor cells infiltrating or invading surrounding healthy brain

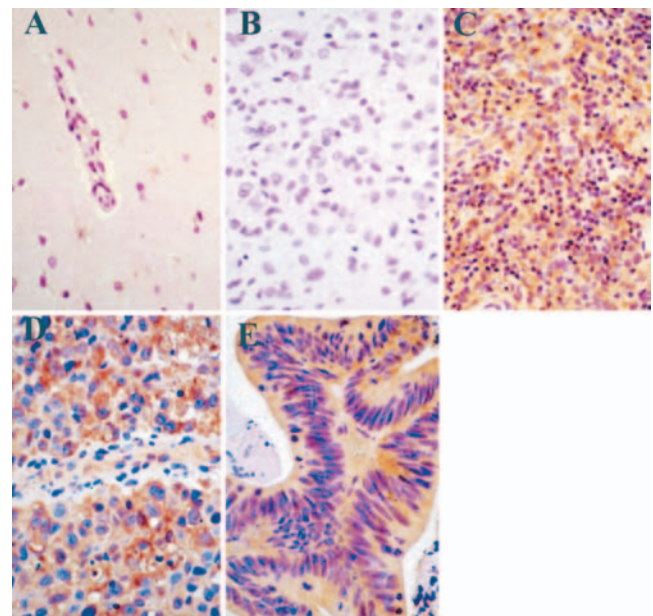


FIG. 2. Paraffin-embedded sections from healthy adjacent brain tissues, GBMs, and metastatic brain tumors were stained with heparanase mAb. Representative photomicrographs for each histological group: healthy white matter (A), GBM (B), metastatic lung adenocarcinoma (C), metastatic breast carcinoma (D), and metastatic colon carcinoma (E). Original magnifications \times 130.

tissue during analysis of adjacent healthy brain samples, whereas tumor biopsies or bulk samples may contain various proportions of tumors and healthy brain tissues.⁴⁰ Oligonucleotide primers in this study were used to amplify 585 bp cDNA for heparanase. No gene products of interest were amplified using PCR when mRNA extracts from these tumor tissues were incubated in reverse transcriptase reactions without reverse transcriptase (data not shown). Direct sequencing analyses of RT-PCR products showed that heparanase transcripts were identical. The PCR amplification of cDNA prepared from frozen tissue samples defined histologically as metastatic lung adenocarcinoma, metastatic breast carcinoma, GBM (Grade IV), and healthy brain tissue demonstrated the presence of the heparanase transcript. The results of cases in which we examined the expression of heparanase mRNA in the same samples are shown in Fig. 1. Amplified 585-bp bands corresponding to the size of amplified heparanase were obtained through RT-PCR in three of three metastatic lung adenocarcinomas (Fig. 1A, lanes 1–3) and three of three metastatic breast carcinomas (Fig. 1A, lanes 4–6). In marked contrast, heparanase mRNA could not be detected on RT-PCR analysis in GBM (Fig. 1B) or healthy white matter adjacent to the tumor (Fig. 1C). Amplified heparanase (585-bp bands) were obtained through RT-PCR in one (7.1%) of 14 GBMs (Grade IV) and in one (16.6%) of six healthy brain tissues. The undetectable heparanase mRNA amplification product in these samples was not related to the absence or quality of mRNA because the presence of intact mRNA in these specimens was demonstrated by amplification of the constitutively expressed GAPDH gene (Fig. 1). These tissues showed strong amplification at levels similar to those generated by the metastatic brain tumor samples. We conclud-

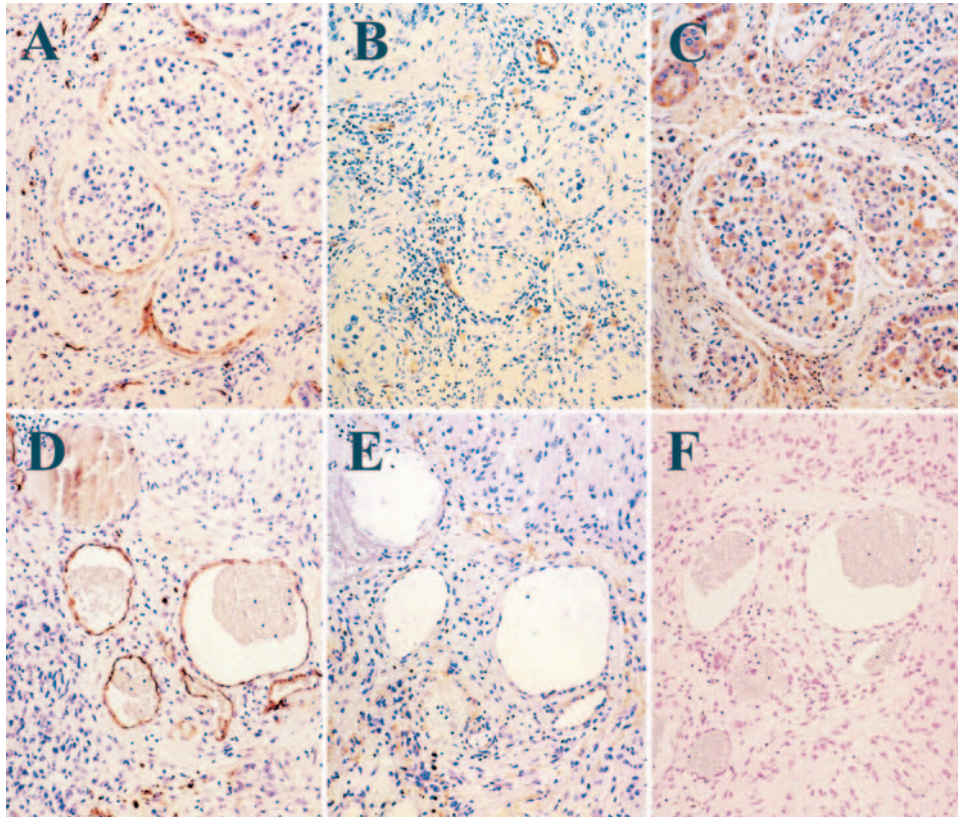


FIG. 3. Paraffin-embedded sequential tissue sections from metastatic brain tumor and GBM stained with CD34 (A and D, respectively), HSPG (B and E, respectively), and heparanase mAb (C and F, respectively). Representative photomicrographs for each histological group: metastatic lung carcinoma (A–C) and GBM (D–E). The blood vessels were identified through CD34 immunostaining in metastatic lung adenocarcinoma (A) and GBM (D), where HSPG was also expressed in the wall of the blood vessels (B and E). There was marked vascular invasion in the metastatic lung adenocarcinoma (C), where the tumor cells strongly expressed heparanase. Note, however, that there were no signals in GBM (F). Original magnifications $\times 130$.

ed that the expression of heparanase mRNA was significantly increased in metastatic brain tumors and that heparanase mRNA was rarely detected in the healthy white matter tissue and GBM examined in this study.

Immunohistochemical Staining of Heparanase in Human Brain Tumors and Healthy Brain Tissues

To detect the expression of heparanase and to identify the cells that express heparanase, immunohistochemical analysis of paraffin-embedded tissue sections was performed. Antibody against heparanase showed undetectable staining in healthy white matter tissue (Fig. 2A) and in GBM (Fig. 2B). On the other hand, heparanase was strongly expressed in tumor cells of metastatic lung adenocarcinoma (Fig. 2C), metastatic breast carcinoma (Fig. 2D), and metastatic colon carcinoma (Fig. 2E). Immunohistochemical staining of heparanase was detected in 12 of 19 metastatic lung carcinomas, six of six metastatic breast carcinomas, and two of two metastatic colon adenocarcinomas. In marked contrast, heparanase was not detected in 20 of 20 GBMs and 12 of 12 healthy brain tissue specimens. There was no detectable staining of metastatic brain tumors in the absence of the primary antibody (data not shown). Tissue sections of primary colon adenocarcinoma and breast carcinoma, which previ-

ously have been reported to express heparanase,³⁸ were used as a positive control in immunostaining for heparanase. In GBM and healthy white matter, tumor cells or healthy astrocytic cells showed no detectable heparanase immunoreactivity, as indicated in Fig. 2A and B, which is consistent with the results of the PCR analysis.

Given that GBM from one patient and healthy white matter from another positively expressed heparanase mRNA by PCR (Fig. 1B, lane 6 and Fig. 1C, lane 6), the expression of heparanase was further examined through immunohistochemical analysis of frozen sections that were sequential to those used for RT-PCR analysis. Heparanase immunoreactivity was not detected in nine cases of GBM (Grade IV) or in six cases of healthy brain tissue, whereas six of seven metastatic brain tumor samples displayed heterogeneous and intense immunoreactivity with the heparanase antibody (data not shown). There was no detectable staining of the tumor in the absence of the primary antibody or with a nonspecific normal mouse serum as a primary antibody. These findings indicated that the sensitivity of the detection system for mRNA or protein of heparanase led to a slight difference in the results between RT-PCR and immunohistochemical analysis or that a small amount of heparanase mRNA may not be translated to protein in GBM or healthy brain tissue. Nonetheless, healthy white matter dis-

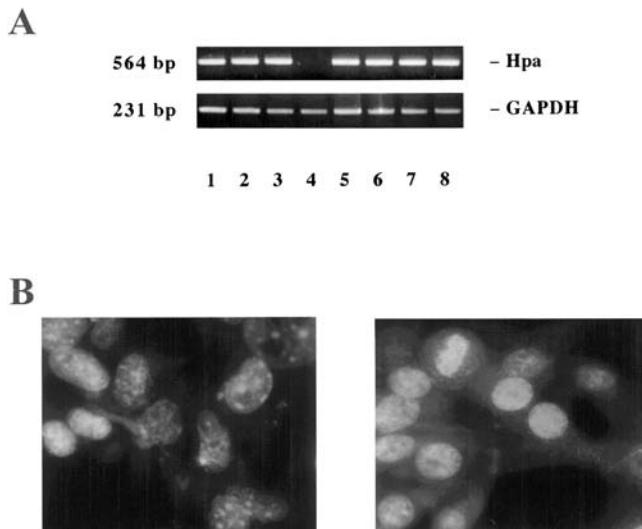


FIG. 4. A: Gel blot demonstrating the differential expression of heparanase mRNA in human glioma cell lines. Equal amounts of total RNA (2.5 μ g) derived from human glioma cell lines and human melanoma and hepatocellular carcinoma cell lines were reverse-transcribed into cDNA as described in *Materials and Methods*. The cDNAs (0.5 μ g) were amplified by 40 cycles of PCR. The upper gel blot shows ethidium bromide staining of a 585-bp PCR product of heparanase mRNA. The lower gel blot shows amplification of the GAPDH mRNA. Samples of each heparanase PCR product (10 μ l) and each GAPDH PCR product (10 μ l) were electrophoresed on 1.5% agarose gels and photographed as ethidium bromide fluorescent bands. Lane 1, U87MG glioma cell; lane 2, A172 glioma cell; lane 3, T98G glioma cell line; lane 4, U105MG glioma cell line; lane 5, U251MG glioma cell line; lane 6, U373MG glioma cell line; lane 7, B16-F1 melanoma cell line; and lane 8, SK-HEP-1 hepatocellular carcinoma cell line. B: Immunofluorescent staining for heparanase was performed using the mouse anti-human heparanase antibody HP-92, followed by fluorescein-conjugated sheep anti-mouse immunoglobulin G. The chromosomes were counterstained using 4,6'-diamino-2-phenylindole-dihydrochloride. *Left*: Positive control, B16-F1 melanoma cell line. *Right*: The U87MG glioma cell line. Negative control included the omission of the specific antibodies and showed no staining. Original magnifications \times 750.

played no immunoreactivity, which indicates that astrocytes and endothelial cells in healthy cerebral white matter do not express heparanase under normal conditions, similar to GBMs.

Immunohistochemical Staining of HSPG and CD34 in Human Brain Tumors and Healthy Brain Tissues

The expression of HSPG, a substrate of heparanase, was determined through immunohistochemical studies. The blood vessels were identified in tumor tissues by performing CD34 immunostaining, and the expression of HSPG and heparanase were examined at the region of vascular invasion of tumor cells in sequential paraffin-embedded tissue sections. Metastatic lung adenocarcinoma demonstrated vascular invasion of tumor cells (Fig. 3A) where HSPG was strongly expressed (Fig. 3B). Tumor cells that invaded blood vessels also expressed heparanase (Fig. 3C). In GBM, there were vascular endothelial proliferations (Fig. 3D), and HSPG was also expressed in the vascular walls (Fig. 3E). In marked contrast, neither the vascular invasion

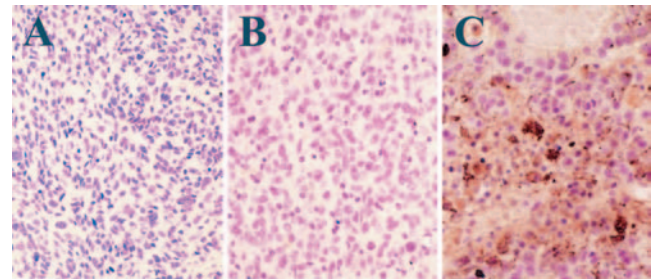


FIG. 5. Photomicrographs of formalin-fixed and paraffin-embedded sections from a subcutaneous xenograft (A) and an intracerebral xenograft (B) of U87MG glioma cells and a subcutaneous xenograft (C) of B16-F1 melanoma cells. The immunostaining signal of heparanase was abundantly expressed in the cytoplasm of tumor cells in the xenograft of B16-F1, whereas signals were faint or absent in the xenografts of U87MG glioma cells. Original magnifications \times 130.

of tumor cells nor the expression of heparanase occurred in tumor cells (Fig. 3F). Furthermore, HSPG was detected in the wall of blood vessels in healthy white matter as well as in metastatic breast carcinoma and metastatic colon adenocarcinoma (data not shown).

Expression of Heparanase in Glioma Cell Lines

Results of immunocytochemical studies for heparanase demonstrated that healthy and neoplastic astrocytes do not express heparanase *in vivo*. To confirm this notion, heparanase expression was analyzed in glioma cell lines. Heparanase mRNA was examined in six human glioma cell lines. One melanoma cell line (B16-F1) and one hepatocellular carcinoma cell line (SK-HEP-1) were used as positive controls (Fig. 4A).³⁸ Heparanase mRNA was present in all but one glioma cell line (U105MG; Fig. 4A, lane 4). Heparanase activity was also measured using a biotinylated HS substrate for heparanase. The assay is highly sensitive and, according to the manufacturer, is capable of measuring enzyme activities as low as 0.1 U/ml in a given sample. Heparanase was detected in U87MG (0.41 U/ml) as well as in the melanoma cell line B16-F1 (0.50 U/ml), although it was not detected in U105MG. In the four other glioma cell lines, low levels of heparanase activity were found: U251MG, 0.26 U/ml; U373MG, 0.28 U/ml; A172, 0.27 U/ml; and T98G, 0.13 U/ml. Immunofluorescent cytochemical analysis of the human glioma cell line U87MG for heparanase was performed using a mouse anti-human heparanase mAb, followed by fluorescein-conjugated sheep anti-mouse immunoglobulin G (Fig. 4B). We found that heparanase was distributed in the cytoplasm in U87MG (Fig. 4B right) as well as in B16-F1 melanoma cells (Fig. 4B left), which were used as a positive control.

Lung Colonization by Glioma Cells

The ability of glioma cells, which were shown to express heparanase in this study, to produce metastases was examined through experimental lung colonization with U87MG and U251MG. The B16-F1 melanoma cells, which were shown to have high metastatic capability,²⁵ were used as a positive control. There were multiple lung metastases of B16-F1 melanoma cells in 10 of 10 mice, whereas metastases were not detected for U87MG (zero of five mice) or U251MG (zero of five mice). These results indicated

Heparanase expression in brain tumors

that heparanase alone is insufficient to enhance the process through which tumor cells adhere to endothelial cells and cross the wall of the target vessels in tumor metastasis and that additional factors may be involved in this process.

Heparanase Expression in Experimental Tumors in Mice In Vivo

Different results regarding the expression of heparanase were seen in human GBM tissue and a human glioma cell line in this study. Heparanase was detected in glioma cell lines *in vitro*, but not in GBM tissue *in vivo*. The expression of heparanase might be accelerated in the *in vitro* culture environment. To examine this hypothesis, changes in the expression of heparanase were examined in experimental murine brain and subcutaneous tumors, in which heparanase-expressing tumor cells had been inoculated. The U87MG cell line, which has previously been shown to have tumorigenicity in mice,²⁸ was inoculated through subcutaneous and intracerebral stereotactic implantation, with B16-F1 melanoma cells as a positive control. Each experimental group consisted of more than five animals. Although heparanase was not detected in either subcutaneous tumor (Fig. 5A) or intracerebral tumor (Fig. 5B) of U87MG, it was strongly expressed in the subcutaneous tumor of B16-F1 (Fig. 5C).

The expression of heparanase mRNA was also examined using RT-PCR. It was detected in subcutaneous tumor (Fig. 6A, lane 1) and intracerebral tumor (Fig. 6A, lane 2) of U87MG as well as in the subcutaneous tumor of B16-F1 melanoma cells (Fig. 6A, lane 3). The expression of heparanase mRNA was quantified through real-time quantitative PCR, and the actual real-time RT-PCR curve is featured in Fig. 6B. Compared with U87MG cells *in vitro*, heparanase mRNA levels were decreased 10-fold in subcutaneous tumor and intracerebral tumor of U87MG cells *in vivo* (Fig. 6C; $p < 0.001$). These results indicated that the expression of heparanase in a U87MG experimental tumor was decreased *in vivo*.

Discussion

In this study, we found that heparanase was rarely expressed in human GBM *in vivo*. The HSPGs, which act as tissue barriers, were detected in the wall of blood vessels in both healthy white matter and GBM, as described previously.¹² On the other hand, heparanase and its mRNA were frequently and increasingly expressed in the cells of metastatic brain tumors (lung, breast, and colon carcinomas), especially in the lesion of vascular invasion by tumor cells *in vivo*. There was also a remarkable difference between the *in vitro* and *in vivo* expression of heparanase in glioma. Heparanase was detected in five glioma cell lines in various amounts. We investigated the expression of heparanase in experimental subcutaneous and intracerebral tumors of mice that had been inoculated with the glioma cell line U87MG to determine whether this tumor cell still produces heparanase *in vivo*. The U87MG cell line showed heparanase expression *in vitro*, but only a small amount of mRNA was found in xenografts. The results of an immunohistochemical study and a real-time PCR analysis revealed that the expression of heparanase in U87MG cells was down-regulated *in vivo* or, alternatively, the expression of hepara-

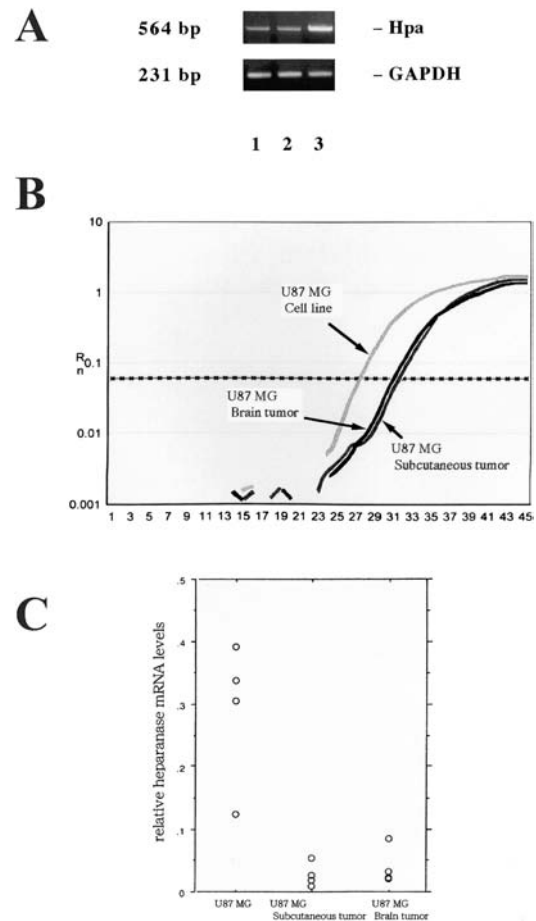


FIG. 6. A: Gel blots demonstrating the expression of heparanase mRNA in subcutaneous (lane 1) and intracerebral (lane 2) tumor of U87MG cells and in subcutaneous (lane 3) tumor of B16-F1 melanoma cells. B: Graph demonstrating the expression of heparanase mRNA as quantified by real-time quantitative PCR. C: Graph displaying results of real-time quantitative PCR analysis, expressed as the ratio of the values for heparanase and 7S in each sample.

nase was enhanced *in vitro*. The U87MG cells exhibited a mean 10-fold increase in heparanase mRNA compared with the xenografts of U87MG cells in brain and subcutaneous preparations. These findings indicated that heparanase expression might be regulated at distinct steps, either at the transcriptional level by a mechanism governing message stability or at a posttranscriptional level. Low-density lipoprotein receptor-related protein reportedly is expressed in the *in vitro* tissue culture environment, shows reduced expression *in vivo*,⁵ and may function in a variety of distinct physiological processes including cancer cell invasion and metastasis.⁴⁰ It is reasonable to conclude that the expression of heparanase is regulated differently in various cell types, and its expression in malignant tumors depends on the origin of the tumors and the heterogeneity of heparanase production in the tumors. In contrast to our results, Zetser, et al.,⁴⁴ demonstrated that the U87MG cell line expressed only low levels of heparanase. This difference might be a consequence of the proliferative state of the cells *in vitro*, the passage number, and the culture conditions. Tissue culture con-

ditions may also affect the expression of heparanase in vitro in glioma. A further understanding of the mechanisms involved in increasing the expression of heparanase in glioma cell lines in vitro will require additional studies.

Human malignant glial tumors constitute the majority of primary intracranial tumors, which are often characterized by rapid growth and aggressive invasion into surrounding healthy brain tissue.¹³ Malignant tumor cell migration and invasion clearly involve a complex interplay of multiple proteolytic enzymes and their inhibitors.²² Many proteolytic enzymes, such as urokinase-type plasminogen activator⁴² and MMPs,⁴¹ have been found to be closely associated with the invasive phenotype of glioma. Glioblastoma multiforme invades healthy white matter more aggressively than does metastatic brain tumor, whereas GBM rarely metastasizes to organs outside the CNS.³³ To explore the mechanism of this biological phenomenon, we investigated the expression of heparanase in GBM in this study. Our results demonstrated that HSPG is expressed in blood vessel walls in GBMs as well as in healthy white matter, as previously reported.¹² The HSPGs are concentrated mainly in subendothelial basement membrane and help to protect against invasion into the blood vessel wall and the extravasation of blood-borne tumor cells.³⁷ In this study, vascular invasion was seen in metastatic brain tumors where heparanase was highly expressed, whereas the tumor cells rarely invaded the vascular wall in GBM. Extracellular matrix-degrading enzymes break down tissue barriers, thus enabling tumor cells to invade through stroma and blood vessel walls.³⁷ Cancer metastasis is produced by the concerted sequential action of matrix-degradation enzymes, such as MMPs, serine and cysteine proteases, and heparanase. Heparanase is required for the degradation of other major components of the ECM and basement membranes, that is, HSPGs, whose enzymatic activity might be involved in the detachment and escape of tumor cells from the primary site.²⁰ Heparanase was first reported to be a human endoglycosidase, which degrades HSPGs in melanoma cells.^{26,39} In studies of cell lines with different metastatic capacities, the ability of heparanase to degrade HSPGs has been shown to be associated with metastatic potential.^{27,29,39} Heparanase plays a key role in degrading the stromal tissue and basement membrane of blood vessels to enable invasion of tumor cells into vessels and in mediating the extravasation of blood-borne tumor cells to form metastatic foci in target organs.^{6,10,16,38} Glioblastoma multiforme cells could not penetrate the barrier that consisted of HSPGs in the basement membrane of the vascular wall in the brain because of a lack of heparanase expression, which might be one of the factors explaining the low metastatic potential of GBM in vivo. The mechanisms for the invasion and migration of malignant glial tumors in local white matter might be different from those of extracranial tumors, mainly because of the profoundly different composition of the extracellular environment within the brain.¹² Heparanase might not be implicated in the invasiveness of GBM into surrounding healthy brain tissue in vivo, given that GBMs invade healthy white matter more aggressively than do metastatic brain tumors without heparanase expression. We are now conducting a study of human heparanase gene overexpression in the U87MG human glioma cell line to confirm this conclusion; that is, heparanase overexpression increases cellular invasion and metastasis in GBM.

Heparanase was also not found in healthy brain tissues in

this study, which was consistent with data from a previous report.⁶ Inflammatory conditions induce both the release and activation of heparanase in platelets and leukocytes.^{6,17,29} Purified astrocytes from rat or mouse cerebral cortices express heparanase, and this expression is upregulated by nerve growth factor or neurotrophin-3.²³ Coculture with purified astrocytes and brain-metastatic melanoma cells stimulated heparanase activity and cellular invasion by melanoma cells, indicating that astrocytes may significantly contribute to the brain colonization of melanoma cells via heparanase secretion from astrocytes as a response to invasion.²³ The invasion of metastatic tumor cells into the brain parenchyma causes the same inflammatory events as mechanical or chemical brain injury.²³ Heparanase has also been shown to facilitate cellular invasion associated with autoimmunity and inflammation.^{37,44} When astrocytes adjacent to the site of neoplastic invasion are activated by nerve growth factors, metastasis might be stimulated by neurotrophins to produce heparanase. The role of heparanase in signal transduction pathways has been reported: heparanase activates FAK, Akt phosphorylation, and Rac activation, which modulates tumor development and progression as well as tumor cell dissemination.⁴⁴ This activation depends on heparanase expression level.

Conclusions

Analysis of our results indicate that heparanase is not expressed in GBM in vivo, which rarely metastasizes to distant organs outside the CNS. The finding of a high expression of heparanase in metastatic brain tumors, especially in the region of vascular invasion, could shed new light on how to prevent cancers in other organs from metastasizing to the brain. Heparanase-inhibiting molecules, including various nonanticoagulant heparan derivatives, may be able to reduce the incidence of brain metastasis.

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