

Modulation of cystatin C expression impairs the invasive and tumorigenic potential of human glioblastoma cells

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Increases in the abundance of cathepsin B transcript and protein with increased tumor grade and changes in subcellular localization and activity of this enzyme. We observed progressive reductions in levels of the protease inhibitor cystatin C, an inhibitor of cathepsin B with corresponding increases in the malignancy of glioma cell lines, implying an inverse correlation between cystatin C and tumor grade. To investigate the role of cystatin C in the invasion of brain tumor cells, we stably transfected SNB19 glioblastoma cells with either a 0.4-kb cDNA construct of human cystatin C in the sense orientation or an empty vector. Clones expressing sense-cystatin C cDNA had higher cystatin C mRNA and protein levels than did control cells. Sense-transfected cells were also markedly less invasive than control cells in a Matrigel invasion assay and in a coculture assay of SNB19 spheroids and fetal rat brain aggregates. Finally, the sense-transfected cells did not form tumors in nude mice upon intracerebral injection. These results strongly implicate cystatin C in the invasiveness of human glioblastoma cells and suggest that sense transcripts of cystatin C may prove useful in cancer therapy.

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Introduction

Proteolytic enzymes are involved in a diverse range of physiological processes, including tissue remodeling during embryogenesis and development, wound healing, antigen presentation, bone resorption, and programmed cell death. Various proteinases have also

been implicated in tumor invasion and metastasis, e.g., plasminogen activators, matrix metalloproteinases, and the lysosomal enzymes cathepsins B, D, and L. In the metastatic process, proteolytic degradation of extracellular matrix components allows malignant cancer cells to penetrate basement membrane barriers that otherwise prevent tumors from spreading to distant sites in the body. Proteinases are also involved in tumor growth and angiogenesis. A key requirement for metastatic cancer cell invasion is proteolysis of basement membrane components. Production of extracellular proteolytic enzymes of different classes is a property strongly associated with the metastatic state of cancer cells (Mignatti and Rifkin, 1993; Qian *et al.*, 1989). Several lysosomal proteinases, including the cysteine proteinase cathepsin B, have been implicated in malignant progression of tumors. Many investigators have demonstrated correlations between increased activity of cathepsin B and increased metastatic capability of animal tumors or the malignancy of human tumors. These increases in cathepsin B activity correspond in part to increased amounts of cathepsin B mRNA transcripts and in part to reduced regulation by endogenous low-molecular-weight cysteine proteinase inhibitors (Sloane, 1990). Stefin A is one of such inhibitors inversely related to malignancy; reduced expression of stein A mRNA and protein has been linked to a reduction in its inhibitory capacity against cysteine proteinases (Sloane, 1990; Sloane *et al.*, 1990). Several reports have focused on the role of cysteine proteases (Terada *et al.*, 1995; Sukoh *et al.*, 1994; Watanabe *et al.*, 1989; Murnane *et al.*, 1991; Sheahan *et al.*, 1989; Mort *et al.*, 1980; Sloane, 1990) or cysteine protease inhibitors (Lah *et al.*, 1992) in cancer. In general, a large number of normal and abnormal processes are controlled by maintaining a balance between proteinases and their inhibitors (Bobek and Levine, 1992). Therefore, if an imbalance between cysteine proteases and their inhibitors leads to cancer invasion, then the levels of cathepsins and their endogenous inhibitors in breast, lung, brain, colon and head and neck tumors, and in body fluids from

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patients with ovarian, uterine, or colorectal cancer or melanoma, may be useful in predicting survival and relapse (Kos *et al.*, 1997; 1998; 2000; Strojnik *et al.*, 1999; Strojan *et al.*, 2000; Foekens *et al.*, 1998; Werle *et al.*, 1999; Lah *et al.*, 2000).

Another group of small-molecular-weight protein inhibitors are the cystatins (Barrett, 1987). These inhibitors form tight, reversible complexes with cysteine proteinases of the papain family, including cathepsins B, H and L. Cystatins are produced by virtually all types of cells, and most body fluids contain detectable levels of cystatin C. Further, the degree of malignancy of some types of cancer cells has been inversely associated with the expression of various cystatins (Corticchiato *et al.*, 1992; Hawley-Nelson *et al.*, 1988). It has been reported that cystatin may be a potentially important physiological inhibitor of cysteine proteinase activity in human extracellular fluids (Abrahamson *et al.*, 1986). We sought to determine whether overexpression of cystatin C would effect glioma tumor invasion and tumor growth. We transfected a malignant glioblastoma cell line (SNB19) with an expression vector containing cystatin C cDNA in sense orientation. This report describes the effects of overexpressing cystatin C on glioblastoma invasiveness and tumor formation in *in vitro* and *in vivo* models.

Results

Cystatin C protein expression differs among three glioma cell lines

To verify that different grades of glioma cells express different amounts of cystatin C, we first grew Hs683 (low-grade), SW1088 (anaplastic), and SNB19 (high-grade) cells to 80–90% confluence and assessed cystatin C protein in the conditioned medium and in the lysate fractions of these cultures by Western blotting. The highest amounts of protein were observed in the conditioned medium and cell lysate of the low-grade Hs683 cell line; less protein was present in the anaplastic SW1088 cell line, and the lowest amounts of protein were found in conditioned medium and lysate

of the high-grade SNB19 cells (Figure 1a,b). Densitometric quantification verified that the amount of cystatin C protein was significantly 7–9 times higher in Hs683 cells, and 3–4 times higher in SW1088 cells, than the amount in SNB19 cells ($P < 0.001$). Similar observation was made in tumor tissue samples and controls (data not shown).

Transfection with sense cystatin C increases cystatin C protein production in SNB19 cells

The eukaryotic expression vector pcDNA 3.1 was used to transfect the high-grade human glioblastoma cell line SNB19 with a 0.4 kb, sense-oriented cystatin C cDNA construct. Stable transfectants were selected with cloning cylinders after 10–15 days and weaned from the selection medium. Clones were grown in medium containing G418 (800 $\mu\text{g/ml}$). Glioma cells were grown in serum containing medium up to 7 days to study the growth rate. Not much difference in growth rate was found among the parental cells, cells transfected with an empty vector, or the cystatin C-sense transfectants (data not shown).

Western blot analysis of the cystatin C produced by the parental cells, the vector, and the sense-transfectants from serum-free conditioned medium and cell lysates showed prominent cystatin C protein expression in the conditioned medium of the SNB19-sense transfectants but not in the parental and vector transfected controls (Figure 1c,d). Densitometric quantification on verified that the amount of cystatin C levels were much higher in sense stable clones compared to parental and vector transfected cells. Transfection with sense cystatin C stable clones also led to a significant reduction in cathepsin B protein levels relative to the control cells (Figure 1E), which was also confirmed by densitometry.

Transfection with sense cystatin C increases cystatin C mRNA expression

Similar experiments were conducted using Northern blotting to confirm the differences in cystatin C mRNA expression in the Hs683, SW1088, and SNB19 cell lines

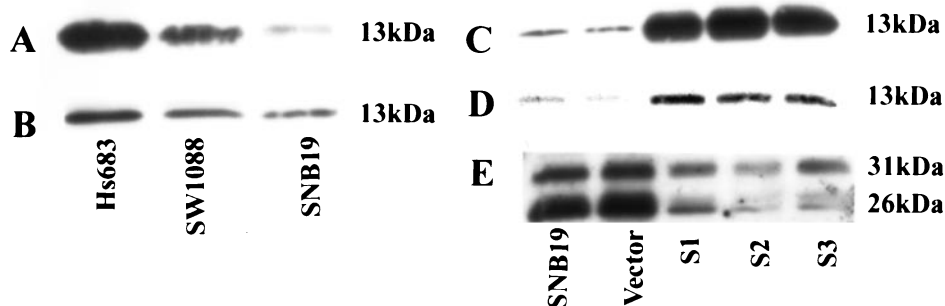


Figure 1 Western blot analysis of cystatin C expression in (a) conditioned medium and (b) cell lysates of human glioma cell lines (Hs683, SW1088, and SNB19). (c) Cystatin C expression in conditioned medium and (d) cell lysates of SNB19 parental cells, SNB19 cells transfected with empty vector, and SNB19 cells transfected with a cystatin C sense-oriented construct (e) Western blot analysis of cathepsin B in conditioned medium of SNB19 parental cells, SNB19 cells transfected with empty vector and SNB19 cells transfected with a cystatin C sense oriented construct

and to investigate the effect of transfection with sense-cystatin C on SNB19 cells. Again, the highest amount of cystatin C mRNA was found in the low grade Hs683 cell line, a lower amount was found in the anaplastic SW1088 cell line, and the lowest amount was found in the high-grade SNB19 cells (Figure 2a). Densitometric quantification verified that the amount of cystatin C message was 6–8 times higher in Hs683 cells, and 4–5 times higher in SW1088 cells, than the amount in the SNB19 cells ($P < 0.001$). With regard to the transfection, the sense-cystatin C-transfected SNB19 cells had much higher levels of cystatin C message than did the parental cells or and vector-only transfectants (Figure 2b). Cystatin C levels were significantly higher (8–10-fold) in sense transfected stable clones compared to parental and vector transfected clones.

Cell invasiveness in Matrigel

Invasiveness of parental SNB19 cells, vector-transfected controls, and stably transfected sense-cystatin C clones was measured in a Matrigel invasion assay. Considerably fewer cells were stained in sense-transfected cells invaded through Matrigel-coated transwell inserts than did parental cells or vector-only controls (Figure 3a). A quantitative MTT assay showed that 95 and 100% of the parental cells and vector controls invaded the Matrigel, significantly more than the invasion of only 15 to 20% of the sense SNB19 clones ($P < 0.001$) (Figure 3b).

Glioma spheroid invasiveness into rat brain aggregates

Invasiveness was also assessed in a 3-dimensional spheroid coculture assay in which fetal rat brain cell aggregates were confronted with spheroids consisting of parental SNB19 cells, vector-only transfectants, or sense-cystatin-C transfected SNB19 clones. Spheroids consisting of the SNB19 sense-cystatin C clones did not

invade the brain aggregates, but the spheroids consisting of parental cells or vector control cells did (Figure 4a). Quantitative analysis revealed that 10 to 15% of the brain aggregates remained in the cocultures with parental cells or vector clones as compared with 85 to 90% in the cocultures with the sense clones ($P < 0.001$) (Figure 4b).

Cerebral tumor formation in athymic mice

Groups of 10 nude mice were intracerebrally injected with one of three types of high-grade SNB19 glioblastoma cells: the parental SNB19 cells, the vector-transfected SNB19 cells, or the sense-cystatin C transfectants. The parental SNB19 cells and vector-control cells formed brain tumors within 4 weeks of their injection; the cystatin C-sense transfectants did not (Figure 4c,d). Analysis, of hematoxylin-and-eosin stained brain sections of GFP sections by a neuropathologist who was blinded as to treatment group revealed no difference in tumor size between the mice injected with the parental cells or the vector-only transfectants; however, tumors, if any, were significantly smaller ($P < 0.001$) in the SNB19 cells that had been transfected with sense-cystatin C (Figure 4e).

Discussion

Invasion and metastasis of cancer cells requires degradation of the proteins that compose basement membranes and connective tissues (Liotta *et al.*, 1980). Proteolysis plays an important role in tumor-cell invasion (Liotta *et al.*, 1991; Schmitt *et al.*, 1992). The most intensively investigated proteolytic systems include the plasminogen-activator system (uPA, tPA) and its inhibitors (PAI-1 and PAI-2) (Schmitt *et al.*, 1992; Heidtmann *et al.*, 1989, 1992), matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs), and the lysosomal cathepsins and their endogenous inhibitors (Schmitt *et al.*, 1992; Heidtmann *et al.*, 1989, 1992; Sloane, 1990; Sloane *et al.*, 1994). The cysteine proteinase cathepsin B has been implicated in the progression of tumors from a premalignant to a malignant state, and it has also been shown to participate in tumor growth, vascularization, invasion, and metastasis (Sloane, 1990). All three types of cysteine proteinase inhibitors (kininogens, stefin A and cystatin C) have been found in ascites from patients with ovarian carcinoma (Lah *et al.*, 1992). In an *in vitro* study of the cysteine proteinase cathepsin B and its physiological inhibitor cystatin C in human colon cancer cells (Corticchiato *et al.*, 1992), cathepsin B activity was totally neutralized by recombinant cystatin C, suggesting a potential interaction between released extracellular cathepsin B and cystatin C. The authors of this study suggested that interactions between these substances may participate in the modulation of the invasive phenotype of colon cancer (Corticchiato *et al.*, 1992). It has been reported

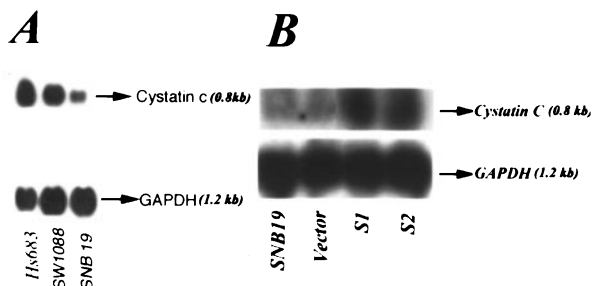


Figure 2 Northern blot analysis of cystatin C mRNA in the Hs683, SW1088, and SNB19 cell lines. (b) Cystatin C mRNA expression in parental SNB19 cells, SNB19 cells transfected with an empty vector, and SNB19 cells transfected with a cystatin C sense fragment. Total cellular RNA was extracted and 10- μ g portions were subjected to electrophoresis on agarose-formaldehyde gels and then transferred to a nylon membrane and labeled with 32 P-cystatin C cDNA. The membranes were then stripped and re-hybridized with GAPDH cDNA as an internal control

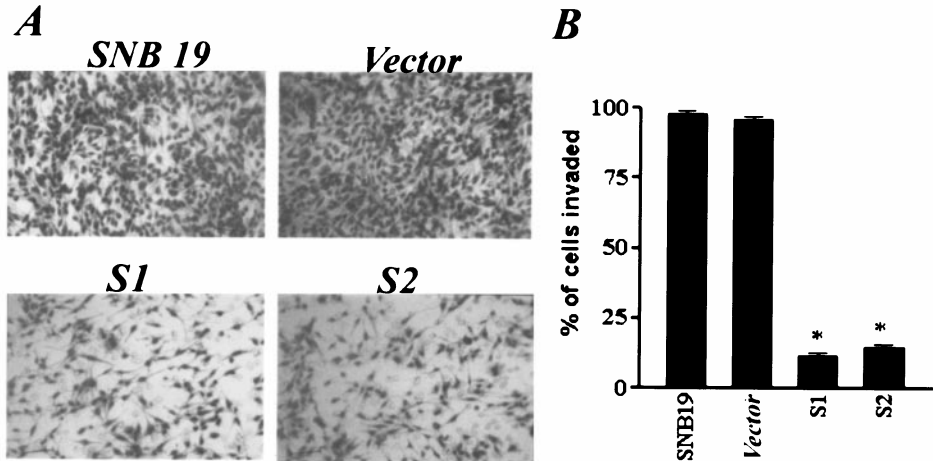


Figure 3 Matrigel invasion of parental SNB19 cells, SNB19 cells transfected with empty vector, and SNB19 cells transfected with a cystatin C sense fragment. Cells (10^6) were layered on Matrigel-coated transwell inserts and the percentage of invasion was calculated as described in Materials and methods. Cells that invaded through Matrigel-coated transwell inserts were stained and photographed (a) and the percentage was quantified (b). Data shown are means \pm s.d. of four different experiments from each clone ($P < 0.001$)

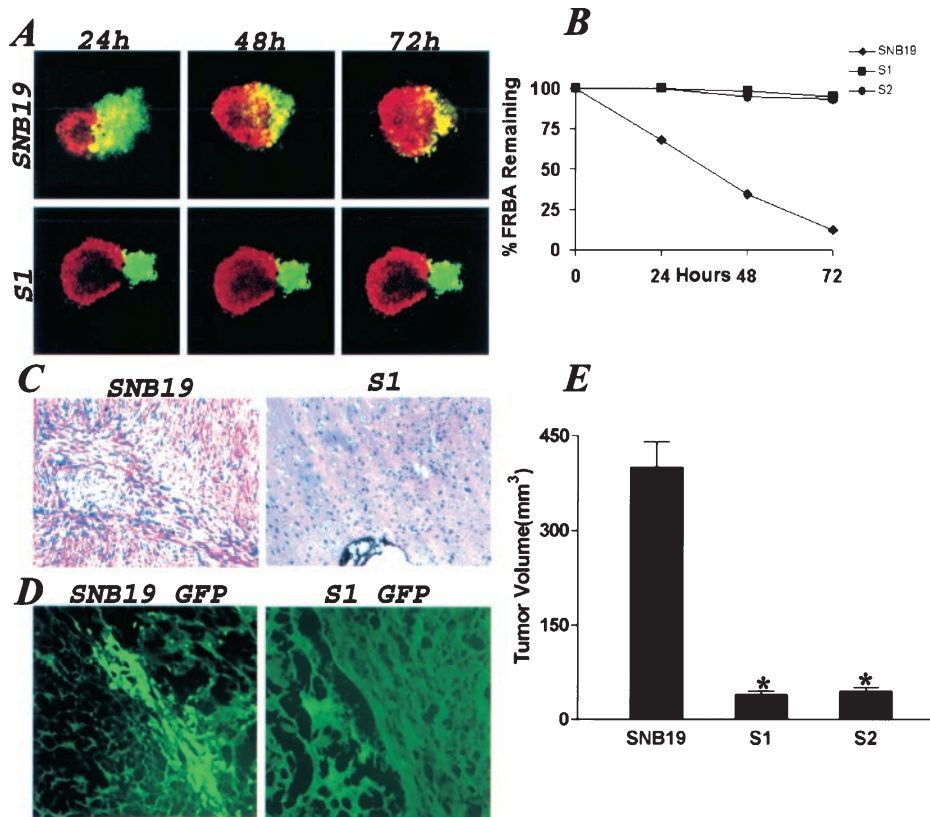


Figure 4 Confocal laser images of DiI-stained tumor spheroids of parental SNB19 cells, SNB19 cells transfected with empty vector, and SNB19 cells transfected with a cystatin C fragment (green fluorescence) cultured with DiO-stained fetal rat brain cell aggregates (red fluorescence). (a) Cocultures were scanned under a laser-scanning microscope after 24, 48, and 72 h of culture. (b) The amount of brain aggregates remaining after SNB19 spheroid invasion was quantified as described in Materials and methods. Tumor formation is by SNB19 and sense-transfected cells in nude mouse brain. Photomicrograph of tumor section stained with hematoxylin and eosin (c) or tumor sections from GFP expressing cells (d) and demonstrating a decrease in tumor formation in brain tissues derived from cystatin C sense transfected cells injected into the mouse brain compared to control SNB19 glioblastoma cells. Semiquantitation of tumor volumes in mice injected intracerebrally with parental SNB19 cells, vector-transfected SNB19 cells, or cystatin C-sense stable transfectants 4 weeks after injection. Data shown are the s.d. values from 10 animals from each group (e). $*P < 0.001$

that a latent form of cathepsin B secreted by colon cancer cells could be activated by neutrophil elastase *in vivo* rather than being controlled by secretion of the cystatin C inhibitor (Keppeler *et al.*, 1994). Alternatively, cystatin C may be inactivated during the early part of its trafficking through the secretory pathway and then reactivated before its secretion through a novel transient dimerization mechanism, in which its uptake by the cell also leads to its redimerization in the lysosomal pathway (Merz *et al.*, 1997).

Increased expression of cystatin C has been shown to inhibit the motility and the *in vitro* invasiveness of B16 melanoma cells by 50% in cells stimulated with autocrine motility factor or laminin and in unstimulated cells (Sexton and Cox, 1997). The human glioma cell line SNB19 is normally highly invasive and produces very little cystatin C protein or RNA. In our study, introducing a sense-oriented plasmid construct of cystatin C into SNB19 cells inhibited the invasiveness of these cells in both a Matrigel invasion assay and a spheroid model. Quantitatively different combinations of cystatins are the major constituents of the inhibition of cathepsin B in squamous cell lung cancer and normal lung tissue (Krepela *et al.*, 1998). Cathepsin B and its endogenous inhibitors may facilitate proteolysis by hepatoma cells and thereby contribute to the invasive phenotype of this type of cancer (Calkins *et al.*, 1998). An inverse correlation between amounts of cathepsin B and cystatin C has been found in a study of human colorectal cancer (Hirai *et al.*, 1999). In our study, we observed that in human glioma cell lines, the relative amounts of cystatin C decreased as the amounts of cathepsin B increased and the degree of malignancy increased.

Increases or changes in the activity of cysteine proteases have been implicated in cancer, rheumatoid arthritis, sepsis, and osteoporosis. Syngenic xenograft model of cystatin C-deficient mouse demonstrated that cystatin C concentrations *in vivo* might influence metastasis in some tissues (Huh *et al.*, 1999). In that study, the decreased metastatic spread of B16-F10 cells in mice without cystatin C resulted from reductions in both tumor seeding and tumor growth in the lungs (Huh *et al.*, 1999). By comparison, in our study, human glioma cells (SNB19) that overexpressed cystatin C did not form tumors upon injection into the brain of nude mice. Several studies of adults and children with renal diseases have suggested that serum cystatin C may be a better marker of glomerular filtration rate than serum creatinine (Randers and Erlandsen, 1999). The invasive properties of squamous-cell carcinomas can be changed by modulation of the balance between cathepsin B and its endogenous inhibitors, a finding that provides further evidence of the involvement of cathepsin B in tumor invasion and metastasis (Coulibaly *et al.*, 1999).

It has been reported that overexpression of cystatin C levels in B16 melanoma affects properties associated with metastasis (Cox *et al.*, 1999). In our study, we also observed that sense-cystatin C stables cloned had more cystatin C, both in the medium and in the cell lysates,

than did the parental cells. We also found that transfection with the sense-cystatin C transcript did not affect cell growth rate relative to the parental and vector-transfected SNB19 cells. The cathepsin B/cystatin C complex has been less abundant in the sera of patients with malignant tumors than in those with benign diseases or healthy control subjects, suggesting an imbalance between the enzyme and its inhibitor in cancer (Kos *et al.*, 2000). Similar observation was observed in another study (Yoshimura *et al.*, 2000), where cathepsin B and cystatin C levels were assessed in cell types constituting human pituitary adenomas and normal adenophypophyseal tissue.

Serum cystatin C measurements are superior to measurements of serum creatinine for detecting a decrease in creatine clearance and potentially for estimating glomerular filtration rate in patients with cancer, independent of the presence of metastases or chemotherapy (Stabuc *et al.*, 2000). Elevated levels of cystatin C have been found in patients with rheumatoid arthritis (Mangge *et al.*, 2000). Cystatins A and C both were shown to inhibit cathepsin B by a two-step mechanism, involving an initial weak interaction followed by a conformational change (Pavlova *et al.*, 2000). Cystatin C expression relative to cathepsin B expression was found to be decreased in breast cancer tissues (Yano *et al.*, 2001). Anaplastic carcinoma of the thyroid, a rapidly growing neoplasm with a very poor prognosis, has osteoclast-like giant cells that express cysteine proteinases and cystatin C. Expression of cathepsins B and K in anaplastic thyroid carcinoma might contribute to the invasive behavior of this tumor, promoting metastasis and destruction of the cartilaginous trachea (Gaumann *et al.*, 2001).

In conclusion, cystatin C is a potent inhibitor for cathepsin B. An inverse correlation was observed between amounts of cathepsin B and cystatin C in many malignant phenotypes. Similar observations were noticed in the present study with different grades of glioma. Further, over-expression of cystatin C in malignant glioma cell lines resulted in decreased invasion both *in vitro* and *in vivo*. These results suggest that cystatin C could be used as a diagnostic marker and adjuvant therapeutic agent in malignant gliomas.

Materials and methods

Cell culture

Human glioma cells of high grade (SNB19), medium grade (SW1088), and low grade (Hs683) were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (pH 7.2–7.4) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were subcultured every 3–5 days.

Preparation of constructs

A 0.4-kb fragment of human cystatin C was amplified from Hs683 cells, which normally express large amounts of cystatin

C, by reverse transcriptase-polymerase chain reaction (RT-PCR) with gene-specific primers. The 400-bp fragment was subcloned into the mammalian expression vector pcDNA3.1 in the sense orientation (Invitrogen, San Diego, CA, USA). The orientation of the fragment was confirmed by restriction digestion, and subsequent sequencing with an automated sequencer (Applied Biosystems Inc., USA) showed 100% homology with the published sequence of cystatin cDNA (Abrahamson *et al.*, 1987).

Transfection of SNB19 cells with human cystatin C sense cDNA construct

SNB19 cells were transfected with either the 0.4-kb (sense) cDNA construct or with the pcDNA3.1 vector alone (as a control) by using lipofectamine (Life Technologies, Gaithersburg, MD, USA). Cells were grown overnight (4×10^5 cells per 60-mm dish), washed with phosphate-buffered saline (PBS), and treated with 1–2 μg of plasmid DNA in 100 μl of serum-free medium. Lipofectamine (6 μl diluted in 100 μl of serum-free medium) was added drop-wise to the DNA. Five to six hours later, the medium was replaced with DMEM containing 10% fetal bovine serum. Selection was begun 48 h after transfection by growing the cells in complete medium containing 800 $\mu\text{g}/\text{ml}$ G418 (Life Technologies). Transfected cells were passaged every 3–4 days and were always grown in the presence of G418. We checked the selected clones periodically for the presence of cystatin C by Western and Northern blotting. Selected stable transfectants were expanded and used for subsequent studies.

Western blotting

Parental cell lines (Hs683, SW1088 and SNB19), empty-vector-transfected SNB19 cells, and sense-cystatin C-transfected SNB19 clones were grown to 80–90% confluence in 6-well tissue culture plates, after which the serum-free medium was collected, cells lysed with RIPA buffer, and the proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels (Laemmli, 1970). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Towbin *et al.*, 1979) and blocked with 5% nonfat dry milk in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.1% Tween-20 (TTBS) for 1 h. The membranes were then incubated overnight at 4°C with anti-human cystatin-C rabbit polyclonal antibody (Bio Vendor Laboratory Medicine, Inc., Czech Republic) that had been diluted 1:2000 in TTBS containing 1% bovine serum albumin. After several washes, the membranes were incubated for 1 h with an anti-rabbit peroxidase-conjugated secondary antibody (also diluted 1:2000 in TTBS and 1% bovine serum albumin). The immunoreactive proteins were identified by using an enhanced chemiluminescence reagent system, according to the manufacturer's instructions (Amersham).

Northern blotting

Steady-state levels of cystatin C mRNA were measured by Northern blot analysis of parental cell lines (Hs683, SW1088 and SNB19), empty-vector-transfected SNB19 cells, and sense-cystatin C-transfected SNB19 clones. Total cellular RNA was extracted as described previously (Chomczynski and Sacchi, 1987), and 10 μg of the RNA was electrophoresed in agarose-formaldehyde gel, transferred to a nylon membrane by capillary action overnight, and then cross-

linked by UV irradiation. The membranes were hybridized at 65°C overnight with a human cystatin C cDNA probe labeled with ^{32}P -deoxycytidine triphosphate by random primer labeling, after which they were washed in 0.5% SSC and 0.1% SDS for 20 min at room temperature, then for 15 min at 65°C, and then exposed to X-ray film at -70°C . The membranes were then stripped and rehybridized with glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as an internal control.

Matrigel invasion assay

The invasiveness of the transfected cells (cystatin C-sense and vector-only SNB19 cells) and the parental SNB19 cells was tested *in vitro* in a Matrigel invasion assay. Briefly, transwell inserts with 8- μm pores were coated with Matrigel (1 mg/ml), and then cells were trypsinized and 20 μl of the cell suspension (3×10^5 cells/ml) was added to triplicate wells. After incubation for 24 h at 37°C, cells that passed through the filters into the lower wells were quantified (Konduri *et al.*, 2001) and expressed as a percentage of the sum of the cells in the upper and lower wells. Cells on the lower side of the membrane were fixed, stained with Hema-3, and photographed.

Three-dimensional spheroid invasion assay

The invasiveness of glioma spheroids was measured in a 3-dimensional model (Pederson *et al.*, 1993) in which the spheroids were stained with the fluorescent dye DiI and cultured with fetal rat brain aggregates stained with another fluorescent dye, DiO. At various culture intervals, serial 1- μm optical sections were obtained with the aid of a confocal laser-scanning microscope from the surface to center of the co-cultures. DiI and DiO fluorescence (Molecular Probes Inc, Eugene, OR, USA) was detected with an argon laser at 488 nm with a band pass filter at 520–560 nm (DiI) and a helium/neon laser at 543 nm with a long pass filter at 590 nm (DiO). The volume of brain aggregate remaining after tumor-spheroid invasion was quantified at 24, 48, and 72 h of coculture as described previously (Konduri *et al.*, 2001).

Intracerebral injection

To examine the *in vivo* effects of the up-regulation of cystatin C produced by the transfection, we injected suspensions of sense-cystatin C-transfected SNB19 cells, vector-transfected SNB19 cells, or parental SNB19 cells into the brains of athymic mice. A total of 40 mice were used, 10 each for the parental or vector-control conditions and 10 each for the two transfectant conditions. Cells were trypsinized and resuspended in serum free medium to a concentration of 2×10^5 cells/ μl . The mice were anesthetized with an intraperitoneal injection of a 0.35–0.45 μl solution consisting of 0.06 M 2,2,2-tribromethanol (Aldrich Chemical Co., Milwaukee, WI, USA), 1.25 isoamyl alcohol, and 98.5% bacteristatic saline and injected intracerebrally with a 10- μl aliquot (2×10^5 cells/ μl) of the specified cell type with the aid of a stereotactic frame as described elsewhere (Konduri *et al.*, 2001). Four weeks later, the mice were killed by intracardiac perfusion, first with PBS and then with 4% paraformaldehyde in normal saline. The brains were removed, placed in 4% paraformaldehyde for 4 h, and then incubated for 2 days in 30% sucrose in PBS at 4°C. The following day, the brains were sectioned, embedded in microscopic slides, and frozen at -20°C . Cryostat sections were stained with hematoxylin and eosin to examine tumor

growth. The sections were blindly evaluated by a neuropathologist who was blinded as to the treatment group and scored semiquantitatively for tumor size. The maximal cross-sectional tumor diameter, measured in each section, was considered a measurement of tumor size, and those measurements were compared between mice injected with control cells and those injected with cystatin C-sense

transfectants. The variation between the sections in each group was less than 10%.

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